

Proteomics in Food Science

From Farm to Fork



Edited by
Michelle L. Colgrave



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Preface

Proteins are the end result of a series of biological processes including epigenetic modifications, transcription, translation, posttranslational processing, and degradation. They are an essential part of the human diet, serving as a fuel source and as the source of amino acids, the building blocks used to make new proteins. Commonly thought of as a main constituent of meat, fish, dairy, and eggs, proteins are also present in the broad spectrum of food ingredients including the grains, legumes, and nuts. The term proteomics typically refers to large-scale comprehensive studies of a specific proteome, that is, the complement of proteins expressed by a cell, tissue, or organism. Proteomics aims to obtain information on protein abundance, protein variations and modifications, and protein interactions. Proteomics has been most frequently applied to study health and disease but is rapidly being adopted in the food sciences to answer questions relating to quality, safety, allergenicity, bioactivity, and the promotion of good health.

The successful application of proteomics relies upon a combination of technologies and approaches, including protein biochemistry, protein (and peptide) separation science, tandem mass spectrometry, and bioinformatic analysis. It can include the simple separation of proteins by electrophoresis using conventional gels and dyes to the advanced bioinformatic analysis of peptide fragments to determine their protein origins. Overall, these techniques entail the merging of physical and chemical principles in an endeavor to advance analytical and investigative science. Sample preparation methods aim to reduce the complexity of the proteome by fractionating, depleting, or enriching for low-abundant proteins. Gel-based techniques allow the visualization of the proteome, in particular when combined with fluorescent dyes or affinity techniques such as Western blotting. Gel-free proteomics employing mass spectrometry are typically termed “bottom-up” when the analysis involves first digesting the proteins into peptide constituents or “top down” when the analysis examines intact proteins. Protein quantification may be achieved through the use of isobaric or chemical tags in the so-called “labeled” approaches or more frequently using “label-free” approaches. In the latter, peptides may be selected that serve as biomarkers that can be used as proxies to quantify their parent proteins or to determine the authenticity of a particular food product.

Various practical applications of proteomics have emerged in the past decade particularly related to their application in food science from production to the point of consumption. In

this book, the term proteomics covers a range of methods for elucidating the identity or composition of specific proteins in foods or cells related to food science: from edible components to spoilage organisms. A variety of analytical platforms are described, ranging from usage of simple electrophoresis to more sophisticated mass spectrometry and bioinformatic platforms. *Proteomics in Food Science: From Farm to Fork* introduces the current state-of-play, the predicted trends for the future and elaborates on the promise of proteomics to wide-ranging applications in the food science arena. The depth of coverage achieved in this book aims will enable those in one food discipline to become familiar with the concepts and applications of proteomics in other disciplines of food science. The book comprises 29 chapters and is divided into five sections: (1) Application to plants—cereals, nuts, pulses, and fruits (8 chapters); (2) Application to farm animals—eats, dairy, and eggs (8 chapters); (3) Application to aquaculture (5 chapters); (4) Processed foods (3 chapters); (5) Food spoilage, pathogenic organisms, and allergens (5 chapters).

The individual chapters deal with quality issues such as the elucidation of quality traits for a wide variety of foods including meat, fish, dairy, eggs, wine, beer, cereals, legumes, nuts, and fruits; as well as the deterioration of quality as in the case of post mortem processes in both plant-based and animal-based foods. Contributions within the book also tackle the identification and characterization of bioactive peptides and proteins which are important from a nutritional perspective. The application of proteomics to understand changing environmental factors such as drought, flood, salinity, and response to abiotic stress, pests, and pathogens is also addressed. Consumers are acutely aware of food safety issues and proteomics can alleviate concerns regarding safety aspects including food authenticity, meat speciation, accuracy in food labeling, and the detection of allergens or pathogenic microorganisms. Moreover, the general public shows an increasing interest in animal health-related aspects, including stress, welfare, and the influence of animal management practices, and the utility of proteomics in this area is also addressed. This book has 74 contributors from 19 countries with vast experience and recognized international reputation, resulting in a compendium of knowledge in the current and future applications of proteomics in the food sciences.

Proteomics is a rapidly evolving area primarily owing to the recent developments in modern analytical instrumentation and bioinformatics solutions that in turn give rise to an unprecedented speed, accuracy, and depth of coverage in proteomic studies. The advent of fast, affordable, and convenient genome sequencing will further support the application of proteomics to the food and agricultural sciences. The emergence of whole-genome sequence data and large-scale transcriptomic data sets, when integrated with comprehensive proteomic analyses will enable systems biology-based studies of agricultural species. With these advances, understanding the mechanisms underpinning complex traits, such as environmental tolerance, pest resistance, yield enhancement, and food quality, will soon be possible paving the way for a safe and sustainable future for the agriculture and food industries.

Michelle L. Colgrave

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Michelle L. Colgrave

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SECTION 1

Application to Plants — Cereals, Nuts, Pulses, and Fruits

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Postharvest Proteomics of Perishables

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1.1 Introduction

Postharvest physiology and technology has been key to maintaining and extending the shelf-life of perishables and reducing food losses. However, postharvest losses are still significant and reduction of such losses would be the easiest, less costly, and most effective method instead of increasing food production (Pedreschi et al., 2013a).

Postharvest strategies or technological implementation, such as temperature reduction, modification of the atmosphere, or chemical treatments, are applied. These serve to reduce respiration rates, retard ripening, decrease ethylene production, and consequently retard senescence, prevent dehydration, and extend the shelf-life thus preserving produce quality. These strategies imply that produce are submitted to abiotic stresses and they need to activate different metabolic pathways to cope with these stresses and reach homeostasis to avoid undesirable quality traits that limit produce shelf-life (Pedreschi and Lurie, 2015).

Proteomics studies on fruits and vegetables have increased in recent years (Li et al., 2015; Buts et al., 2014). Proteins play key roles in fruit development, ripening, and senescence and are involved in key metabolic pathways and networks related to resistance to biotic and abiotic stresses. Numerous proteomics studies focused on understanding ripening, development, and senescence of different commodities such as tomato, grapes, citrus, strawberry, peach, papaya, mango, and banana are available and the reader is encouraged to review these papers to gain further insight (Rocco et al., 2006; Kok et al., 2008; Faurobert et al., 2007; Deytieux et al., 2007; Negri et al., 2008; Giribaldi et al., 2010; Katz et al., 2007, 2010; Bianco et al., 2009; Prinsi et al., 2011; Nogueira et al., 2012; Magalhaes Andrade et al., 2012; Torres-Toledo et al., 2012). Proteomic studies focused on microbial/fungal diseases and mechanisms during postharvest have been extensively reported. For example, Buron-Moles et al. (2015) studied the apple defense response to wounding, *Penicillium expansum* and *Penicillium digitatum* infection at the proteome and oxi-proteome (protein carbonyl) levels. The proteins Mal d 1.03A, Mal d 1.03E, and EF-Tu were specifically induced in response to *P. digitatum* infection. In addition, 27 oxidized proteins were identified as reactive oxygen species (ROS)-sensitive targets and were suggested to play a leading role in the response against biotic and abiotic stresses. Comparative proteomics and functional analysis have

revealed that defense-related proteins, energy metabolism, and antioxidant-related proteins play key roles in fruits in response to storage conditions and elicitor treatments. For more studies on microbial/fungal diseases using proteomics approaches, the reader is referred to [Gonzalez-Fernandez and Jorrin-Novo \(2012\)](#), [Shah et al. \(2012\)](#), [Chan \(2013\)](#), and [Taylor et al. \(2008\)](#) and for a complete review of the mechanisms related to protein level changes in both the host and pathogen, the reader is referred to [Chan \(2013\)](#).

Numerous proteomics studies reported up to date have focused on understanding either desirable or undesirable postharvest quality traits ([Urbany et al., 2011](#); [Pedreschi et al., 2007](#); [Nilo et al., 2010](#); [Minas et al., 2016](#); [Miyasaka et al., 2016](#)) to find potential initial quality markers or early stage biomarkers of such quality traits. But these postharvest quality traits are strongly influenced by preharvest abiotic stresses (e.g., temperature, radiation, light exposure, dehydration) and enhanced or buffered by postharvest abiotic stresses. Thus, this book review focuses upon mainly commercially important fruits and vegetables with focus on how abiotic stresses (temperature, dehydration, atmosphere modification, light and chemical exposure, ozone treatments, etc.) influence certain produce quality traits or characteristics.

1.2 Factors Affecting Postharvest Quality

The type of stress and characteristics (severity, exposure, duration) and the characteristics of the different produce (genotype, maturity stage, organ type) will influence the response of the different produce to acclimate and withstand the different imposed abiotic stresses. It has been extensively reported that preharvest factors (e.g., growing conditions and crop management) and the stage of development influence the response of the different produce to abiotic stresses ([Fig. 1.1](#)) and thus on the postharvest phenotype obtained.

Some studies using other omics platforms have been reported on this topic but proteomics studies are still limited. [Abdi et al. \(2002\)](#) carried out a 2D-PAGE proteomics experiment for determining optimal harvest of stone fruits. Commercial harvest indices are skin color, firmness, soluble solids, and size. These parameters are affected not only by the cultivar but also by growing conditions and seasonal climatic factors. The authors proposed to use four allergenic proteins that were synthesized in the fruit a few days before the optimal harvest time in plums, peaches, and nectarines as a harvest index for stone fruits. [Ye and Dilley \(1992\)](#) and [Dilley et al. \(1995\)](#) by using a gel-based approach proposed 1-amino cyclopropane carboxylate-oxidase (ACO) as a biomarker for apple harvest. Other omics approaches employing transcriptomics and metabolomics have proposed potential initial quality markers for Hass avocado ripening heterogeneity and potatoes cold sweetening ([Pedreschi et al., 2013b, 2014](#)). Recently, [Buts et al. \(2016\)](#) studied at the proteome level using a gel-free approach, an apple browning disorder in the cultivar Braeburn related to preharvest application of calcium, potassium, and triazole as fertilizers. Calcium and potassium have been previously reported to reduce the incidence of this disorder while triazole displayed the

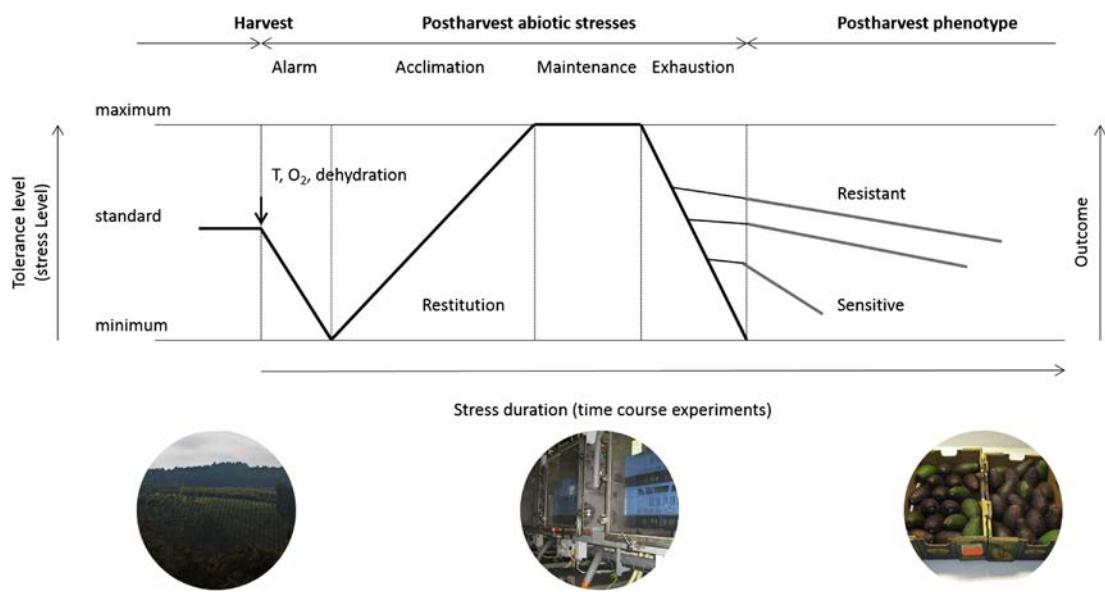


Figure 1.1

A generalized scheme of the dynamics that perishables undergo over the supply chain showing the relationship between preharvest factors and the final postharvest phenotype and shelf-life. Adapted and modified from Pedreschi, R., Lurie, S., Hertog, M., Nicolai, B., Mes, J., Woltering E., 2013a. Post-harvest proteomics and food security. *Proteomics* 13, 1772–1783. In this particular example, preharvest factors (growing conditions and cultural practices) determine the potential of the fruit at harvest and shelf-life. In addition, postharvest abiotic stresses (such as temperature or controlled atmosphere) might influence the obtained quality trait (for example the ripening heterogeneity) of avocados.

opposite effect. Results from this study revealed that calcium and triazole application resulted in significant effects at the proteome level with 29 and 63 differentially expressed proteins after the treatment application. Key antioxidant enzymes were correlated with calcium fertilization and respiration and ethylene-related proteins were correlated with the triazole treatment. The authors, in addition, postulated that an early proteomics imprint (at harvest) could be used as an early decision tool of the postharvest performance of a batch to develop this browning disorder.

1.3 Proteome Level Responses of Perishables to Main Postharvest Abiotic Stresses

1.3.1 Cold Storage and Heat Treatments

Temperature controlled chains are widely used to delay perishable ripening and senescence and thus extend shelf-life. Cold and heat share some similar molecular mechanisms and heat is used before cold storage on some commodities to delay ripening, to confer protection against chilling injury, or as quarantine treatment (Lurie and Pedreschi, 2014). Proteomics

studies related to cold storage and heat treatments or combination of treatments (heat followed by cold storage) are available for different commodities. [Lliso et al. \(2007\)](#) reported on the proteome changes of the albedo of Murcott tangor after exposure at 4°C for 2 weeks with an increase in cysteine proteinase and a decrease in ascorbate peroxidase. [Yun et al. \(2012\)](#) examined pommelo stored at 8°C for 72 and 120 days and reported 108 differentially expressed proteins of the fruit sacs. Of these, 63 were identified and classified as involved in sugar and carbohydrate metabolism, protein destination and storage, response to stimulus, for example, cold response proteins (COR), heat shock proteins (HSP), and temperature-induced lipocalins (TIL), which were higher in cold-stored fruit besides secondary metabolism proteins. [Giraldo et al. \(2012\)](#) reported on nectarine fruit that developed chilling injury at 4°C for 5 weeks. Out of the 350 detected spots, 17 proteins were differentially expressed compared to fruit one day after harvest. Some of these differentially expressed proteins that were increased in cold-stored fruit corresponded to pathogenesis-related (PR) proteins and glutathione peroxidase, whereas a small HSP was downregulated. [Yuan et al. \(2014\)](#) reported proteome changes on *Vitis labruscana* stored at 2°C for 50 days and 95% relative humidity (RH). Changes included downregulation of enzymes related to glycolysis and the citric acid cycle (TCA) and upregulation of cell wall degrading proteins, HSPs, antioxidant enzymes, and proteasomes. [Sánchez-Bel et al. \(2012\)](#) based on a 2-DIGE proteomics approach on pepper stored at 10°C for 21 days and 80% RH reported downregulation of glycolytic, TCA and Calvin cycle, and catalase enzymes.

Heat treatments can be used before cold storage to prime the commodity for cold stress. [Yun et al. \(2013\)](#) reported proteome changes in *Citrus unshiu* cv. Marc submitted to 52°C for 2 min. Upregulated proteins corresponded to glucanases, chitinases, low-MW HSPs, and reduced redox metabolism (isoflavone reductase, oxidoreductase, and superoxide dismutase). [Bustamante et al. \(2012\)](#) submitted *Prunus persica* L. Batsch cv. Dixiland to 39°C and 90% RH for 3 days and by a 2-DIGE approach reported reduced ethylene-related enzyme (ACO), downregulation of 12 cell wall-modifying enzymes, and increased glyceraldehyde 3-phosphate dehydrogenase.

1.3.2 Dehydration

The vapor pressure deficit, defined as the difference of vapor pressure of the produce and atmosphere, determines the amount of water that is lost due to transpiration. Minimizing produce water loss is important not only from an economical point of view but also from a quality stand point since small water losses can induce a series of metabolic responses that might end up in undesirable quality traits such as wilting, loss of color, or peel damage.

Abscisic acid (ABA) is the most important regulator of the dehydration response in plants and the ABA and MAPK perception and signaling pathways are involved in any abiotic stress that involves decrease of turgor pressure and water loss ([Danquash et al., 2014](#)). Most of the

studies reported on dehydration and the underlying physiology using postgenomic approaches are on citrus and grapes (Rizzini et al., 2009; Romero et al., 2013) but the only reported study using a proteomics approach correspond to Lliso et al. (2007). These authors reported on protein changes of *Murcott tangor* in the albedo during storage in high (99% RH) and low (60% RH) for 15 days. Water stress-induced accumulation of cysteine protease which might be involved in the modulation of programmed cell death triggered by oxidative stress; ascorbate peroxidase (APX) and manganese-dependent superoxide dismutase (MnSOD) are antioxidant enzymes that help protect against stress and ATPase which might be involved in ion transport in order to maintain osmotic homeostasis.

1.3.3 Controlled and/or Modified Atmosphere

A complementary strategy to cold storage to extend shelf-life of perishables is controlled atmosphere (CA) and/or modified atmosphere packaging (MAP). Generally, it involves reducing the oxygen and increasing the carbon dioxide levels to retard respiration and thus senescence.

Reported studies can be classified as (1) short anaerobiosis shocks (N₂ flush, high CO₂) to induce positive responses and metabolic changes such as delayed ripening, delayed physiological disorders, and acclimation during storage disorders (Lara et al., 2009); (2) short-term hypoxic conditions with no signals of physiological disorders (Pedreschi et al., 2009); and (3) long-term hypoxic conditions that lead to anoxic conditions and disorders associated with low oxygen (Pedreschi et al., 2007).

Shi et al. (2008) reported a gel-based peel and pulp proteomics study on citrus fruit (grapefruit-tolerant and mandarin-sensitive) exposed to N₂ flush for 24 h. Only five proteins were suppressed in grapefruit pulp, but the nitrogen shock significantly affected protein accumulation in mandarins such as glycolytic enzymes (alcohol dehydrogenase) and stress proteins (HSPs and ascorbate peroxidase). In the peel, most of the proteins were involved in cell rescue defense and only a few (6%) were involved in energy production contrary to that observed in the pulp.

Short-term exposure to low oxygen and high carbon dioxide conditions (air vs. 10 kPa CO₂ for 5 days at 1°C) resulted in downregulation of respiration involved enzymes (enolase and malic enzyme) and upregulation of malate dehydrogenase and transketolase. Additionally, PR and protein synthesis-related enzymes were downregulated. Ascorbate peroxidase remained unchanged while polygalacturonase-inhibiting protein, 14-3-3 and ACO were upregulated (Pedreschi et al., 2009). Long-term exposure of Conference pears submitted to browning inducing conditions (1 kPa O₂ and 10 kPa CO₂ at 1°C for 6 months) revealed that the brown tissue was associated with a total collapse of the antioxidant system with very marked downregulation or disappearance of ascorbate-glutathione proteins, downregulation of respiratory, protein synthesis and other proteins involved in defense mechanisms (Pedreschi et al., 2007).

The tissue submitted to the same conditions but not developing this browning disorder were capable of maintaining an adequate ATP pool, antioxidant and other defense-related mechanisms. Recently, [Li et al. \(2015\)](#) by using a label-free quantitative proteomics approach reported on protein expression changes of strawberry (*Fragaria ananassa* Duch cv. Akihime) submitted to controlled atmosphere conditions of (2 kPa O₂ and 12 kPa CO₂), air (room temperature) and low temperature (air) storage for 9 days. Differentially expressed proteins were associated with carbohydrate and energy metabolism, volatile biosynthesis, phenylpropanoid activity, cellular stress responses, protein synthesis, and degradation and folding during fruit senescence.

1.3.4 Light Exposure

Sun exposure and/or artificial light exposure of perishables have been related to a lower incidence of physiological disorders such as superficial scald in apples. Another light regime is that of UV treatment. This has been found to enhance resistance to decay and enhance pigment accumulation. Treatment with UV-C (100–280 nm)-induced synthesis of phenolic compounds in strawberry ([Erkan et al., 2008](#)) and tomato ([Jagadeesh et al., 2011; Bravo et al., 2012](#)). The prevention of decay in strawberries following UV-C irradiation is associated with activation of defense genes including 1,3-glucanases, chitinases, peroxidases and phenylalanine ammonia lyase ([Bravo et al., 2012](#)). It was also noted that UV-C enhanced resistance to decay in tomato, and studies found enhanced activity of antioxidant enzymes, higher lycopene and polyamine in treated fruit ([Barka, 2001; Bravo et al., 2012; Charles et al., 2008; Liu et al., 2009](#)). Therefore the wavelengths of 100–280 nm appear to induce PR proteins (thau-matin-like, glucanases, chitinases) as well as other metabolites involved in plant defense (e.g., phenylpropanoids) and also metabolites which may retard senescence (e.g., polyamides) ([Tiecher et al., 2013](#)). Recently, a UV-C postharvest treatment on mangoes resulted not only in quality retention but extension of shelf-life for up to 15 days and increased antioxidants. The gel-based proteomics approach revealed 24 proteins of which 20 could be identified and classified as involved in processes related to stress response, energy and metabolism, and ripening and senescence ([George et al., 2015](#)).

1.3.5 Mechanical Stress and Wounding

Most studies on mechanical stresses have been based on observations of symptoms rather than attempting to understand the mechanisms that rule these symptoms. Not only are increased respiration and ethylene production observed, but loss of water/dehydration, loss of flavor and texture, loss of nutrients, formation of brown coloration, and increased susceptibility to pathogens ([Hodges and Toivonen, 2008](#)). The fresh-cut industry has to deal with many of the these problems due to a series of abiotic stresses on the product, starting from wounding in its different ways (e.g., slicing, dicing, chopping, trimming, peeling), low temperature,

dehydration and change in the atmosphere composition due to packaging (MAP) and respiration of the product. Wounding is not only associated with the release of volatiles but with the release of phenylpropanoids, lipoxygenase derived compounds, terpenoids, jasmonic acid, auxin, abscisic acid, and active oxygen species (Hedges and Toivonen, 2008).

Early wounding response genes involve regulatory proteins such as transcription factors (e.g., MYB/Myb-like, WRKY, AP2/ERF) which have been identified in different crop types such as persimmon, tobacco and the rubber tree, *Hevea brasiliensis*, respectively (Akagi et al., 2010; Hara et al., 2000; Chen et al., 2012). These transcription factors are involved in the modulation of late responsive genes that encode proteins that improve the capacity to recover from the stressful conditions such as HSPs, cell wall modifying enzymes, secondary metabolites and PR proteins (Cheong et al., 2002). In the pulp of ripe banana submitted to wounding, an upregulation of phenylalanine ammonia lyase (PAL) and different HSPs has been reported (Chen et al., 2009). Many of the wound-induced transcription factors (TFs) are involved in the cross-talk among the different signaling cascades for the different abiotic stresses and also act as modulators in response to the different hormones (Reymond et al., 2000).

Reported studies using postgenomics approaches in fleshy fruits and perishables in response to mechanical stress and wounding are scarce (Strehmel et al., 2010; Buron-Moles et al., 2014; Tosetti et al., 2014). At the proteome level, induction of PR proteins in apple submitted to wounding (3 mm wide and 3 mm depth) and kept at 20°C and 80%–85% RH for up to 48 h were reported (Buron-Moles et al., 2014). Recently, Tosetti et al. (2014) studied the molecular and biochemical responses of ripe tissue of two cultivars (melting vs. slow melting) submitted to wounding (8 wedges/apple containing both epicarp and mesocarp) and kept at 4°C, 90%–95% RH for up to 72 h. The differential transcriptomics analysis revealed some regulatory genes/elements involved in stress response such as: AP2/ERF and WRKY TFs as reported for plants, but revealed a specific secondary metabolic pathway (triterpenoids) in fruit. In addition, several transcripts were differentially expressed between sound and wound tissue and are proposed as candidates to target the study of the role of the different hormones and their cross-talk in the modulation of the responses to wounding. The slow melting cultivar displayed less reactivity to wounding with a higher tolerance to wounding expressed with reduced induction of genes associated with PAL and phenylpropanoid metabolism and also delayed activation of stress-related responses.

1.3.6 Ozone

Ozone use in perishables is an effective alternative to the use of traditional sanitizers (e.g., chlorine- and bromide-based products which have been questioned in terms of safety) but that can also induce dramatic changes in the physiology of the fruit. Ozone has been shown to activate an oxidative burst in plant tissue, similar to plant pathogen attack, which results in the accumulation of ROS (Rao et al., 2000). Ozone-induced ROS appears to activate distinct

signaling pathways dependent on salicylic acid, jasmonic acid and ethylene, and to induce a wide array of defense reactions including PR proteins and antimicrobial defenses (Overmyer et al., 2000; Rao et al., 2000).

Moderate ozone enrichment (0.2 $\mu\text{mol/mol}$) has been reported to induce accumulation of antifungal compounds such as resveratrol and pterostilbene in table grapes and to have a positive effect on shelf-life (Sarig et al., 1996; Tzortzakis et al., 2007). The postharvest application of ozone in perishables has been associated with delay of the onset of senescence as well as preventing decay development. Minas et al. (2012) applied an ozone treatment (0.3 $\mu\text{L/L}$) on kiwi fruit (*Actinidia deliciosa* cv. “Hayward”) stored for up to 5 months at 0°C and 95% RH in comparison to a control (without ozone application). Ozone treated fruit had blocked ethylene production, delayed senescence, and increased antioxidant and antiradical activities. The ozone treatment, in addition, decreased the stimulation of protein carbonylation during ripening as evidenced by using gel-based proteomics. Ozone treated fruits retained firmness, had similar titrable acidity (TA) content but lower soluble solid content (SSC) and lower water loss compared to the control fruit. Tzortzakis et al. (2013) found that tomatoes exposed to ozone treatment had a higher protein yield and induction of proteins involved in detoxification such as thioredoxin peroxidase (TPX). However, ripening-related proteins including ACO were reduced in ozone treated fruit, suggesting that reduced ethylene production is partly involved in the mechanism of ozone increased resistance to pathogens.

1.3.7 *Chemicals and Exogenous Treatments and Combinations*

The chemical 1-methylcyclopropane (1-MCP) is used to inhibit the action of ethylene by delaying the onset of ripening and associated problems. Commercially, 1-MCP is widely applied in apples to preserve and extend fruit quality and shelf-life. 1-MCP binds to the ethylene receptor, thus impeding the ethylene action. 1-MCP can prevent some disorders in apples but can enhance carbon dioxide injury and flesh browning (Tsantili et al., 2007; Jung and Walkins, 2011). Gapper et al. (2013) searched at the transcriptomic level and for DNA methylation changes to identify biomarkers of development of external CO₂ injury in “Empire” apples. The apples were treated with 1-MCP (1 $\mu\text{L/L}$), diphenylamine (1 g/L) or untreated and subsequently stored at 5 kPa CO₂ and 2 kPa O₂. The methylation state of the ACS1 correlated with the occurrence of this disorder and indicated that epigenetic regulation of ethylene biosynthesis was a possible mechanism leading to the development of this disorder.

Huerta-Ocampo et al. (2012) studied by using gel-based proteomics the application of 1-MCP in papaya fruit at the green-ripe maturity stage (300 ppb for 12 h at 20°C) and then stored at 20°C and allowed to ripen. 1-MCP-treated fruit showed downregulation of cell wall-degrading enzymes, enzymes related to respiration and involved in the synthesis of flavonoids (e.g., cyt b5) which explains the reported effects of 1-MCP on color and taste for 1-MCP-treated

fruits (Huerta-Ocampo et al., 2012). The application of 1-MCP (900 or 1800 nL/L for 12 h at 1°C followed by 12 days storage) in Tsai Tai (*Brassica chinensis*) leaves during cold storage was successful in inhibiting leaf etiolation and weight loss, thus retarding senescence.

Observed protein changes for 1-MCP-treated leaves involved accumulation of proteins related to photosynthesis (e.g., photosystem II, oxygen-evolving enhancer protein 2, high molecular weight Rubisco), downregulation of key TCA cycle enzymes (e.g., malate dehydrogenase) (Wang et al., 2014). In general, 1-MCP-treated leaves displayed enhancement of proteins involved in carbon assimilation, inhibition of the TCA cycle and enhancement of giberellins (GAs), and scavenging of ROS resulting in a prolonged shelf-life. A heat treatment combined with 1-MCP in peach fruit was demonstrated through a gel-based proteomics approach to increase the antioxidant and defense capabilities of the fruit, thus delaying ripening and senescence. The combination of both treatments demonstrated synergism rather than additive effects (Jiang et al., 2014). The application of 10 µL/L of 1-MCP in commercially mature peach fruit resulted in inhibition of starch degradation, glycolysis, and expression of ethylene enzymes and in the induction of defense-related enzymes such as chaperonin 60 and HSPs as demonstrated by Zhang et al. (2012) using a gel-based proteomics approach.

External application of ethylene is a common commercial practice to speed up ripening of climacteric fruit harvested at the green-mature stage and to induce color change in the nonclimacteric citrus fruit. Zheng et al. (2013) reported changes induced in apples exposed to an ethylene gas treatment of 36 µL/L for 36 h at a flow rate of 0.41–0.5 mL/s versus an air-treated sample. The external ethylene gas treatment not only induced the typical changes such as increased respiration rate, ethylene production, SSC/TA, and reduced firmness, but also enhanced proteins involved in carbon and energy metabolism, defense mechanisms (several PR Mal d 1 isoforms), amino acid metabolism (cysteine synthase, arginase), and ethylene (ACO isoforms). A unique group of proteins was expressed only in ethylene-treated fruit whose function and specific role remains to be further studied. Peach fruit dipped in 2000 µL/L ethephon for 10 min and held at room temperature for several days, displayed induction of ethylene-related enzymes, increased abscisic acid, and cell wall-related enzymes and induction of glycolytic and amino acid proteins as demonstrated using a gel-based proteomics approach by Zhang et al. (2012). Recently, Minas et al. (2016) by using a coupled physiological and 2-DE proteomics approach studied the mechanisms of action of ethylene (100 µL/L at 20°C for 24 h) and chilling induced (0°C for 10 days) kiwifruit ripening syndrome. Ten proteins were specific to the exogenous ethylene application, two to chilling, and twelve to their combination. These proteins were involved in disease/defense, energy, protein destination/storage, and cell wall structure. This study revealed a link between ethylene and chilling signaling during kiwifruit ripening. Chilling was postulated to regulate kiwifruit ripening by controlling the ABA levels.

Ma et al. (2014) carried out a comprehensive analysis at the transcriptomic and proteomic levels of citrus fruit treated with the antistalling agent 2,4-dichlorophenoxyacetic acid

(2,4-D). The changes induced in the peel of treated fruit (500 ppm 2,4-D for 2 min, stored at room temperature and 85%–90% RH for up to 2 months) included upregulation of transcription factors (AP2/ERF, WRKY, and NAC family members) which possibly amplified hormone signaling. Endogenous levels of the phytohormones ABA and SA were induced by 2,4-D, while ethylene production was inhibited. In addition, transport and hormone metabolism genes were also upregulated 24 h posttreatment. Stress defense genes and proteins were upregulated 48 h posttreatment enhancing the fruits resistance to stresses. The final response was the downregulation of cell wall metabolism genes which was linked to the reduced respiration and energy consumption observed. At all of the time points observed posttreatment, genes related with secondary metabolism (phenylpropanoid and lignin) were upregulated.

1.4 Perspectives

As more genomes are sequenced and with increasing use of transcriptomics, it is also expected that more profound studies at the proteome level are carried out. Most of the proteomics studies reported in the postharvest area are based on two-dimensional electrophoresis (classical and/or differential gel electrophoresis, DIGE) for protein separation and quantitation followed by LC-MS/MS for protein identification either by cross-species identification or by species-specific database search if available. Few postharvest studies are reported using a gel-free approach for separation and identification. Gel-free approaches are considered more powerful in terms of coverage of the proteome, time, and resources.

Proteomics and related omics studies in the postharvest arena need to be complemented by appropriate phenotyping strategies in order to be able to transform research results into a practical implementation. Careful and considered experimental design is required to comprehensively answer the questions proposed in each study. Targeted studies focused on proteins in specific pathways (e.g., the role of glutathione-ascorbate, respiration pathways) are needed to validate preliminary findings.

Certainly, as the proteome is only one of the cellular mechanisms of control, its complement with other omics platforms (transcriptomics and metabolomics) is necessary to accomplish the state of a systems biology approach. Metabolic control is exerted not only on metabolites but proteins and includes their modification (by phosphorylation, glycosylation, carbonylation) in order to exert function. Additionally, epigenetic modifications directly impacting the phenotype cannot be disregarded and studies in this area are also needed.

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Proteomics of Rice—Our Most Valuable Food Crop

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2.1 Introduction

Water and nutrient supply, salinity, and temperature fluctuations all affect agricultural production around the world. As the impacts of human civilization on our planet increase, the changes to the environment caused by our activities are becoming clear. Among these changes, climate change, whether naturally occurring or due to anthropogenic agents, has led to increased frequency of drought, flood, and extreme temperature events (Groisman et al., 2005). These changes will have profound impacts on natural and agricultural ecosystems. In addition to the global climatic changes, diseases caused by various fungal, bacterial, and viral diseases have become another major factor affecting agricultural production worldwide (Li et al., 2012a,b). With the rapid growth of world population and the changes in global climate, food security has become a high priority concern. Producing more food at affordable prices, while facing climatic and pathogenic threats, is becoming ever more essential. Confronting these challenges requires the development of various approaches to boost agricultural production under environmental threats.

An important challenge for crop breeding is to identify the genes responsible for important crop traits, especially in food crops. The complexity of crop traits are usually conferred by polygenes, which are difficult to analyze. To get over this obstacle, an alternative approach through the application of high-throughput technologies is essential. However, study of the proteins expressed in an organism is not as easy as study of the genes. The difficulty is mainly because proteins are highly dynamic and mobile, and expression levels frequently differ from individual to individual. As technologies have advanced in the food industry in recent years, proteomics has been applied to address the growing demand for food by providing fundamental molecular-level knowledge that can be used in understanding the phenotypic properties of various plants, and also as molecular markers for use in selective breeding programs.

Rice is the most important food crop we have, feeding over half of the world's population, and prices for rice have risen to record highs due to global demand in recent years. In conjunction with this, proteomic analysis has been applied to study stress-responsive proteins

which are regulated by, for example, extreme temperature, water deficit, salinity, and pathogenic infection in rice (Gammulla et al., 2011; Li et al., 2012a; Mirzaei et al., 2012a; Sarhadi et al., 2012). Understanding the molecular basis for stress responses will have great impact on breeding rice tolerant to stresses, and thus contribute significantly to future global food security.

2.2 Chemical and Physical Principles

Sample preparation is critical for the following quantitative proteomic analysis approaches, as it can eliminate the interfering compounds. As techniques in sample preparation have developed, two major approaches have been used to extract the proteins in different tissues from rice. Both of them start with mechanical disruption of the tissue by grinding in a mortar and pestle under liquid nitrogen. Proteins can then either be precipitated with trichloroacetic acid (TCA)/acetone, which removes interfering compounds, and solubilized in a buffer containing a mixture of chaotropic and reducing agents and detergent (Gammulla et al., 2010; Mirzaei et al., 2014) or, alternatively, proteins can be extracted with phenol followed by ammonium acetate/methanol precipitation (Collado-Romero et al., 2014; Wang et al., 2013).

Different plant tissues have diverse biophysical properties; hence sample preparation may need to be optimized for each type of tissue. Each tissue has its own inherent difficulties; leaf tissue has very high pigment content and contains an extremely high abundance of the storage of protein ribulose bisphosphate carboxylase (Rubisco). To remove the pigment content, leaf tissue can be washed with acetone several times (Mirzaei et al., 2012a). Rubisco is almost impossible to remove completely, but the reduction of levels of Rubisco present during sample preparation can be performed with two main methods: use of an anti-Rubisco antibody affinity column (Cellar et al., 2008) or polyethylene glycol (PEG) fractionation (Kim et al., 2001). Seeds contain high starch content and abundant storage proteins; thus to extract the proteins, seeds need to be homogenized in extraction buffer before the TCA/acetone extraction (Yang et al., 2013b). For the study of root tissues, the major difficulty is to obtain clean material from roots grown in soil. To overcome the contamination from soil, rice can be grown hydroponically or aeroponically (Neilson et al., 2013). Despite the various difficulties, the number of rice proteomics papers published in recent years is increasing. Research shows that we are gradually improving the techniques used, which is making the study of rice proteomics more accessible than ever before.

2.3 Synopsis of Analytical Techniques

A large variety of quantitative proteomic techniques have been used in rice research. These techniques have undergone a number of advances that help overcome the experimental challenges. In this section, we briefly discuss selected quantitative proteomic approaches used in rice research, with a focus on recent work.

Traditional two-dimensional electrophoresis (2-DE) techniques have been used as the major methods for comparative quantitative proteomic studies in rice, as they can provide a visual output for protein profiling and comparative mapping of expressed proteins between biological samples. Briefly, the classical 2-DE proteomic method begins with protein extraction, followed by protein separation using 2-DE. The gel is stained and visualized to identify and quantify the protein spots of interest. After protein in-gel digestion, the peptides can be analyzed by mass spectrometry (MS) (Jagadish et al., 2011; Muthurajan et al., 2011).

Alternatives to 2-DE-based methods used in rice study are usually divided into two categories: label-free quantitation and stable isotope labeling approaches. Label-free quantitation involves several techniques for identification and quantitation of differentially expressed proteins in two or more complex biological samples. It is subdivided into two different strategies: one type is known as spectral counting (SpC) (Liu et al., 2004) and the other is area under the curve (AUC) (Podwojski et al., 2010). In label-free quantitative proteomics, each sample is analyzed individually by LC-MS/MS and protein quantitation is performed on either peak intensity of the same peptide or the number of spectral counts for the same proteins (Zhu et al., 2010). In the SpC approach, peptides from the most abundant proteins will be chosen more often for further fragmentation, thus the abundance of generated MS/MS spectra in data-dependent acquisition is proportional to the protein amount, while in the AUC approach the protein abundance is estimated from the measurement of the changes in ion intensity of chromatographic peak areas for individual peptides.

Stable isotope labeling is a powerful quantitative proteomic approach which is becoming more widely used in rice studies. This approach is generally further divided into two categories: chemical labeling and metabolic labeling. In chemical labeling [ICAT, iTRAQ, TMT and dimethyl labeling, among others (Patterson et al., 2007; Zhang et al., 2015)], the introduction of isotopically distinct labels to proteins is applied after protein extraction and preparation. In metabolic labeling (for example SILAC and SILIP) (Lewandowska et al., 2013; Schaff et al., 2008), the labels are added to the growth media to be metabolized by the cell and ultimately label the whole plant. The application of stable isotope labeling of both types has been reported in a growing number of recent rice studies; for example, iTRAQ and TMT labeling have been used to study cold stress in rice (Neilson et al., 2011; Neilson et al., 2013). Given the large number of quantitative proteomic techniques available, it is not possible to list all the methods which have been used to study rice. In Table 2.1 we have included some selected examples of key studies in rice proteomics using different techniques.

2.4 Proteomic Analysis of Stress Response in Rice

2.4.1 Rice Is Important as Both a Food Crop and a Model Species

Rice is one of the most important staple foods for the world's population and it is a model plant for the monocots. Except for *Arabidopsis*, rice may be the most extensively studied

Table 2.1: Quantitative proteomic techniques and application in rice.

| Biological Study | Technique | References |
|-------------------------------|-----------------------------|------------------------|
| Seed germination | 2D | Xu et al. (2016) |
| Natural aging | 2D | Gao et al. (2016) |
| Nutritional compositions | 2D | Gayen et al. (2016) |
| Seed priming | 2D | Cheng et al. (2017) |
| Cold stress | 2D | Huo et al. (2016) |
| Aroma compound biosynthesis | 2D | Wongpia et al. (2016) |
| Iron deficiency | 2D | Chen et al. (2015) |
| Defense response to pathogens | 2D-DIGE | Chen et al. (2016) |
| Detoxification | 2D-DIGE | Fang et al. (2015) |
| Radiation stress | 2D-DIGE | Hayashi et al. (2015) |
| Heat stress | Label-free quantitation | Kim et al. (2015) |
| Cold stress | Label-free quantitation | Lee et al. (2015) |
| Drought stress | Label-free quantitation | Mirzaei et al. (2012a) |
| Drought stress | Label-free quantitation/TMT | Wu et al. (2016) |
| Pistil responses | iTRAQ | Li et al. (2016) |
| Endoplasmic reticulum stress | iTRAQ | Qian et al. (2015) |
| Defense response to pathogens | iTRAQ | Wang et al. (2015) |
| Salinity stress | iTRAQ | Xu et al. (2015) |
| Cadmium tolerance | iTRAQ | Ma et al. (2016) |

plant species, including in the field of proteomics. A large number of proteomic research studies have been performed in rice. Essentially, these studies can be divided into three major categories: nutrition component, growth and development, and stress responses.

2.4.2 Nutritional Component

Rice grain is a primary source of proteins for humans. Compared with other cereals, rice has a relatively low protein content ranging from 7% to 10% of the grain dry weight (Khush, 1997).

Thus, there is potential to raise the nutritional value of rice by increasing its protein content. In recent years, enhancing rice seed storage proteins to improve rice nutritional value has gradually become one of the critical targets for rice quality breeding. Proteomics is an ideal tool to study the nutritional content as it can be used to analyze complex mixtures of proteins. To understand the difference among different rice cultivars, Teshima et al. (2010) analyzed 10 varieties of rice and found that the protein expression differences between six Japanese rice cultivars were relatively small. However, differences in protein content between four cultivars from other countries (especially India and Thailand) and a Japanese cultivar were large. In addition, they discovered the profiles of the major rice allergens of the RAG2 family were slightly different between some cultivars. These results suggest that profiling of rice seed proteins could be useful for future crop breeding. Jiang et al. (2014) compared the seed storage proteins in three wild rice species and two cultivated rice varieties and discovered

remarkable differences in protein expression profiles. Also, the numbers of detected protein spots of the three wild rice species were significantly higher than those of the two cultivars. Their results imply that wild rice species are important genetic resources for improving nutritional quality to rice.

2.4.3 Growth and Development

Vegetative growth is a vital factor that determines the productivity of rice. As seed germination is the first committed step of growth, the vigor of the seed and seedling is of paramount importance. To uncover the mechanisms of rice development, extensive proteomic studies for establishing a global view of protein expression during six developmental stages have been conducted in recent years. Profiling and dynamic analysis of germinating seeds have shown that during germination, there were alterations in the embryo proteome in metabolism, antioxidant enzymes, protein degradation and processing, and stress or defense proteins (Kim et al., 2009). In a different study, comparative analysis of germinating rice embryo and endosperm proteomes has found different proteins expressed in embryo and endosperm (Xu et al., 2016). In order to elucidate the molecular processes that are related to leaf functions during rice growth, Zhao et al. (2005) analyzed the proteomic changes of rice during six development stages. They found that most proteins in rice leaf are constitutively expressed from the vegetative phase throughout the early stage of the ripening phase. The main purpose of rice production studies is to acquire high yields of good-quality seeds, which provide the main food resource. Recently, a number of proteomic experiments have been performed to understand the machinery behind the reproductive stage. Anthers, which are crucial for plant sexual reproduction, are one of the rice tissues that has been extensively studied. Ye et al. (2015) used proteomic and phosphoproteomic analyses to investigate the molecular mechanism modulating protein activities during anther development. Proteins related to DNA repair, transcription regulation, and signaling were all found overrepresented in the phosphorylated proteins. Taken together, proteomic studies of rice at different developmental stages have shown that the development of rice is a highly coordinated process that relies on expression of a large range of different kinds of proteins in the right place at the right time.

2.4.4 Temperature Stress in Rice

Low temperature is one of the most serious stresses for field grown plants, causing reduced plant growth, rolled and withered leaves, and overall reduction in yield. Proteomic analyses have helped to identify many proteins responsive to cold stress (Table 2.2). Neilson et al. (2011) employed both label-free and labeled shotgun proteomics to analyze rice leaf exposed to low temperature and discovered cold-responsive proteins involved in transport, photosynthesis, generation of precursor metabolites, and energy. More specifically, histones and vitamin B biosynthetic proteins were also observed to be affected by cold stress.

Table 2.2: Proteins differentially expressed in response to cold stress.

| Name | Tissue | References |
|--|--|---|
| Upregulated Protein | | |
| Aconitate hydratase | Leaf, root | Cui et al. (2005) and Lee et al. (2009) |
| Adenylate kinase A | Anther, root, leaf | Hashimoto and Komatsu (2007), Imin et al. (2006), and Yan et al. (2006) |
| Ascorbate peroxidase | Anther, leaf | Imin et al. (2004), Lee et al. (2007a), and Yan et al. (2006) |
| Calreticulin | Basal leaf sheath, root | Komatsu et al. (2009) and Lee et al. (2009) |
| Cysteine proteinase | Root, leaf | Hashimoto and Komatsu (2007) and Lee et al. (2007a) |
| Cysteine proteinase inhibitor | Anther, leaf | Imin et al. (2006) and Yan et al. (2006) |
| Enolase | Leaf, basal leaf sheath | Komatsu et al. (2009) and Yan et al. (2006) |
| Nucleoside diphosphate kinase 1 | Anther, leaf, leaf sheath | Hashimoto and Komatsu (2007), Imin et al. (2004), and Lee et al. (2007a) |
| Oxalyl-CoA decarboxylase | Leaf, root | Cui et al. (2005), Lee et al. (2009), and Yan et al. (2006) |
| Oxygen-evolving complex protein (photosystem II) | Leaf | Cui et al. (2005) and Yan et al. (2006) |
| Phosphogluconate dehydrogenase | Leaf, root | Cui et al. (2005) and Lee et al. (2009) |
| Rubisco (large and small chains, precursor) | Leaf | Hashimoto and Komatsu (2007) and Yan et al. (2006) |
| UDP-glucose pyrophosphorylase | Anther, root, leaf | Cui et al. (2005) and Imin et al. (2004) |
| Downregulated Protein | | |
| Ascorbate peroxidase | Anther, leaf | Imin et al. (2004) and Yan et al. (2006) |
| Calreticulin (precursor) | Anther, basal leaf sheath, leaf sheath | Hashimoto and Komatsu (2007), Imin et al. (2006), and Komatsu et al. (2009) |
| Elongation factor (G, 1-b0) | Leaf | Hashimoto and Komatsu (2007) and Yan et al. (2006) |
| Rubisco subunit binding-protein a subunit | Leaf sheath, basal leaf sheath | Hashimoto and Komatsu (2007) and Komatsu et al. (2009) |
| UDP-glucose pyrophosphorylase | Leaf sheath, basal leaf sheath | Hashimoto and Komatsu (2007) and Komatsu et al. (2009) |

Currently, the overall global temperature is steadily increasing compared to the preceding decades due to a rapid increase in atmospheric greenhouse gas concentration. In order to overcome the influence of global warming, scientists have been using proteomics to discover heat-responsive proteins in rice (Table 2.3). Lee et al. (2007b) identified 48 proteins, among

Table 2.3: Proteins differentially expressed in response to heat stress.

| Name | Tissue | References |
|-------------------------------|-------------------|---|
| Upregulated Proteins | | |
| ATP synthase | Leaf, anther | Kim et al. (2015) and Lee et al. (2007b) |
| Ascorbate peroxidase | Grain, leaf | Han et al. (2009), Liao et al. (2014), and Timabud et al. (2016) |
| Chaperone | Anther, leaf | Kim et al. (2015) and Lee et al. (2007b) |
| Inorganic pyrophosphatase | Anther, spikelets | Jagadish et al. (2011) and Jagadish et al. (2009) |
| UDP-glucose pyrophosphorylase | Leaf, grain | Lee et al. (2007b) and Liao et al. (2014) |
| Small heat shock proteins | Leaf, grain | Lee et al. (2007b) and Timabud et al. (2016) |
| Downregulated Proteins | | |
| ADP-glucose pyrophosphorylase | Grain | Liao et al. (2014), Mitsui et al. (2013), and Timabud et al. (2016) |
| Granule-bound starch synthase | Grain, caryopsis | Liao et al. (2014) and Timabud et al. (2016) |

which were proteins related to heat shock proteins, energy and metabolism, redox homeostasis, and regulatory proteins. In addition, a group of low molecular small heat shock proteins were found to be newly induced by heat stress. Jagadish et al. (2009) used three contrasting rice varieties to evaluate the effect of high temperature during anthesis on the rice spikelet. They found that the greater heat tolerance of rice cultivar N22 could be due to the accumulation of stress-responsive cold and heat shock proteins in the anthers. As more proteomic studies of rice exposed to cold or heat stress are published, it is becoming clear that low temperature and high temperature stresses cause distinct molecular response in rice tissues. In general, low temperature stresses cause changes in chloroplast components, energy production, and secondary metabolism, while high temperature stresses commonly result in increased abundance of heat shock proteins.

2.4.5 Drought Stress in Rice

Among all the abiotic stresses, drought is the most extensively studied stress that affects rice development, due to the fact that large amounts of water supply are required for the growth of rice. A number of studies have been performed to investigate the response of the rice proteome to water stress at the cellular and molecular levels (Table 2.4). Mirzaei et al. (2012b) studied the long-distance drought signaling in rice roots using an ingenious experimental design employing split-rooted plants. By comparing proteins in well-watered root tissue and adjacent droughted roots, 126 proteins were downregulated and 90 proteins were upregulated.

Table 2.4: Proteins differentially expressed in response to drought stress.

| Name | Tissue | References |
|-----------------------------------|-----------------|--|
| Upregulated Protein | | |
| Aquaporins | Leaf | Dong et al. (2014) and Mirzaei et al. (2012a) |
| Ascorbate peroxidase | Anther, leaf | Liu and Bennett (2011) and Rabello et al. (2014) |
| Catalase isoenzyme | Leaf | Mirzaei et al. (2012b) and Mirzaei et al. (2014) |
| ClpD1 protease | Leaf | Mirzaei et al. (2014) and Wu et al. (2016) |
| Small G proteins | Root, leaf | Mirzaei et al. (2012a,b) |
| Small heat shock proteins | Root, spikelets | Dong et al. (2014) and Mirzaei et al. (2012b) |
| Superoxide dismutase | Flag leaf, root | Dong et al. (2014), Ji et al. (2012), and Mirzaei et al. (2012b) |
| Downregulated Protein | | |
| Chitinase | Root, spikelets | Dong et al. (2014) and Paul et al. (2015) |
| Peroxidase | Leaf, anther | Liu and Bennett (2011) and Paul et al. (2015) |
| Ribulose bisphosphate carboxylase | Leaf, flag leaf | Ali and Komatsu (2006), Ji et al. (2012), and Mirzaei et al. (2012a) |

Surprisingly, there was a large change in the proportion of total gene expression between the well-watered roots with and without a vascular connection to droughted roots. This leads to the conclusion that water supply can alter gene expression remotely. Besides root growth, water deficit also led to reduction in leaf area, reduced net photosynthetic assimilation and stomatal closure in leaves (Chaves et al., 2003; Farquhar and Sharkey, 1982; Ghannoum et al., 2003; Xu and Zhou, 2005). Salekdeh et al. (2002) compared two contrasting rice genotypes and found that 42 spots out of more than 1000 protein spots visualized showed a significant change in abundance under stress, with 27 of them exhibiting a different response pattern in the two cultivars. Mass spectrometry enabled the identification of 16 drought-responsive proteins, including an actin depolymerizing factor, which was one of three proteins detectable under stress in both cultivars, but undetectable in well-watered plants or in plants 10 days after rewatering. In a different study, Liu and Bennett (2011) examined the effect of drought on the anther proteome of two rice genotypes. They found that there were 13 newly drought-induced proteins in cultivar Moroberekan, 10 of which were reversible on re-watering, while in cultivar IR64 there were 6 drought-induced protein spots that were not reversible on rewatering. A range of drought conditions have been applied to different cultivars of rice, and proteomics has assisted researchers in identifying the main families of drought-responsive proteins, which include ABA-responsive proteins, heat shock proteins, detoxification and defense proteins, transport proteins, and many other proteins involved in various metabolisms.

These findings will not only be useful in identifying regulated pathways and developing biomarkers for drought stress but will also lead to yield enhancement in agricultural production.

2.4.6 Salinity Stress in Rice

High salinity causes growth inhibition in plants that do not tolerate high salt, including rice (Li et al., 2014). Yan et al. (2005) exposed rice roots to salinity stress to examine in detail the rice root response to such conditions. MS analysis and database searching were used for the identification of 10 different stress-responsive proteins, which included UDP-glucose pyrophosphorylase, cytochrome *c* oxidase subunit 6b-1, glutamine synthetase root isozyme, putative nascent polypeptide-associated complex alpha chain, putative splicing factor-like protein, and putative actin-binding protein. In another study, Abbasi and Komatsu (2004) exposed rice seedlings to salinity stress and identified five proteins responsive to 50 mM NaCl for 24 h, which included fructose bisphosphate aldolases, photosystem II oxygen-evolving complex protein, oxygen-evolving enhancer protein 2, and superoxide dismutase (SOD). The elevated expression of SOD was maintained throughout the different treatments while the increased expression of most proteins seen in the 50 mM NaCl treatment group was less pronounced in the groups receiving 100 or 150 mM NaCl for 24 h. Dooki et al. (2006) employed a proteomic approach to understand the mechanism of rice responses to salinity at the early reproductive stage. They discovered that the expression pattern of 13 proteins significantly changed in response to stress. These studies suggest that salt stress has a negative effect on rice photosynthesis, which leads to increased photorespiration and reduced plant yield. However, rice can survive under salt stress by increasing the expression of proteins involved in defense, detoxification, and osmotic adjustment (Table 2.5). Salt tolerance is a multigenic trait, which involves a complex of responses at the metabolic, cellular, molecular, physiological, and whole-plant levels. As such, one of the main focuses of the future work will be to integrate the large-scale data sets and undertake the systems biology-based approaches needed for the in-depth analysis of protein networks.

2.4.7 Biotic Stress in Rice

In addition to abiotic stress, global climate change has also led to more frequent disease breakouts in rice. One of the most common diseases is rice blast disease, which is caused by the fungus, *Magnaporthe grisea*. Recently, proteomics have been applied in several studies with the aim of understanding the interaction between rice and *M. grisea*. Kim et al. (2004) used 2-DE to identify proteins from rice that are differentially expressed in response to *M. grisea*. They discovered eight proteins that were induced by the fungus which included two receptor-like protein kinases, two β -1,3-glucanases, thaumatin-like protein, peroxidase, probenazole-inducible protein and rice pathogenesis-related protein 10. Proteomics approaches have also been employed to study other rice diseases, such as black-streaked

Table 2.5: Proteins differentially expressed in response to salinity stress.

| Name | Tissue | References |
|---|------------------------|--|
| Upregulated Protein | | |
| Cysteine synthase | Leaf, root | Ghaffari et al. (2014) and Liu et al. (2014) |
| Fructokinase-2 | Anther, root | Liu et al. (2014) and Sarhadi et al. (2012) |
| Heat shock proteins | Cell, anther, leaf | Ghaffari et al. (2014), Liu et al. (2013), and Sarhadi et al. (2012) |
| Peroxidase protein family | Shoot stem, root, leaf | Liu et al. (2014) and Song et al. (2011) |
| Downregulated Protein | | |
| 2,3-Bisphosphoglycerate-independent phosphoglycerate mutase | Leaf, root | Ghaffari et al. (2014) and Liu et al. (2014) |
| ATP synthase | Leaf, root | Ghaffari et al. (2014), Liu et al. (2014), and Xu et al. (2015) |
| Elongation factor 2 | Seed, root | Liu et al. (2014) and Nakamura et al. (2014) |
| Glyceraldehyde-3-phosphate dehydrogenase | Cell, anther | Liu et al. (2013) and Sarhadi et al. (2012) |
| Phosphoglycerate kinase | Leaf, root | Liu et al. (2014) and Liu et al. (2013) |

dwarf virus (RBSDV) infection (Xu et al., 2013) and rice stripe disease (Yang et al., 2013a). Compared to the abiotic stress studies of rice, few published reports are available on investigation of rice infected with pathogens. However, proteomic studies in the published literature have shown that rice elicits various molecular events, such as strengthening of the cell wall, evoking antimicrobial activity through expression of pathogenesis-related proteins and production of a large variety of hydrolytic enzymes, for survival. A list of published literature related to diseases of rice is included in Table 2.6.

2.4.8 Highlights From Selected Recent Studies in Rice Proteomics

The nutritional component from various cultivars of rice was analyzed by Teshima et al. (2010). They found that the protein expression profiles of six Japanese rice cultivars are similar to each other. In contrast, the proteins contained in four cultivars from other countries (especially India and Thailand) were quite different from the Japanese cultivars. They identified some allergenic proteins of rice, whose sequences were very similar to those of the RAG2 family. The profiles of RAG2 family-allergenic proteins are also known to vary among these rice cultivars.

An interesting study by Neilson et al. (2013) discovered that chilled roots in rice seedlings resulted in decreased shoot growth. Proteins responded to transmitted chilling-induced signals, for example, microtubule-associated proteins were found upregulated in shoots. The

Table 2.6: Application of quantitative proteomic techniques in rice diseases.

| Pathogen | References |
|--|---|
| <i>Magnaporthe grisea</i> | Kim et al. (2004) and Li et al. (2015) |
| <i>Magnaporthe oryzae</i> | Jung et al. (2012), Kim et al. (2013), and Wang et al. (2011) |
| <i>Xanthomonas oryzae</i> pv. <i>oryzae</i> | Chen et al. (2016), González et al. (2012), and Hou et al. (2012) |
| Rice stripe virus | Wang et al. (2015) and Yang et al. (2013a) |
| Southern rice black-streaked dwarf virus/rice ragged stunt virus | Kim et al. (2014) and Xu et al. (2013) |
| <i>Cochliobolus miyabeanus</i> | Kim et al. (2014) |
| <i>Rhizoctonia solani</i> | Anderson et al. (2016) and Lee et al. (2006) |

induction of the sucrose signaling pathway was supported by the identification of sucrose synthase in leaf and a protein with 98% identity compared to a vacuolar invertase in the shoot growing zone. The substrates and products of these enzymes are both nutrients and signaling molecules in plants. Metabolism and carbohydrate demand were reduced by root chilling which in turn presents the long-distance signaling for stress response and altered shoot development.

Recently, Wu et al. (2016) identified that the difference between two phenotypically distinct rice cultivars could be observed in the level of photosynthetic machinery and the ClpD1 protease under drought stress. The ClpD1 protease, a protein known from numerous previous studies to be strongly linked to stress tolerance (Mishra et al., 2016; Rosano et al., 2011; Singh et al., 2010), was found to be only expressed in the drought-tolerant cultivar. The increased abundance of ClpD1 protease is correlated with increased drought tolerance in rice as it was greatly increased in abundance in response to severe drought stress. This protein represents a potentially valuable biomarker for use in the development of more drought-tolerant rice varieties.

A rice cyclophilin, OsCYP2, was identified by Ruan et al. (2011) when comparing different varieties under salt stress. This protein functions as a key regulator that controls ROS levels by modulating activities of antioxidant enzymes at the translation level. Besides salt stress, this protein was also induced by changes in circadian rhythm. In addition, it also plays a role in signal pathways of other types of stresses such as heat, cold, or ABA. OsCYP2 may function as a key integrator in response to multiple stresses.

Two pathogenesis-related proteins, including allene oxide synthase, were identified by Li et al. (2012a) when studying rice infected with *Xanthomonas campestris* pv. *oryzicola*. This protein is involved in the biosynthesis of jasmonic acid, which is a signaling molecule in plant defense. The other pathogenesis-related protein is an ascorbate peroxidase that detoxifies peroxides and plays an important role in controlling the H₂O₂ concentration during intracellular signaling under pathogen attack.

2.5 Conclusions and Future Outlook

Temperature stress, including cold and heat stress, has been extensively studied in rice. Changes in expression of proteins can be found either under cold or heat stress. Low temperature and high temperature stresses result in distinct molecular response in rice tissues. Low temperature stresses cause changes in chloroplast components, energy production, and secondary metabolism. However, high temperature stresses commonly result in increased abundance of heat shock proteins. Drought stress in rice has been comprehensively examined due to the fact that a huge amount of water supply is needed for the growth of rice. A number of proteomic studies have been performed to understand the mechanism of drought response in different rice cultivars and rice tissues. Despite all the different stress conditions that have been applied to different cultivars of rice, the main families of drought-responsive proteins have been identified using proteomics. High salinity causes growth inhibition in rice and proteomic analysis has helped to identified salinity stress-responsive proteins in different rice tissues. The major protein responses to salinity stress are related to defense, detoxification, and osmotic adjustment. These proteins could be considered essential targets for engineering salt tolerance into rice. As salt tolerance is a multigenic trait, in-depth analysis of protein networks is required for the future development of highly salt-tolerant rice.

Proteomic analysis approaches have also been employed to study the interaction between rice and pathogens. The pathogens that have been investigated include *M. grisea*, *Magnaporthe oryzae*, *Xanthomonas oryzae*, and some other common diseases found in rice. A limited numbers of reports are available on investigation of rice infected with pathogens, but proteomic studies in the published literature have shown that rice can rearrange various molecular events to help ensure survival.

The nutritional component from various cultivars of rice is different and has been investigated using proteomics. The protein expression differences between commercial rice cultivars were relatively small. However, the differences between cultivated rice and wild rice are relatively large. Hence, wild rice species are important genetic resources for improving nutritional quality of rice. Moreover, the growth and development of rice is a complex process. It requires the coordination of protein expression at different stages. The proteins expressed in different tissues are distinct from each other. Some proteins are only present for a short period during the life cycle of rice, while the expression of some other proteins is maintained at a higher level for a relatively long period.

There has been a notable increase in the number of studies of rice proteomics appearing in the scientific literature in recent years. As more studies are published, more information is accumulated regarding the molecular mechanisms of how rice plants respond to the imposition of external stresses. Understanding how this works is an essential first step in trying to improve how it works. The findings of these studies will be valuable in future selective breeding programs aimed at enhancing yield and increasing sustainability of rice production.

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Proteomics as a Tool to Understand Maize Biology and to Improve Maize Crop

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3.1 Introduction

With the world's population rapidly growing, safeguarding food security is becoming critical. Development of crops with enhanced agricultural traits will continue to be an even more important objective. Maize (*Zea mays* L.) is the most grown agricultural commodity. According to the US Department of Agriculture, corn is being produced on 178.64 million acres worldwide, providing a yield of 1011 million tons, as projected in the World Agricultural Supply and Demand Report of May 10, 2016. Due to its nutritive value and versatility of usage, maize and maize-derived foods have become important ingredients in both human and animal diets, making maize one of the most studied biological systems. Proteomics has greatly contributed to maize research, significantly advancing our knowledge about many aspects of maize biology. Using advanced proteomic tools, mass spectrometry instrumentation, and maize genomic data, scientists were able to make significant progress in unraveling the maize proteomes of particular tissues or stages and under various conditions. Large-scale, high-throughput state-of-the-art approaches enable identification of thousands of proteins that might ultimately lead to the development of better maize varieties, either by traditional breeding or genetic engineering. Those would include varieties of higher yield, more nutritive value, increased tolerance to environmental stresses, and higher resistance to pathogens and pests. In addition, the body of knowledge gained from proteome-based maize research can also be applied to other cereal crops.

In this chapter, we summarize findings of numerous research groups that employed both gel-based and gel-free proteomic approaches to elucidate the maize proteome and its changes attributed to different developmental stages, diverse environmental challenges, nutrient imbalance, heterosis, safety of GM varieties, aging of the maize seeds, and maize potential allergenic properties. The typical workflow of maize proteomic research is depicted in [Fig. 3.1](#).

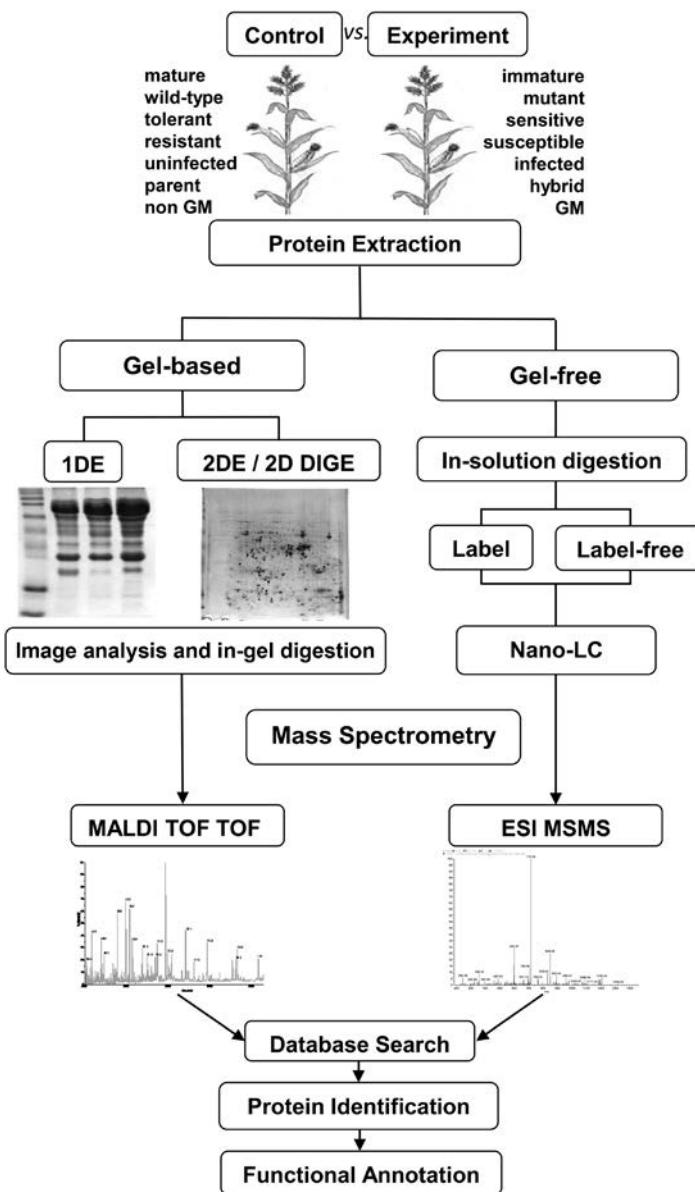


Figure 3.1
Typical experimental flow utilized in maize proteomic research.

3.2 Proteomics of Maize Development

Numerous proteomic investigations were devoted to dissect proteome changes in diverse maize organs, tissues, and cell types that are behind the physiological changes during maize growth and development.

3.2.1 Leaf and Chloroplast Development

Perhaps the most comprehensive studies were performed to elucidate partitioning of chloroplast photosynthetic activities between morphologically and biochemically distinct mesophyll (M) and bundle sheath (BS) cells. Multiple proteomic approaches exposed a wide variety of differential M/BS distributions. For instance, enzymes of the Calvin cycle, detoxification, and lipid metabolism were preferentially found in the M stroma, while starch synthesis enzymes were more abundant in BS stroma (Majeran et al., 2005). In chloroplast membranes, differential expression revealed strong underrepresentation of PSII complexes, subsequent reduction in electron transport in the BS thylakoids, and increased protein import and synthesis of thylakoid proteins in M chloroplasts (Majeran et al., 2008). Structural and metabolic transitions during C4 leaf development were described in a truly impressive system analysis approach (Majeran et al., 2010). The authors exploited a developmental continuum along the maize leaves that spans from undifferentiated cells at the leaf base to the highly specialized M and BS cells at the tip. They established a leaf and BS five-phase developmental gradient via 4430 identified and functionally annotated proteins. The most robust transitions along the leaf gradient were observed for extraplastidic protein synthesis and homeostasis, followed by signaling/regulation, thylakoid electron transport chain, and carbon metabolism. A survey of M and BS chloroplasts uncovered robust qualitative and quantitative differences in protein expressions, metabolic pathways, and homeostasis machineries (Friso et al., 2010). In-depth proteome analysis was also carried out to elucidate functions of chloroplast nucleoids by comparing nucleoids from immature (proplastids) and mature maize chloroplasts (Majeran et al., 2012). The core nucleoid proteome contained proteins from DNA replication, quality control, organization, repair, and transcription. Strikingly, proteins involved in mRNA processing and ribosome assembly were highly overexpressed in the nucleoid. RNA metabolism was more characteristic for proplastid nucleoids proteome, while photosynthetic and transport proteins largely contributed to the nucleoid proteome from fully photosynthetic mature chloroplasts. To unfold the light regulation in C4 plants, molecular processes during the greening of etiolated maize seedlings were studied by label-free quantitative proteomics (Shen et al., 2009). As expected, a large repertoire of chloroplast proteins, including thylakoid and stroma, dramatically changed in expression upon light exposure. Rapidly responding were enzymes from photosynthesis and carbon assimilation, such as subunits of PSI, PSII, ATP synthase, and Rubisco. Transition from dark to light was further accompanied by rapid accumulation of chlorophyll synthesis proteins as pigment concentration is critical during the transformation of etioplasts to fully matured chloroplasts.

The studies earlier provided a new insight into the chloroplast's spatial and temporal specialization of C4 photosynthetic metabolism through identification of chloroplast proteins using the most advanced mass spectrometry tools.

3.2.2 Grain Tissue Development

Endosperm constitutes a major portion of maize kernel. Its high starch content supplies nutrition to germinating seeds. Endosperm development is a complex trait where early stages are dedicated to cellularization, cell division, and cell wall deposition (Mechin et al., 2007). They are accompanied by robust accumulation of actins, tubulins, and proteins related to cell division, detoxification, respiration, and energy production. Peak expression of proteases at the mid-late accumulation phase suggested an increased protein turnover and an onset of the switch point from growth to storage. Anoxic conditions during starch accumulation were characterized by increased abundances of the glycolytic enzymes. Finally, starch–protein balance seems to be obtained by the specific late-stage accumulation of the pyruvate orthophosphate dikinase.

Optimal starch and protein deposition to the maize grains is an agronomic trait of immense importance, thus kernel filling is being extensively studied. For example, all the core starch synthesis enzymes exhibited low or undetectable activity levels during the lag phase at 14 days postpollination (Prioul et al., 2008), while they progressively increased to a maximum at 30 days postpollination, in parallel with starch and amylose accumulation.

Seed germination is critical for the establishment of future plant and ultimately a crop yield. Signaling processes behind the activation of metabolic activities upon seed imbibition were studied in germinating embryos (Lu et al., 2008). Thirty-nine kinases, 16 phosphatases, and 33 unique phosphoproteins containing 36 *in vivo* phosphorylation sites were identified via shotgun phosphoproteomic approaches. This universal posttranslational modification (PTM) affects nearly all aspects of cell biology, and Lu's study confirmed that it also plays a role in maize seed germination. Phosphorylated proteins were involved in DNA repair, transcription, RNA processing, protein translation, and protein folding.

3.2.3 Pollen Tube Development

Pollen germination and tube growth are processes fundamental for reproduction of flowering plants. Germinating and mature maize pollens were compared via gel-based proteomic tools (Zhu et al., 2007). Proteins present at higher amounts in mature pollen were mainly those associated with tube wall modification, actin cytoskeleton organization, energy metabolism, signaling, protein folding, and degradation. Specifically, pectin methylesterase, inorganic pyrophosphatase, and glucose-1-phosphate uridylyltransferase were profoundly upregulated, demonstrating their role in pollen tube growth. Proteins present in declining amounts included detoxifying enzymes as well as a few pollen allergens and metabolic enzymes.

3.2.4 Wild-Type Versus Mutant Developmental Proteomics

Induced or natural mutagenesis is a tremendously useful and vastly used tool that allows researchers to study genes affecting particular traits (pleiotropy). Comparative protein profiles of mutants and their corresponding wild-type genotypes helped researchers to measure the impact of pleiotropic genes on maize phenotype, which can be used for development of better maize varieties.

Mutants were employed to study development of the maize root system, which is composed of several root types. Primary and seminal roots are formed during embryogenesis, while shoot-borne roots are formed in postembryonic phase, as are the lateral roots, which are initiated from pericycle cells of other types of roots (Hochholdinger et al., 2008). Lateral roots span throughout a large underground area allowing the plant to extract nutrients from the soil. Proteome of *lrl1* mutant, that does not initiate lateral roots, differed from its wild-type genotype in significantly higher expression (up 16.4-fold) of several lignin metabolism-related proteins, such as caffeoyl-CoA-3-O-methyltransferase, beta-glucosidase, L-ascorbate-peroxidase, and 1,4-benzoquinone reductase (Hochholdinger et al., 2004). An analogous pattern was observed in a *rum1* mutant (Liu et al., 2006), which is defective in both seminal and lateral root initiation. Lignin metabolism, stress response, and citrate cycle were the predominantly altered metabolic pathways in the primary roots of the *rum1* mutant at the developmental stage prior to lateral root initiation. As in the *lrl1* mutant, 1,4-benzoquinone reductase was also preferentially expressed in the *rum1* mutant. In addition, the enzyme catalyzing the final biosynthesis step of benzoxazinoid defensive compounds was absent in *rum1*, indicating that these compounds are made before lateral root initiation. These data indicate that lignin metabolism might be important before and after lateral root emergence. Tissue-specific protein accumulation was also observed in *rum1* embryo, cortical parenchyma, and stele when compared to a wild-type (Saleem et al., 2009). Particularly distinct was the expression of 11 embryo globulin spots, probably due to the qualitative differences of a Hageman factor inhibitor that regulates their degradation. This inhibitor was expressed only in wild-type embryos. Differential expression of carbohydrate metabolism and defense proteins in cortical parenchyma and stele respectively, is very likely linked to the higher meristematic activity of wild-type embryos to support and protect an initiation of lateral roots.

The pleiotropic effect of the *RTCS* gene on shoot-borne root initiation in maize coleoptilar nodes was studied in the *rtcs* mutant and impaired the initiation of seminal and shoot-borne roots (Sauer et al., 2006). Actin, profilin, calmodulin, and histone H2B.2 were predominantly expressed in wild-type coleoptilar nodes that are, unlike *rtcs*, mitotically active and display enhanced meristematic activity during crown root development. On the contrary, G-protein, auxin-binding protein, ethylene-inducible protein, sulfate adenyllyltransferase 4, and protein disulfide isomerase were enriched in *rtcs* coleoptilar

nodes. Similarly, immature wild-type embryos preferentially amassed energy-producing proteins of carbohydrate metabolism, also likely due to high embryonal meristematic activity (Muthreich et al., 2010).

Maize mutants affected in starch biosynthesis assisted researchers in gaining a greater understanding of the starch granule-associated proteome (Grimaud et al., 2008). Such proteins can be located within starch granules or on their surfaces. The study confirmed previous findings that granule-bound starch synthase GBSS, starch synthases SSI and SSIIa, and branching enzyme BEIIb are internal granule-associated proteins. More importantly, four additional proteins (SSIII, BEIIa, BEI, and starch synthase) that were not previously known to be located within starch granules were now also determined to be internally granule associated. In addition, GBSS, BEIIb, and starch phosphorylase were phosphorylated, indicating that phosphorylation seems to be regulating maize starch biosynthesis.

ABA-deficient mutant *vp5* is unable to synthesize ABA, a major plant hormone that regulates a number of physiological processes during the plant life cycle. *vp5* was used to dissect proteome alterations due to the absence of ABA as well as to identify ABA-dependent proteins during maize seed maturation (Wu et al., 2014). The most notable differences were observed for LEA proteins and small heat shock proteins (HSPs) in mutant embryos. ABA deficiency greatly inhibited the accumulation of six LEA proteins, while it promoted the accumulation of nine sHSPs, indicating their dissimilarity on ABA dependency.

3.3 Stress-Associated Maize Proteomics

3.3.1 Maize Response to Abiotic Stress

Maize often faces diverse abiotic challenges that negatively affect its growth, depending on the severity and duration of stress. With global climate changes, maize losses due to environmental stresses, mostly heat and drought, are expected to increase. Understanding the adaptation processes leading to the development of abiotic stress-tolerant maize varieties is, therefore, of fundamental importance.

3.3.1.1 Temperature Stress

Extreme temperatures are one of the most frequent abiotic stresses adversely affecting the maize proteome. For example, cold pretreatment of maize anthers and induced androgenesis resulted in increased expression and activities of detoxifying enzymes ascorbate peroxidase (APX) and superoxide dismutase (SOD) (Uvackova et al., 2012), implying their role in controlling oxidative stress during androgenesis induction. In another study, thylakoid membranes from chloroplasts of maize grown in different temperatures (13°C and 24°C) and light (high and low) exhibited substantial differences in accumulation of both the major light harvesting complex of photosystem II (LHCII) and minor antenna chlorophyll proteins CP29, CP26, and CP24 (Caffarri et al., 2005). For example, at low temperature and high light

intensity, content of LHCII proteins strongly increased, while CP29, CP26, and CP24 were less affected in accumulation. These data clearly show that temperature and light have an impact on photosystem II and photoprotection.

One of the necessary requirements under high temperature conditions is accumulation of HSPs, chaperones that maintain proper folding and function of proteins in plants enduring heat stress. Three heat shock proteins, HSP17.4, sHSP17.2, and sHSP26, were induced in maize leaves subjected to 42°C or under conditions of heat and drought, while almost undetectable under drought alone (Hu et al., 2010), confirming their pivotal role in plant adaptation to heat stress.

3.3.1.2 Drought Stress

Drought is one of the major causes of maize yield reductions. To understand root-to-shoot communication under drought, maize xylem sap was investigated 7, 10, and 12 days after water withholding (Alvarez et al., 2008). Variations were detected in hormonal, metabolite, and protein abundances. For instance, levels of ABA and cytokinins were adjusted, very likely to regulate stomatal aperture. Changes in the levels of metabolites (coumaric, caffeic and ferulic acids) and proteins (peroxidase, endotransglycosylase, pectin methylesterase, and xyloglucan) indicated reduction of lignin biosynthesis of the root xylem vessels and increase of cell wall stiffening as root and leaf growth decelerate during drought. Also differentially expressed were several defense proteins. While chitinases and glucanases decreased under water stress, many isoforms of thaumatin were significantly elevated.

During development, maize seeds acquire desiccation tolerance, which they subsequently lose once they start to germinate to a new plant. Developing embryos (immature and mature) and mature embryo after imbibition (germinating) were, therefore, investigated for desiccation tolerance (Huang et al., 2012), with 11 proteinaceous key players identified. Globulin 2, 17.4 kDa Class I HSP, LEA, putative cystatin, NBS-LRR resistance-like, stress-responsive protein, and a few others accumulated during embryo maturation and desiccation, but decreased during germination. Low-molecular-weight HSP precursor and Rhd6-like 2 exhibited the opposite pattern.

An important mechanism of plant adaptation to water deficit is adjustment of their root growth. Drought conditions induced alterations in soluble and ionically bound cell wall proteins of the primary root elongation zone (Zhu et al., 2007). Increased levels of apoplastic ROS as well as altered expressions of SOD, oxalate oxidase, peroxidases, alpha-L-arabinofuranosidase, alpha-1,4-glucan-protein synthase, beta-D-glucosidase, xyloglucan endotransglycosylases/hydrolases, xylosidases, etc. indicate that ROS metabolism and cell wall remodeling enhance cell wall loosening, thus regulating root cell elongation during the water-limited conditions. ROS scavenging enzymes were also found among differentially responsive proteins to drought and ABA treatment in a study that exploited roots of ABA-deficient mutant *vp5* and its counterpart wild-type genotype (Hu et al., 2011). The same

mutant was used to elucidate the effect of ABA on the maize leaf proteome enduring abiotic stress conditions (Hu et al., 2012). Seedlings of *vp5* and wild-type plants subjected to drought and different light conditions (normal and dim) responded with altered expression of proteins from ATP synthesis, protein and chlorophyll synthesis, CO₂ fixation, gluconeogenesis, antioxidant defense, and signal transduction. The majority of these proteins were predicted to be localized in the chloroplast, implying ABA's strong effect on the drought and light regulation of chloroplast proteins.

Water-deficit causes diverse changes in maize physiology, one of which is stomata closure to limit transpiration. When moderate drought conditions (6 days) were imposed on young maize plants of contrasting tolerances to drought, premature stomatal closure in sensitive genotype was very likely the cause of the photosynthesis suppression, which subsequently led to unproductive detoxification and defense (Benesova et al., 2012). Diverse protective proteins (chaperones, HSPs, dehydrins, etc.) responded the strongest to the drought, but to a much lesser degree in the drought-susceptible genotype.

3.3.1.3 Salinity

Excessive salt significantly reduces plant growth, especially in salt-sensitive crops such as maize (Munns, 2002). Regulation of apoplastic proteins responsible for cell wall loosening and growth is, therefore, critical under saline conditions. Expansins, for example, were reduced after 8-day salt stress in the salt-sensitive genotype Pioneer 3906 when compared to salt-resistant hybrid SR03, in which they exhibited a slight increase (Pitann et al., 2009). This was very likely due to salt-induced increase in apoplastic pH that inhibited expansin activities in susceptible hybrid, while it was preserved in resistant hybrid.

Moderate short-term salt stress conditions (up to 4 h) caused rapid and strong accumulation of sodium ions in the chloroplast of salt-resistant maize genotype (Zorb et al., 2009), which triggered a robust increase of 12 chloroplast proteins. Specifically three proteins, ferredoxin NADPH reductase, 23 kDa polypeptide of the photosystem II, and FtsH-like protein were overexpressed to lessen the detrimental effects of salt on the photosynthetic apparatus. On the other hand, calcium-sensing receptor decreased in abundance. Calcium is a critical secondary messenger in plants during normal and stress conditions, salinity included (Tuteja and Mahajan, 2007). As reported for other species (Wang et al., 2014), the potential function of this regulator of extracellular calcium-induced stomatal closure might also be associated with salinity stress signaling in maize. Calcium-mediated signaling was also postulated to occur in maize roots subjected to mild 1-h-long salinity (Zorb et al., 2010). Calmodulin, a central transducer of calcium signaling, was heavily dephosphorylated. Salt-inducible changes to the phosphoproteome included phosphorylation of fructokinase, UDP-glucosyltransferase BX9 and 2-Cys-peroxyredoxine and dephosphorylation of isocitrate-dehydrogenase, maturase, and 40-S-ribosomal protein, implying that this central PTM plays an integral role in maize adaptation to saline conditions. In another study (Cui et al., 2015), the roots of salt-tolerant

genotype F63 reacted to salt treatment by enhancing proteins from signal processing, water conservation, protein synthesis, and biotic cross-tolerance, implying their roles as main contributors to stress tolerance.

Finally, a protective role of nitric oxide (NO) against salinity stress was defined in maize seedlings (Bai et al., 2011). This important signaling and antioxidant molecule induced salt tolerance via multiple mechanisms that were mediated by G-protein signal transduction. NO-induced salinity tolerance was characterized by an increase in antioxidant enzyme (e.g., SOD, APX, GR) activity, acceleration of the cell division rate, cytoskeleton remodeling, and boosting the rate of secondary metabolism.

3.3.2 Maize Response to Biotic Stress

Maize losses due to pathogen damage continue to be a serious worldwide problem. Understanding maize–pathogen interactions is, therefore, critical for the development of resistant varieties. Proteomics has largely assisted in elucidation of genetic basis of host resistance through identification of numerous defense-related proteins. This was achieved through comparative analyses of pathogen-resistant and susceptible maize varieties as well as after subjection to a pathogen challenge.

3.3.2.1 Fungal Pathogens

Aspergillus flavus

A. flavus causes *Aspergillus* ear rot and preharvest and postharvest contamination of maize grains with aflatoxins, which predominantly accumulate during heat and drought. As potent carcinogens, aflatoxins pose a great threat to worldwide economies and human health.

Therefore, maize infestation by *A. flavus* continues to attract the attention of research teams (Pechanova and Pechan, 2015). Resistance to *A. flavus* infection and/or aflatoxin accumulation in kernel tissues (embryo and endosperm) is believed to be contributed by constitutive expression of three major groups of proteins: storage proteins (e.g., globulin 1 and 2, LEA 3 and 14), stress-related proteins (e.g., HSPs, anionic peroxidase, glyoxalase 1, peroxiredoxin antioxidant, and aldose reductase), and antifungal proteins (e.g., trypsin inhibitor and PR-10) (Chen et al., 2002, 2004). Involvement of PR-10 was further confirmed via overexpression and RNAi studies (Chen et al., 2006, 2010). More importantly, the proposed association between abiotic stress tolerance and disease resistance (Chen et al., 2004a) is a novel approach that can be exploited to enhance aflatoxin resistance in maize.

Dissimilar defense mechanisms between aflatoxin-resistant and aflatoxin-susceptible inbreds were reported for maize rachis (cob) (Pechanova et al., 2011). This tissue has been shown to assist in aflatoxin resistance via halting the *A. flavus* movement from the cob interior to the kernels (Alfaro, 1999; Magbanua et al., 2013). Comparative studies showed that resistant rachis almost exclusively relies on constitutive defenses throughout the whole life cycle.

At young age, it uses abiotic stress-related proteins and detoxifying enzymes (HSPs, APX, thioredoxin, etc.) to withstand heat and drought, which in turn help it to resist aflatoxin production. Additionally, phenylpropanoid pathway proteins (PAL, caffeoyl-CoA-3-*O*-methyltransferase1, and chalcone flavonone isomerase) also aid resistance through enhanced lignification. As the ear matures, resistant rachis strongly accumulates a variety of PR proteins to preserve the resistance. Most notably, PRm3 chitinase accumulated 40-fold during resistant rachis maturation, versus 7.3-fold increase in the susceptible inbred. Susceptible rachis, on the other hand, is mostly dependent on inducible defenses, which are vigorously triggered 35 days postinfection. The most inducible were multiple isoforms of chitinases, glucanases, protein P21, permatin, PR-1, PR-5, PRm3, and PRm6b.

Finally, maize silks were also inspected for aflatoxin resistance (Peethambaran et al., 2010). Due to their specific function and location on the maize ear, silks are believed to serve as a main entrance point for *A. flavus* (Payne, 1992) and simultaneously, a first line of defense during preharvest contamination. Comparative studies showed that antifungal properties of silk from aflatoxin-resistant inbreds are facilitated by PRm3 chitinase, chitinase I, and chitinase A proteins, which was also supported by antifungal assays.

Fusarium pathogens

Fusarium species, especially *F. graminearum* and *F. verticillioides* are widespread maize pathogens that cause substantial damage to its ear and foliage, including contamination of grains with mycotoxins. Fusaria-elicited diseases occur worldwide and are of great concern due to their deleterious effects on human and animal health.

F. graminearum causes Gibberella ear rot and stalk rot leading to contamination of grains with deoxynivalenol and zearalenone (Munkvold, 2003). Forty-eight hours postinoculation, developing kernels from resistant CO441 inbred displayed higher abundances of many defense proteins than susceptible B73 (Mohammadi et al., 2011). Examples include chitinase, xylanase inhibitors (XIP), thaumatin-like protein, PR-10, zearatin precursor and peroxidase as well as cinnamyl alcohol dehydrogenase, 4-coumarate-CoA ligase, and phenolic *O*-methyltransferase. The B73, on the other hand, strongly induced PAL. In addition, B73 silks also responded to *F. graminearum* by strong induction of PRm3 chitinase, PR-10, PR-5, and several others.

The elicitor of *Fusarium* ear and stalk rot, *F. verticillioides*, causes contamination of grains with fumonisins, one of the most common maize contaminants. After a 24 h challenge with *F. verticillioides*, germinating embryos from susceptible maize genotype W64A upregulated numerous protective proteins (Camp et al., 2004). For instance, while almost entirely missing in uninfected embryos, detoxifying enzymes catalase 2, SOD, and glutathione-S-transferase (GST) were highly abundant in fungus-infected embryos serving to protect them from oxidative damage. Similarly, HSP 17.2, peptidylprolyl *cis*–*trans* isomerase and

cyclophilin were upregulated in infected embryos to avoid possible impairment of the protein structures. In addition, differential expression of several housekeeping proteins suggested that *F. verticillioides* considerably influenced carbohydrate metabolism by activating gluconeogenesis and repressing glycolysis.

The studies earlier support the evidence of a common resistance mechanism in maize against both *Aspergillus* ear rot/aflatoxins and *Fusarium* ear rot/fumonisins that was found by QTL mapping (Robertson-Hoyt et al., 2007). Thus, knowledge of the mechanism of maize–*A. flavus* interaction might be applied to the Fusaria resistance research, and vice versa.

The earliest host–pathogen interactions occur in extracellular matrix (ECM) through pathogen recognition followed by rapid onset of downstream defenses. Simulation of pathogen attack on cell culture with a fungal elicitor was undertaken to investigate this critical event (Chivasa et al., 2005). A 6 h treatment resulted in triggering three main defense-related events, including secretion of six putative XIP isoforms into the ECM culture medium to inhibit fungal-secreted xylanases. Dephosphorylation of several extracellular peroxidases implied that phosphorylation regulatory machinery was involved in this interaction, likely by strengthening the cell walls via peroxidase-mediated cross-linking. Finally, the enrichment of cytosolic GAPDH and HSP in the cell wall fraction indicated their targeting into the cell wall during the infection.

3.3.2.2 Viral Pathogens

Rice black-streaked dwarf virus (RBSDV) and sugar cane mosaic virus (SCMV) are the two most common viral pathogens associated with maize disease. RBSDV causes dwarfism and severe damage to maize plants. Long-term adjustment (50 days) to this causal agent of maize rough dwarf disease (MRDD) was studied by Li and colleagues (Li et al., 2011). Significant proteome changes in leaves of the susceptible genotype revealed a large assortment of responsive mechanisms toward the RBSDV that might be behind the drastic morphological changes. Severely compromised by virus were the fundamental metabolic pathways, such as glycolysis, TCA cycle, glycogenesis, pentose phosphate metabolism, starch metabolism, amino acid metabolism, and photosynthesis. Upregulation of peroxidase 39, APx2-cytosolic ascorbate peroxidase, and catalase 3 specifies the need for removal of ROS generated by oxidative stress. Cell wall modifications via enhanced levels of cinnamyl alcohol dehydrogenase and caffeic acid 3-*O*-methyltransferase also occurred as infected plants had rough enation-containing leaves. Accumulation of UDP-glucosyltransferase BX9 in diseased plants showed that benzoxazinoids might be participating in defense. Lastly, Ran-A1 of GTPase superfamily and several lipoxygenases were likely accumulated to modulate defense-related signaling pathways.

Inoculation of maize with SCMV facilitated the exploration of its interaction with foliar virus that causes a mosaic disease in maize plants (Wu et al., 2013a,b). Multiple pathways were affected 6 and 12 days postinfection in both resistant and susceptible varieties, with energy-yielding metabolism, stress/defense, and photosynthesis predominantly compromised. Among

them, carbohydrate metabolism seemed to undergo the most dramatic changes. Specifically after longer exposure time, enzymes of glycolysis and gluconeogenesis were strongly enhanced in the resistant line but to a lesser extent in the susceptible genotype. Proteins associated with stress/defense, signal transduction, and transcription were upregulated to a greater degree in the resistant line. More importantly, during both time points of infection, proteins that were previously not known as virus-responsive were identified. Examples include bZIP transcription factor ABI5, ATP synthase CF1 alpha subunit, glutamate dehydrogenase, remorin, ferredoxin-NADP reductase, nucleolar RNA helicase 2, serine/threonine-protein kinase, and abscisic acid (ABA) ripening protein. Lastly, defenses arbitrated via phytohormone signaling were also likely involved as differences in ethylene, ABA, salicylic, jasmonic, and azelaic acids were observed between resistant and susceptible plants, as well as between mock-inoculated and SCMV-inoculated plants.

Proteomic analyses of maize-pathogen interactions undeniably showed that maize resistance is a very complex trait. It engages a wide spectrum of cellular processes and signaling pathways within multiple tissues and organs. Proteome-based findings (Fig. 3.2) continue to help

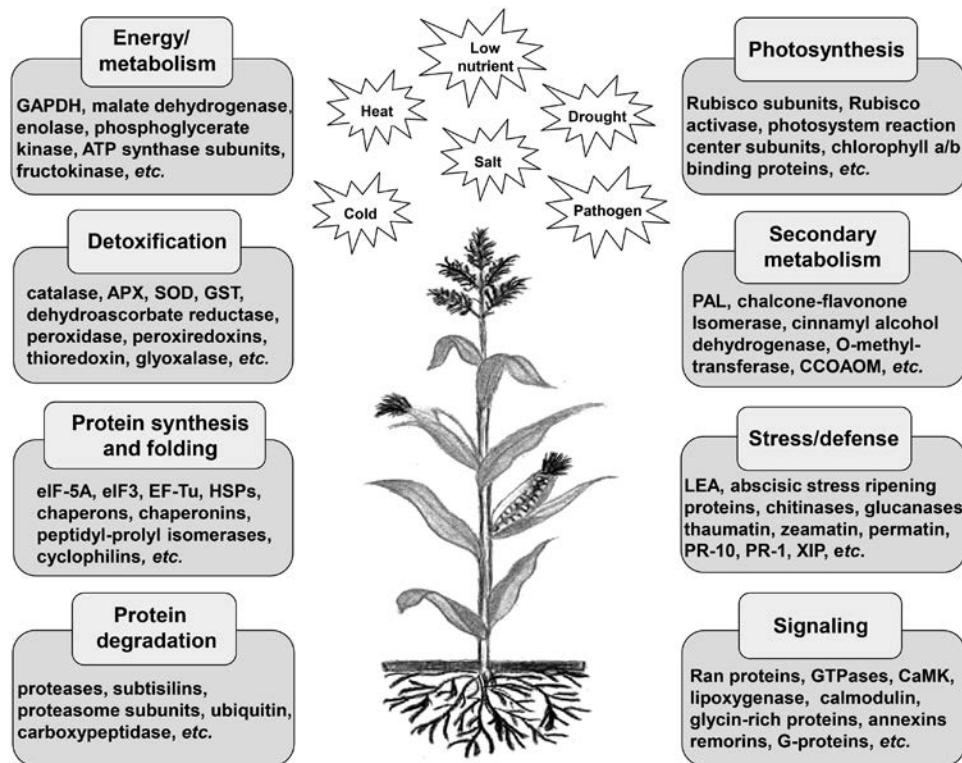


Figure 3.2

A simplified overview of maize proteins identified in different maize organs, tissues, and cell types under diverse environmental stimuli. Proteins were detected by gel-based and/or gel-free approaches coupled to mass spectrometry and database searches.

geneticists and breeders in the development of maize-resistant varieties either via marker-assisted breeding or via genetic engineering.

3.4 Nutrient Imbalance

Nutrient imbalance has serious consequences on maize production. Nitrogen (N) nutrition, in particular, is one of the major factors in plant growth and its deficiency considerably reduces kernel set and cob biomass (Liao et al., 2012). In developing maize ears, both N deprivation and N excess triggered general stress responses via altered expression of HSPs, APX, glutathione reductase, cinnamoyl-CoA reductase, etc. (18 in total) (Liao et al., 2012). A more exciting impact was seen on hormonal metabolism regulation. While N deficit repressed sex determination protein Tasselseed2, which might have stimulated female flowers on the tassel, N surplus induced its expression to suppress this abnormality. Limited N also regulated jasmonic acid levels through higher lipoxygenase production. Finally, numerous modifications of carbon/nitrogen metabolism resulted in further developmental reprogramming, such as prolonged ear development.

Maize seedlings exposed to N starvation were heavily affected in several physiological traits including height, root length, leaf tolerance index, root/shoot ratio, and chlorophyll content (Jin et al., 2015). Carbohydrate, amino acid, and nucleotide metabolism were the most affected pathways in roots, where upregulation of glutamine synthetase (GS) lead to a higher efficiency of N assimilation. In leaves, the major impact was observed for photosynthetic carbon fixation and carbohydrate metabolism, but also for a few stress/disease-related proteins (e.g., PRm 6 B, alpha-1,4-glucan-protein synthase).

Maize responses to high nitrate exposure after previous N starvation were also examined (Prinsi et al., 2009). The majority of proteome changes were directly associated with assimilation of this mineral nutrient, which seemed to be in a strict relationship with carbon metabolism. Beside accumulation of nitrite reductase and GS, roots also amassed proteins of energy and redox status, such as phosphoglycerate mutase, glucose-6-phosphate dehydrogenase, 6-phospho-gluconate dehydrogenase, and monodehydroascorbate reductase. Leaves, on the other hand, increased the photosynthetic rate to reactivate photosynthesis. Finally, phosphorylation of phosphoenolpyruvate carboxylase in leaves and its monoubiquitination in roots suggests that these two PTMs are influenced by N nutritional status.

Adaptive processes to multiple macro- and micronutrient deprivation in root hairs were recently reported (Li et al., 2015). Overall, large nutrient-specific differential expressions were detected. For instance, N scarcity increased amino acid synthesis but repressed their degradation. Glycolysis was induced by N, P, and K deprivations, while Fe and Zn withdrawal resulted in its suppression. Secondary metabolism was upregulated under P, Mg, and K scarcity, but it was downregulated by Fe deficit. Low Fe and Zn downregulated transcription factors. Interestingly, almost no impact on root hair proteome was observed under Mn deprivation.

Phosphorus (P) deficit resulted in growth arrest and accelerated aging of maize roots (Li et al., 2007). It was linked to changes in multiple mechanisms, such as increased proteolysis and cellular organization via strong upregulation of ubiquitin/26S proteasome pathway, actins and tubulins. Accumulation of PAL, caffeate *O*-methyltransferase, *O*-methyltransferase, and UDP-glucosyltransferase BX9 implied a shift to secondary metabolism. As one of the key elements in nucleic acid biosynthesis, P starvation also altered regulation of nucleic acid metabolism by switching to AMP de novo biosynthesis.

The studies reviewed earlier clearly demonstrate how nutritional imbalance affects maize metabolism and physiology resulting in substantial yield reductions.

3.5 Heterosis-Associated Maize Proteomes

Crossing for hybrid superiority (hybrid vigor, heterosis) helped maize breeders to improve numerous agronomically important traits and resulted in nearly all currently grown maize hybrids exhibiting hybrid vigor. Proteomics was utilized to clarify this phenomenon through the comparisons of proteins from hybrids and their parental lines.

Such investigations revealed that heterosis can be detected on a molecular level during very early stages of plant development. For instance, for length, weight, and the time point of seminal root primordia formation, heterosis was detected during embryo development (Marcon et al., 2010). Gel-based analysis of immature embryos before (25-day-old) and after (35-day-old) seminal root primordia formation showed that 24% of differentially expressed proteins were nonadditively accumulated between the parental lines and at least one reciprocal hybrid. They mainly related to development, protein metabolism, redox-regulation, amino acid metabolism, glycolysis, and TCA cycle, leading to the more robust hybrid embryos, which was also supported by morphological parameters.

Nonadditive protein expression pattern was also exhibited by embryos of germinating maize seeds from five elite maize hybrids. Fifty-four nonadditive proteins were mainly associated with metabolism, detoxification, and chaperoning (Fu et al., 2011). For instance, catalase, GST, and hydroxyacid oxidase were upregulated in hybrid embryos to minimize cell damage from ROS during imbibition, while glyoxalase was required to detoxify methylglyoxal. Cupins and 14-3-3 protein indicated a cross-talk between gibberellic acid and ABA signaling pathways that play a critical role in seed germination and other developmental events in higher plants.

Proteome-associated heterosis was also inspected in the developing root system whose performance is absolutely critical for the establishment and growth of a new plant (Hoecker et al., 2008). One hundred and fifty proteins were found nonadditively accumulated in 3.5-day-old primary roots of hybrids, of which metabolism and disease/defense-related proteins comprised two of the most prominent groups. They included

alpha-1,4-glucan-protein synthase, several HSPs, GST, mitochondrial SOD (Mn) 3.4, UDP-glucosyltransferase BX8, and others. Interestingly, lower expression in hybrids was observed for six out of seven PAL isoforms, caffeoyl-CoA 3-*O*-methyltransferase 1, and chalcone-flavonone isomerase of the phenylpropanoid pathway. PAL, however, likely plays a role in enhancing lateral root density (Hoecker et al., 2006) as it was highly abundant in the proteome of pericycle cells (Dembinsky et al., 2007) from which lateral roots are formed.

The studies earlier greatly contributed to the understanding of the genetic mechanisms and principles of heterosis. They clearly showed that multiple pathways are implicated in this complex trait that leads to the growth and development of high-yielding heterotic hybrids.

3.6 Seed Viability

Seed fitness is critical for germination and ultimately a crop yield. Reports, however, show that during prolonged storage, seeds tend to age due to alterations of their proteomes, which consequently leads to the reduction of their germination vigor (Rajjou et al., 2008). Two gel-based proteomic studies on artificially aged maize seeds helped to elucidate this event. Embryos of seeds subjected to 50°C for 5 and 13 days were severely affected in mobilization of energy supplies due to robust downregulation of carbohydrate metabolism and energy-yielding pathways (Xin et al., 2011). Decreased levels of malate dehydrogenase, phosphoglucomutase, 3-phosphoglycerate kinase, triosephosphate isomerase, and six ATP synthase subunits obviously resulted in an insufficient energy stream. Embryos further exhibited changes in signal transduction, transcription, and degradation of storage proteins due to elevated proteases. Breakdown of globulin 2 and vicilin-like protein resulted in poor supply of amino acids for synthesis of proteins essential for germination. Deteriorated seeds also showed abnormal cell division and growth due to altered expression of cyclin-dependent kinase A and mitogen-activated protein kinase, two important cell-division control proteins. Finally, the failure to efficiently protect seeds from oxidative damage due to distorted detoxifying mechanism further contributed to their deterioration.

In another study, embryos of highly viable and dead seeds were compared after exposure to 41°C for 1–6 days (Wu et al., 2011). Remarkable abundance differences were detected for proteins related to stress response, protein folding and stabilization, nutrient reservoir, and metabolism. Highly abundant in viable embryos were defense-related proteins such as small HSPs, LEA proteins, and antioxidant enzymes such as glyoxalase, thioredoxin peroxidase, SOD, and GST. Small HSPs and LEA proteins are known seed protectants during seed development, maturation, storage, and germination (Kalemba and Pukacka, 2007). Scavenging of ROS is critical for seed longevity (Rajjou et al., 2008). Therefore,

detoxifying enzymes play a critical role in maintaining seed vigor by protecting the cells from aging. As in previous studies, proteolysis seemed to be active during accelerated aging. Two isoforms of CAAX prenyl protease were present at higher levels in dead seeds, where they very likely promoted the deterioration by hydrolyzing embryonal proteins (Wu et al., 2011).

Seed viability is of paramount agronomic importance and both proteomic studies considerably contributed to the understanding of the seed aging mechanism. The knowledge obtained could be applied in efforts to prolong seed longevity and improve their germination vigor.

3.7 Safety Assessment of Genetically Modified Maize

Proteomics has also proved valuable as a tool for safety assessment of GM maize. Insect-resistant MON810 variety repels European corn borer via expression of highly specific insecticide encoded by *Cry1Ab* gene from *Bacillus thuringiensis*. Despite its widespread use, MON810 and foods derived from it have been the subject of public concerns about their potential negative impact on human health and environment. Safety assessments are necessary to evaluate possible unintended modifications to the maize metabolism provoked by the foreign gene insertion. They were done by 2DE-based proteome comparisons of GM plants with their non-GM counterparts of the same genetic background.

Kernel flour from MON810 differed from its near-isogenic non-GM line by upregulating triosephosphate isomerase 1 and globulin-1 S and downregulating cytosolic 3-phosphoglycerate kinase and aldose reductase (Albo et al., 2007). More interestingly, glucose and ribitol dehydrogenase homolog appeared to be unique to MON810, while endochitinase A appeared to be unique to its non-GM counterpart. Very few differences with minimal quantitative change-ratios in protein expression were also found in milky-starchy grains of two MON810 and two corresponding near-isogenic non-GM lines ($\leq 1.2\%$) (Coll et al., 2011). Proteins overaccumulated in GM varieties included two HSPs, putative sorbitol dehydrogenase, homocysteine S-methyltransferase, and putative WD-40 repeat protein. The authors concluded that all differences were variety-specific and could not be directly attributed to the genetic manipulation.

In another study, authors attempted to elucidate the role of natural plant-to-plant variability in GM safety assessment (Batista and Oliveira, 2010). Due to extreme variability between individual samples, as well as between pools of samples, differences in protein expressions could not be produced solely by *Cry1Ab* insertion. However, a serial cross-comparison of seeds of two subsequent generations of MON810 and isogenic non-GM control plants grown under environmentally controlled conditions yielded more dramatic results (Zolla et al., 2008). Detection of 100 and 43 differentially expressed proteins regulated by environment

and transgene respectively were noted. Seven proteins were newly expressed in GM line, among them a known maize allergen 50 kDa gamma zein. The overall conclusion confirmed that environment impacts protein expression more strongly than gene manipulation.

To determine the extent of environmental variations, three different locations and three growing seasons were used to compare gene, protein, and metabolite expression between non-GM control and two transgenic lines, MON810 and RR maize (herbicide-tolerant Roundup Ready) (Barros et al., 2010). Five proteins, 65 genes, and 15 metabolites were differentially expressed during three growing seasons at one location. None of the five proteins was identified, however; and transcript levels of LPT allergen were significantly lower in GM line. A greater number of identified variations were produced by the environment than by genetic engineering.

Brazilian varieties of MON810 were examined during growth in two contrasting settings, the growth chamber (Balsamo et al., 2011) and the field (Agapito-Tenfen et al., 2013), to measure changes in leaf proteomes for environmental risk assessments. Twelve newly synthesized variety-specific proteins were detected in two out of four GM versus non-GM pairs (Balsamo et al., 2011). However, the only two proteins identified were putative NAD-dependent epimerase/dehydratase and phosphatase 2C. Several nonconsistent differences in physiological parameters (aerial part weight, main leaf length, chlorophyll, and total protein content) were also declared but were not considered as reliable pointers of gene insertion event. When field-grown in two different agroecosystems, GM and near-isogenic non-GM leaves differentially accumulated 32 proteins, 16 per location (Agapito-Tenfen et al., 2013). They were grouped into three major categories: carbohydrate and energy metabolism, genetic information processing, and stress response. Moreover, eight and seven proteins were unique to the GM line for each of the two geographic locations. They contained fructose-bisphosphate aldolase, ferredoxin–NADP reductase, H (+)-transporting ATP synthase, adenine phosphoribosyltransferase, Apx2-cytosolic ascorbate peroxidase, etc. In summary, differentially expressed proteins represented less than 3.1% of the analyzed spots, and the environment was the major source of changes in protein expression in GM line.

From comparative safety assessments, it has become evident that major changes in proteomes of GM maize lines are strongly modulated by maize immense natural genotypic variations and strong environmental impacts, which may negatively affect the safety assessment data and subsequent marketing of GM cultivars.

3.8 Maize Allergenic Proteins

Food allergies affect many people and in highly allergic individuals can have life-threatening impacts. Since the discovery of the first maize allergen, 9 kDa lipid transfer protein (LTP) by N-terminal sequencing (Pastorello et al., 2000), the search for maize allergens has been

greatly facilitated by advances in mass spectrometry and maize genome sequence. Preceded by immunoblotting of gel-resolved maize extract with sera of maize-allergic patients, they allowed for unambiguous identifications of many proteinaceous allergens. For example, using 2-DE and an Orbitrap mass analyzer, six previously unknown maize allergens were identified in kernels of the B73 inbred (Fasoli et al., 2009). They comprised vicilin, globulin-2, 50 kDa gamma zein, endochitinase, trypsin inhibitor, and thioredoxin. Interestingly, endochitinase and thioredoxin are known for their antifungal and antioxidant properties, respectively.

Recently, the 2-DE MS/MS approach was utilized to unravel the possible allergenic potency of MON810 maize (Fonseca et al., 2012). Several known allergens were detected, such as globulin-1 S allele precursor, 50 kDa gamma zein, enolase, vicilin-like embryo storage protein, chitinase, and endochitinase A. No statistically significant differences between MON810 and non-GM seeds during three developmental stages were found for LTP, trypsin inhibitor, thioredoxin, 50 kDa zein, and glutelins. Fourteen novel allergens were identified in both MON810 and non-GM kernels, including several naturally occurring stress/defense proteins, such as LEA, dehydrin, chaperonin CPN60-1, adenosine kinase, and cysteine proteinase 1 precursor. Added to the maize allergenic repertoire were also malate dehydrogenase, triacylglycerol lipase, UDP-glucose pyrophosphorylase, prolamin PROL 17 precursor, and a few others.

Lastly, the allergenic potency of maize pollen was also inspected (Petersen et al., 2006). Only a few proteins were detected through comparison of pollens from maize and native timothy grass (*Phleum pratense*). They involved expansin, profilin, and polygalacturonase with expansin as a dominant pollen allergen. Unlike in maize flour, no LTPs or globulins were detected in maize pollen. It was concluded that maize pollen is a weaker sensitizer than pollen of common grasses. These high-throughput proteomic approaches have helped researchers to reveal a more complete understanding of the allergenic potential of maize.

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Proteomics of Wheat Flour

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4.1 Introduction

Wheat is a major food crop grown on more than 215 million hectares of land throughout the world. Wheat flour provides an important source of protein for human nutrition and is used as a principal ingredient in a wide range of food products, including many different types of breads, noodles and pasta, breakfast cereals, baked goods and tortillas. This is largely because wheat flour, when mixed with water, has unique viscoelastic properties that make it possible to produce a cohesive dough. The viscoelastic properties are conferred by the complement of proteins accumulated in the grain during development and form the basis of wheat flour quality. Certain proteins in wheat flour also are responsible for human health problems including allergies, food intolerances, and sensitivities. In recent years, proteomics methods have facilitated the detailed study of wheat flour proteins and provided new insights into the molecular basis of flour quality and immunogenic potential. These studies are essential to develop novel approaches to improve flour functional properties, reduce immunogenicity, and maintain wheat flour quality in the face of climate change.

4.2 Wheat Flour Proteins

The protein composition of wheat flour is very complex. Wheat flour proteins are often separated on the basis of solubility. When flour is mixed with water or a dilute salt solution, only a small percentage of the protein is solubilized. The majority of protein forms an insoluble mass referred to as gluten. The major proteins in this mass, referred to as gluten proteins, comprise about 70%–80% of the total flour protein and consist of ~70–100 different, but closely related proteins, all of which have large regions of repetitive sequences. One characteristic of all gluten proteins is that they have unusual amino acid compositions with an abundance of glutamine (Q) and proline (P) that likely contribute to their insolubility in aqueous solutions. Glutamine and proline comprise from 43% to 73% of the total amino acids in these proteins.

The gluten proteins can be divided into two groups on the basis of their functions, referred to as gliadins and glutenins (Wieser, 2007) (Fig. 4.1). The gliadins are soluble in 60% ethanol and generally are present as monomers in the flour. These proteins are believed to

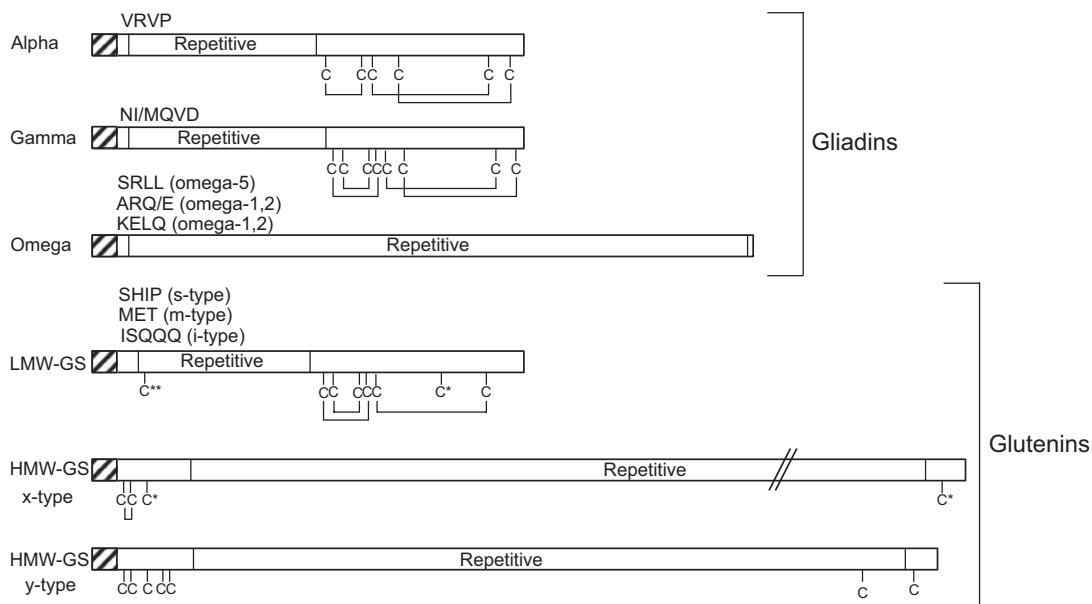


Figure 4.1

Schematic structures of the wheat gluten proteins showing the large regions of repetitive motifs that are characteristic of these proteins. With the exception of the x-type HMW-GS, the relative sizes of proteins in the various classes are drawn to scale. Signal peptides are denoted by hatched boxes. N-terminal sequences are shown for the various gliadins and LMW-GS. Subtypes within a protein group are shown in parentheses. Conserved cysteine residues are shown below each diagram with connecting lines denoting intrachain linkages (if known). Cysteine residues involved in interchain linkages are indicated with * (if known). In the LMW-GS, C** denotes the cysteine whose position varies among different LMW-GS.

contribute viscosity and extensibility to wheat flour dough. Gliadins are separated into alpha, gamma, and omega subgroups, each with distinct primary sequences. Within each subgroup are numerous closely related proteins some of which differ by the substitution, insertion, or deletion of only a few amino acids. Alpha and gamma gliadins range from ~28 to 35 kDa. These proteins have N-terminal sequences beginning with VRVP- (alpha) and NI/MQVD- (gamma), followed by repetitive regions containing motifs rich in P and Q and C-terminal regions containing either six (alpha) or eight (gamma) conserved cysteine residues. The cysteine residues form three (alpha) or four (gamma) intramolecular bonds (Fig. 4.1). Omega gliadins range from ~40 to 50 kDa, consist almost entirely of repetitive sequences, and do not contain cysteine. Omega gliadins are divided into two subtypes. The omega-5 gliadins begin with SRLL- and have repetitive sequences with the motifs FPQQQ and QQIPQQ, while the omega-1,2 gliadins begin with ARQ/E- or KELQ- and have QQPFP repeats. In contrast to the gliadins, the glutenins are present in the flour as large insoluble polymers that range in size from about 500,000 to over 10 million MW and are

poorly soluble in alcohols. The glutenin polymers contribute elasticity to dough and consist of two protein types, high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) that are linked together by interchain disulfide bonds. There are two subtypes of HMW-GS, the x-types that range from 83 to 88 kDa and the y-types that range from 67 to 74 kDa. Both consist of a central repetitive region flanked by small regions of unique sequence that include most of the cysteine residues involved in intramolecular and intermolecular linkages. The LMW-GS are similar in size and structure to gamma gliadins. These proteins are sometimes divided into s-type, m-type, and i-type LMW-GS on the basis of the first amino acid of the mature protein, either serine, methionine, or isoleucine, respectively. LMW-GS contain eight cysteine residues, seven with conserved positions in the C-terminal region and one that varies among different LMW-GS (indicated by C** in Fig. 4.1). Six of the cysteines are involved in intramolecular bonds while the remaining two cysteines are available for interchain bonds with HMW-GS or other LMW-GS. In addition to the typical LMW-GS, some proteins with sequences similar to alpha, gamma, and omega gliadins contain an extra cysteine residue that enables them to be incorporated into the glutenin polymer and thus function as LMW-GS (not shown). While the typical LMW-GS are able to extend the polymer chain, the gliadin-like LMW-GS proteins act as chain terminators of the polymer chain and likely decrease its size (Kasarda, 1989; D’Ovidio and Masci, 2004). These proteins will be referred to here as alpha, gamma, and omega chain-terminators.

It has been estimated that a typical hexaploid bread wheat contains genes or pseudogenes for six HMW-GS, at least 30 LMW-GS, 30 or more gamma gliadins, and up to 150 alpha gliadins (Anderson et al., 1997; Lee et al., 2016; Sabelli and Shewry, 1991). Additionally, there is considerable allelic variation among different wheat cultivars that contributes to the overall complexity of the gluten proteins.

The remainder of the flour protein consists of a diverse collection of salt-soluble proteins collectively referred to as the nongluten proteins. Many nongluten proteins play critical roles in metabolism, controlling grain development, maturation, and desiccation, or protect the grain against abiotic or biotic stress. Other nongluten proteins are likely to serve a storage function in the grain. These include proteins that lack repetitive regions but are otherwise similar to gluten proteins, proteins that are analogous to the major globulin storage proteins of dicot seeds, and the amylase/protease inhibitors that also play a defensive role in the grain.

4.3 Wheat Flour Quality

The viscoelastic properties of the flour provide the basis for its economic value and define flour quality. Thus, it is essential to understand the contributions of the different flour proteins to quality. Various physical tests can be used to evaluate flour functional properties. For bread wheat, these include mixing studies that measure extensibility and elasticity of the dough as

well as baking studies that assess volume and texture of the loaf. Relating functional properties to specific proteins in the flour has been far more challenging. Genetic studies have shown that certain HMW-GS are associated with good bread-making quality while others with only minor sequence variations are associated with poor bread-making performance. However, the basis for the differential effects of the proteins on quality remains unknown (Shewry et al., 2003). Certain LMW-GS also have been associated with good flour quality, but these studies are far more complicated because of the large number of proteins within this group (D’Ovidio et al., 1999; Luo et al., 2001; Zhang et al., 2012). Several studies have correlated the amounts of the glutenin polymers to flour quality and suggest that the ratios of glutenins to gliadins in the flour are important (Gianibelli et al., 2001; Southan and MacRitchie, 1999). The sizes of the glutenin polymers also are likely to be critical for flour quality, but are difficult to assess because of the insolubility of the large polymers. Nonetheless, it is likely that ratios of HMW-GS to LMW-GS and the proportion of chain terminators in the flour influence the size and structure of the polymers and hence flour quality.

4.4 Immunogenic Potential of Wheat Flour

Wheat flour proteins have been reported to trigger celiac disease (CD), IgE-mediated food allergies (FA), nonceliac wheat sensitivity (NCWS), and baker’s asthma (BA). CD, FA, and NCWS result from the ingestion of wheat flour and will be discussed in greater detail because of their relevance to food science. BA is triggered by the inhalation of flour and is considered to be an occupational allergy found in millers and bakers who handle large quantities of wheat flour (Salcedo et al., 2011).

CD is a complex autoimmune disease triggered in genetically susceptible individuals by the ingestion of wheat gluten proteins and similar proteins from barley and rye (Wieser and Koehler, 2008). Indeed, the triggering proteins are some of the same proteins that determine the functional properties of flour. CD causes damage to the lining of the intestine and results in malabsorption of nutrients that is manifested in a wide range of clinical symptoms. A number of studies have identified epitopes in gluten proteins that stimulate T-cells of celiac patients (Sollid et al., 2012). While sequences in all classes of gluten proteins have been associated with CD, the gliadins generally are the most immunogenic. Tye-Din et al. (2010) used peripheral blood from CD patients after a gluten challenge and determined that the most important epitopes were found in alpha gliadins and omega-1,2 gliadins. Two of the immunodominant epitopes from alpha gliadins, PFPQPQLPY and PQPQLPYPQ, are found in a 33-mer protease-resistant peptide that has been shown to be particularly toxic for CD patients (Shan et al., 2002). It is interesting that the immunodominant epitopes and the 33-mer peptide are found in only some of the alpha gliadins despite sequence similarities among these proteins (Van Herpen et al., 2006). The immunodominant epitope from the omega-1,2 gliadins, QFPFPQPQQPFPW, is similar to T-cell stimulatory epitopes identified in barley and rye.

In contrast to CD, food allergies elicit rapid immunoglobulin E (IgE)-mediated responses. Symptoms of FA to wheat can include atopic dermatitis, gastrointestinal problems, urticaria, asthma, and anaphylaxis. IgE-binding studies using fractionated wheat proteins have shown that food allergies are triggered by both gluten and nongluten proteins (Battais et al., 2005). More recently, proteomic approaches have been used to identify many allergenic proteins and it has become clear that most wheat allergens belong to a few protein families with structural or functional properties similar to food allergens characterized in other plants. These include the prolamin and cupin superfamilies of storage proteins that are characteristic of monocots and dicots, respectively, and the pathogenesis-related proteins that protect the plant against pathogens and environmental stress but include proteins with diverse structures (Breiteneder and Radauer, 2004; Mills et al., 2004).

NCWS is a relatively new wheat-related syndrome that is used to describe both intestinal and extraintestinal symptoms that occur upon ingestion of wheat in patients who do not have confirmed cases of CD or FA (Catassi et al., 2013). While symptoms may be similar to CD, NCWS does not result in intestinal damage and does not involve the adaptive immune system. Symptoms generally disappear after withdrawal of wheat from the diet and recur when wheat is reintroduced. Recently, Schuppan et al. (2015) suggested that the amylase/trypsin inhibitors from wheat may be involved in triggering NCWS.

CD, FA, and NCWS are managed with avoidance diets. Although these conditions affect a few percent of the population, they have fueled the much larger gluten-free and wheat-free dieting trend. This has led to the development of many gluten-free and wheat-free products and created a demand for sensitive analytical methods to detect wheat proteins in food products. Currently, there remains a critical need for more detailed information on the immunogenicity of wheat flour proteins to develop better diagnostic tools, novel therapies, and new wheat varieties with reduced immunogenic potential.

4.5 Developing Proteomic Maps of Wheat Flour

During the past 10–15 years, proteomic approaches have advanced research on the wheat flour proteins. The first phase of proteomics research is primarily descriptive and involves the separation of individual flour proteins by two-dimensional gel electrophoresis (2-DE) coupled with their identification by mass spectrometry (MS). To develop an overall picture of the wheat flour proteins, both gluten and nongluten proteins are extracted from flour using a buffer containing 2% SDS and 50 mM dithiothreitol (Dupont et al., 2011). Fig. 4.2A shows total flour proteins from the US wheat cultivar Butte 86 separated by 2-DE using a wide pH range for the first dimension isoelectric focusing gel. The gluten proteins are the most predominant proteins. HMW-GS and omega gliadins are well separated in the gel. However, there is considerable overlap among the alpha and gamma gliadins and the LMW-GS due to their similar MWs and isoelectric points.

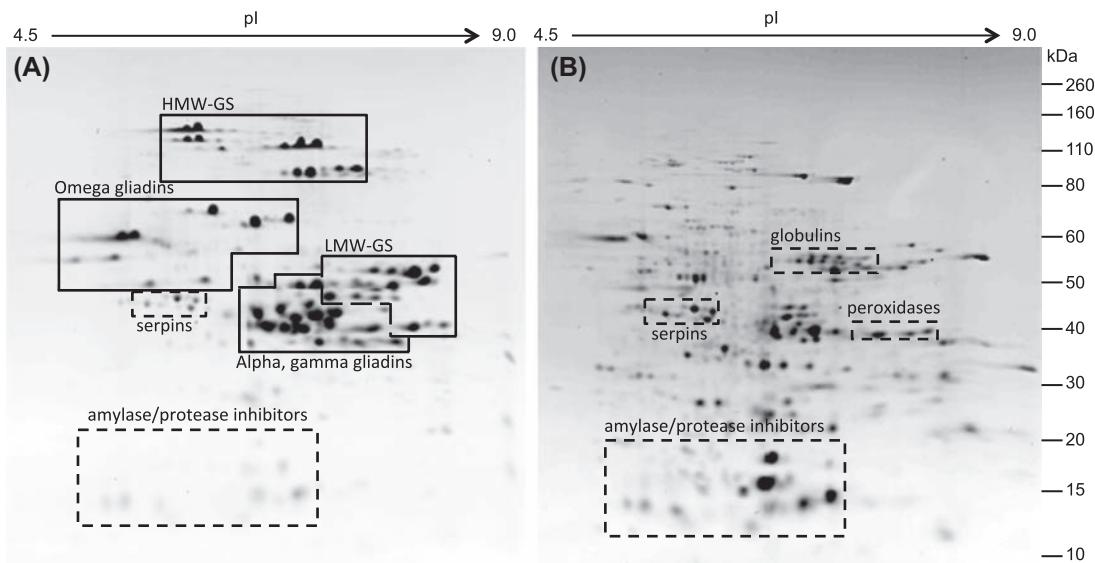


Figure 4.2

Separation of wheat flour proteins by 2-DE. (A) Total flour proteins extracted in a buffer containing SDS and dithiothreitol. Solid boxes show the positions of the major types of gluten proteins. (B) Nongluten proteins preferentially extracted with KCl. The dashed boxes show the positions of the amylase/trypsin inhibitors and serpins in both the total protein and the nongluten protein fractions. The positions of the globulins and peroxidases are shown only in the KCl fraction.

To characterize the low abundance proteins in the flour, the nongluten proteins are preferentially extracted using a buffer containing 100 mM KCl (Hurkman and Tanaka, 2004). This fraction is also very complex when analyzed by 2-DE (Fig. 4.2B). Although nongluten proteins such as the amylase/trypsin inhibitors and serpins are apparent in both fractions, the nongluten protein fraction reveals many other proteins that are not readily visible in a total protein fraction (Fig. 4.2A). For example, the globulin storage proteins and peroxidases, although present in both fractions, are obscured by the gluten proteins in the total protein extract (Fig. 4.2A).

Most nongluten proteins can be identified with a high degree of certainty using standard MS techniques. As a result, proteomic maps of nongluten fractions were the first to be developed (Hurkman et al., 2009; Vensel et al., 2005; Wong et al., 2004). In contrast, the gliadins and glutenins are very difficult to identify. In early studies, very few peptides were identified from the gluten proteins and, at best, it was possible to identify the gluten protein group, but not individual protein sequences within the group (Dupont et al., 2006). This was in part because gluten proteins generally yield few peptides when cleaved with trypsin, the enzyme used in most MS studies. Additionally, the considerable heterogeneity of sequences that is found both within each gluten protein subgroup and among wheat cultivars may not be adequately reflected in protein databases used to analyze the spectra

(Altenbach et al., 2010a,b; Mamone et al., 2005, 2009). Vensel et al. (2011) solved these problems by digesting each protein with chymotrypsin and thermolysin in addition to trypsin and including cultivar-specific sequences of gluten proteins in databases searched with spectral data. The numbers of peptides identified from each protein also were increased by using multiple search engines for the analysis of spectral data. These improvements made it possible to significantly increase the sequence coverage of individual gluten proteins and to develop a detailed 2-DE map of wheat flour proteins from the US wheat cultivar Butte 86. In this map, more than 230 protein spots were associated with specific gene sequences, many from Butte 86. These included 5 HMW-GS, 22 LMW-GS, 13 gamma gliadins, and 23 alpha gliadins (Dupont et al., 2011). In addition to the gluten proteins, a number of farinins, purinins, triticins, globulins, grain softness-related proteins, amylase/protease inhibitors, serpins, beta-amylases, tritins, and numerous enzymes were identified in the flour. Although deeper proteome coverage is certainly possible and may be desirable, it is likely that many of the proteins responsible for flour functional properties and immunogenic potential were identified in this study.

Of particular significance is that the high sequence coverage obtained in the Dupont et al. (2011) study made it possible to distinguish very closely related proteins with different functional properties or immunogenic potentials. As an example, Fig. 4.3 shows similarities in 10 alpha gliadins deduced from contigs assembled from a collection of expressed sequence tags from Butte 86 developing wheat grains (Altenbach et al., 2010b). MS/MS sequence coverage for these proteins ranged from 55% to 80%, making it possible to differentiate alpha gliadin #2 that contains an extra cysteine and may be incorporated into the glutenin polymer from similar proteins that contain six cysteines and are likely present in the flour as monomers. In a similar manner, alpha gliadins #1 and #3 that contain the 33-mer toxic fragment implicated in CD (double underline) were distinguished from alpha gliadins without these sequences. Alpha gliadins containing other CD epitopes (underlined) also were distinguished from alpha gliadins #12, 23, and 27 that do not contain described epitopes.

4.6 The Progression From Descriptive Proteomics to Comparative and Translational Proteomics

The comprehensive map of the wheat flour proteome provides the basis for both comparative and translational proteomic studies aimed at understanding the roles of specific proteins in wheat flour quality and immunogenicity (Fig. 4.4). When coupled with the power of quantitative 2-DE, the proteomic map makes it possible to determine the proportions of individual proteins or groups of proteins in a single flour sample and to quantify relatively small changes in the levels of individual proteins between samples. As a result, the proteomic map can be used to define the precise changes in flour composition that occur when wheat plants are grown under different environmental conditions. Similarly, a comparative proteomic approach

Figure 4.3

Discrimination of closely related alpha gliadins by MS/MS. Protein sequences were deduced from contigs assembled from expressed sequence tags from the wheat cultivar Butte 86 (Altenbach et al., 2010b). Peptides identified by MS/MS are shown in bold. The vertical line shows the cleavage site of

the signal peptide. Conserved cysteine residues are shown in boxes. The position of the extra cysteine in alpha gliadin #2 is indicated by the *arrow*. CD epitopes summarized in [Sollid et al. \(2012\)](#) are underlined. The 33-mer CD toxic peptide that is found in alpha gliadins #1 and 3 is indicated by the *double underline*. Mass spectrometry data are from [Dupont et al. \(2011\)](#).

is valuable in deciphering the protein composition of various fractions of the glutenin polymer, providing the foundation for understanding the complex protein linkages within the polymer and the importance of polymer structure for quality. Because proteins in the map are linked to specific gene sequences, comparative proteomic studies identify specific genes or groups of genes that might be targeted in translational proteomic studies that combine breeding or biotechnology approaches with proteomics to define and improve flour quality and reduce immunogenic potential. The precise effects of genetic modifications on the flour

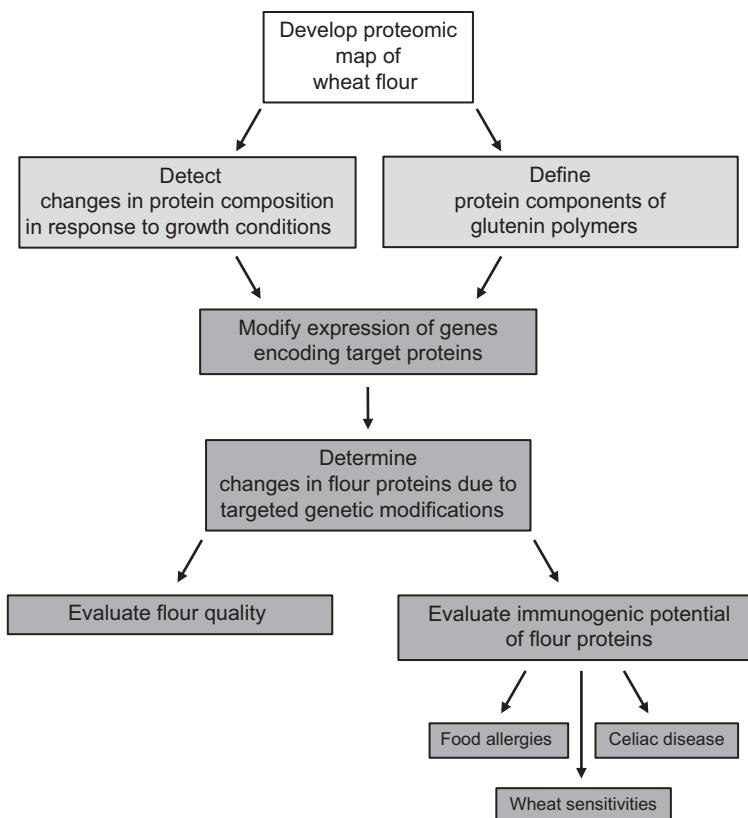


Figure 4.4

Phases of proteomics research in wheat. Descriptive proteomics (white box) involves the development of 2-D maps of wheat flour proteins that serve as the basis for future studies. Comparative proteomics studies (light gray boxes) couple the power of quantitative 2-DE with proteomic maps to identify target proteins that may be important in flour quality. Finally, translational proteomic approaches (dark gray boxes) are used to draw links between specific flour proteins and flour quality and to determine the immunogenic potential of specific proteins.

proteome can be analyzed in detail and related to flour quality. Additionally, the immunogenic potential of flour proteins in FA, CD, and NCWS can be evaluated. These applications of proteomics will be discussed in more detail using a series of studies conducted in the US wheat Butte 86.

4.7 Understanding How the Growth Environment of the Plant Affects Protein Composition of Wheat Flour

Variability of flour quality is a major problem for millers and bakers and is due in part to the environmental conditions under which the wheat is grown. It is well known that temperature,

water, and fertilizer influence flour protein content, composition, and quality. However, environmental factors have interacting effects in the field, can occur at any time during the life cycle of the plant, and are superimposed on the nutritional status of the plant.

Comparative proteomic approaches that combine controlled growth studies with quantitative 2-DE make it possible to define the precise effects of individual environmental variables on flour protein composition. This approach was used to evaluate the effects of a postanthesis fertilizer regimen on flour protein composition (Altenbach et al., 2011). Plants were grown in a greenhouse under a moderate temperature regimen with or without postanthesis fertilizer and total proteins from the resulting flour samples were analyzed by quantitative 2-DE. The results were surprisingly complicated. One hundred and fifty-five 2-DE spots representing 54 unique proteins exhibited statistically significant responses to fertilizer. Among the gluten proteins, the omega gliadins showed the largest increases in response to fertilizer, increasing 144% overall. All five HMW-GS also increased from 10% to 54%. In comparison, the LMW-GS and alpha gliadins showed variable responses to fertilizer. Of the LMW-GS, one s-type LMW-GS and two m-type LMW-GS showed ~25% decreases with fertilizer while the remaining LMW-GS did not change. Of the alpha gliadins, six showed increases with fertilizer that ranged from 33% to 64%. Since these included proteins that contained CD epitopes as well as those that did not, the proportions of alpha gliadins containing CD epitopes in the flour were not altered. Gamma gliadins showed little change in response to fertilizer. Of the chain-terminators, only the omega chain-terminators increased. A number of nongluten proteins also showed changes in response to fertilizer. The serpins increased 37% while the amylase/protease inhibitors decreased 57%. Other nongluten proteins that decreased included farinin, chitinase, globulin-2, lipid transfer protein, thaumatin-like protein, beta-amylase, glucose and ribitol dehydrogenase, triosephosphate isomerase, elongation factor EF1A, and puroindoline. It is notable that several of these proteins are suspected in FA (omega-5 gliadins, serpins, globulin-2, chitinase, lipid transfer protein), BA (amylase/protease inhibitors, lipid transfer protein, thaumatin-like protein), or NCWS (amylase/protease inhibitors).

The effects of high temperature on the composition of gluten proteins in the flour also were investigated (Hurkman et al., 2013). In these experiments, plants were grown under regimens with either moderate temperatures (24/17°C day/night) or high temperatures (37/28°C day/night) with or without postanthesis fertilizer. Surprisingly, most of the gluten proteins that showed significant increases or decreases with postanthesis fertilizer showed a similar response to high temperatures and high temperatures plus fertilizer. In a separate study that examined the effects of high temperatures on the nongluten proteins, Hurkman et al. (2009) also found that a number of proteins that showed significant changes with temperature were immunogenic. Among the proteins that increased significantly with heat were chitinase, globulin-2, and lipid transfer protein while serpins decreased. The data suggest that plant growth conditions influence not only the functional properties of wheat flour but also the immunogenic potential of the flour.

4.8 Defining Protein Components of Glutenin Polymer Fractions

Numerous studies point to the importance of the glutenin polymer in flour quality. However, the size and insolubility of the large glutenin polymers have made these difficult to study. Comparative proteomics makes it possible to define the types and amounts of individual proteins that link together to form the polymers and to determine whether there are changes in the composition of the polymers when plants are grown under different conditions. In flour, there is a continuum of polymer sizes. For these studies, protein fractions containing large glutenin polymers and small glutenin polymers were prepared using the method of [Gupta et al. \(1993\)](#). Total flour protein was separated into fractions extractable with dilute SDS (SDS-soluble) and fractions extractable with SDS only after sonication (SDS-insoluble). These fractions then were separated by size-exclusion chromatography into polymeric and monomeric proteins with the SDS-soluble fraction yielding the small glutenin polymers and the SDS-insoluble fraction yielding the large glutenin polymers. The protein compositions of the large and small polymer fractions from Butte 86 flour were analyzed by quantitative 2-DE ([Altenbach et al., 2016](#); [Vensel et al., 2014](#)). HMW-GS and LMW-GS comprised greater proportions of the large polymer than the small polymer fractions, 24.5% and 46.4% versus 18.1% and 41.6% respectively, and the ratio of HMW-GS to LMW-GS in the large polymer fraction was greater than in the small polymer fraction. Within the LMW-GS, the m-type and s-type LMW-GS were more abundant in the large polymer fraction, while the i-type LMW-GS were more abundant in the small polymer fraction. It is interesting that the position of one of the cysteine residues involved in disulfide bonding differs between the i-type LMW-GS and the other LMW-GS, suggesting that small changes in the structures of these proteins may influence their assembly into polymers. The chain-terminators also comprised more than 26% of the LMW-GS in the small polymer fraction, but only 9.1% of the large polymer fraction. Surprisingly, a number of nongluten proteins, including globulins, triticins, and serpins, were found in both polymer fractions, but were more abundant in small polymer fractions. When plants were grown with and without postanthesis fertilizer, the ratio of HMW-GS to LMW-GS increased in both polymer fractions in response to fertilizer. Both alpha and omega chain-terminators and serpins also increased in both polymer fractions. However, increases in chain-terminators were more pronounced in the large polymer fraction, while increases in the serpins were more pronounced in the small polymer fraction. Triticins also increased, but only in the large polymer fraction. The data point to the need for further studies to provide insight into the roles of the different types of LMW-GS, the chain-terminators, and the serpins in the glutenin polymer and flour quality.

4.9 Combining Genetic and Proteomic Approaches to Establish Links Between Specific Proteins and Flour Quality

While comparative proteomic studies can be used to identify target proteins that change in response to plant growth conditions or are differentially accumulated in small and large

glutenin polymer fractions, translational proteomic studies can test hypotheses about the functions of the target proteins in the flour. One approach involves using genetic approaches such as gene silencing to create transgenic wheat lines that are missing specific flour proteins. Such an approach was used to determine the relationships between flour quality and proteins that showed the largest responses to temperature and fertilizer. The first set of experiments targeted a subset of omega gliadins, the omega-5 gliadins. These proteins also have been reported to increase in response to water stress in durum wheat (Giuliani et al., 2015). It is notable that the omega-5 gliadins are the major sensitizing allergens in a serious food allergy called wheat-dependent exercise-induced anaphylaxis (WDEIA) that occurs in certain individuals when the ingestion of wheat is followed by physical exercise (Palosuo et al., 1999). An RNA interference plasmid that targeted all omega-5 gliadin sequences from the cultivar Butte 86 was constructed and used to genetically transform Butte 86 plants (Altenbach and Allen, 2011). Quantitative 2-DE was used to determine the precise effects of the genetic modifications on the flour proteome in homozygous lines in which the omega-5 gliadins were significantly reduced (Altenbach et al., 2014a). In some lines, there were very specific changes in the proteome and only the omega-5 gliadins were suppressed. However, other lines showed both off-target and compensatory effects in addition to the suppression of the omega-5 gliadins. Mixing and baking studies were performed to assess flour quality of two transgenic lines in which there were few changes in the proteome other than the omega gliadins (Altenbach et al., 2014b). These lines showed improved mixing properties with significant increases in mixing times and mixing tolerances, suggesting that the omega-5 gliadins have a negative effect on wheat flour quality and that changes in flour quality as a result of growth conditions may be due in part to changes in the levels of these proteins. The same approach is being used to establish links between other target proteins and flour quality.

Since a major source of environmental variability was eliminated from the flour, it also was of interest to determine how the transgenic lines would respond to the environment in the absence of the omega-5 gliadins. An obvious question was whether other proteins would compensate for the loss of the omega-5 gliadins. To this end, both control and transgenic lines were grown in greenhouses with and without postanthesis fertilizer and total flour proteins were analyzed by quantitative 2-DE (Altenbach et al., 2014b). It is interesting that the omega-1,2 gliadins did not compensate for the decrease in omega-5 gliadins in the transgenic lines. In fact, with the exception of the omega gliadins, changes in the flour proteome in response to postanthesis fertilizer were similar in both nontransgenic and transgenic lines. Thus, the elimination of the omega-5 gliadins may result in plants with fewer changes in flour protein composition when grown under different levels of fertilizer. This suggests that the transgenic plants may have more consistent flour quality under changing environmental conditions, a hypothesis that could be tested further by growing plants in the field.

4.10 Evaluating Immunogenic Potential of Wheat Flour

Since the omega-5 gliadins trigger a serious food allergy, a proteomic approach also was used to evaluate the allergenic potential of flour from nontransgenic and transgenic lines. Two-dimensional immunoblot analyses were performed using sera from a set of 11 patients with confirmed cases of WDEIA and specific proteins that reacted with patient sera were identified by comparison to the Butte 86 proteomic map (Altenbach et al., 2015). Immunological responses were complex. Sera from 8 of 11 patients showed strong reactivity to the omega-5 gliadins in flour from the nontransgenic plant and little or no reactivity to omega-5 gliadins in the transgenic lines, indicating that the transgenic lines could be considered “reduced-allergen.” However, there were also low levels of reactivity with other proteins in the transgenic lines, particularly with certain LMW-GS. Sera from three WDEIA patients reacted primarily with alpha gliadins, HMW-GS, or nongluten proteins. For these patients, the immunogenic potentials of the transgenic lines were similar to the control line. The study highlights the range of immunological responses in patients with specific allergies to wheat flour and suggests that “reduced-allergen” wheat may not be suitable for consumption by patients already diagnosed with FA. However, “reduced-allergen” wheat could decrease the numbers of people that become sensitized to the omega-5 gliadins and enable more people to consume wheat products without serious allergic reactions. Importantly, the allergenic potential of wheat flour was reduced without negative effects on flour quality.

Gene silencing is an efficient method for eliminating immunogenic proteins, particularly when the goal is to target multiple related proteins. However, the resulting plants are considered genetically modified (GM). Thus far, GM wheat has yet to reach the marketplace because of issues with consumer acceptance. In future experiments, it may be advisable to use alternate approaches such as tilling (Uauy et al., 2009) or genome editing (Belhaj et al., 2013) to eliminate specific proteins from the flour since these methods result in plants that are not considered GM and could be rapidly deployed in breeding programs.

4.11 Other Proteomic Studies

While the previous studies highlight the types of detailed information that can be obtained once a comprehensive map of the flour proteome has been generated for a single cultivar, these studies also complement a large body of work done using other wheat cultivars. For example, numerous reports have focused on the responses of different wheat cultivars to a variety of environmental conditions (Altenbach, 2012 and references therein; Ge et al., 2012; Giuliani et al., 2015; Jiang et al., 2012; Pompa et al., 2013). While the results from the studies may not be directly comparable, they nonetheless provide important information and make it possible to begin to unravel the complex effects of the environment on wheat flour quality. Likewise, “allergenomics” approaches have proven more and more valuable for

understanding the immunogenic potential of wheat flour proteins. The combination of 1-D or 2-D immunoblotting and mass spectrometry has been a powerful strategy for identifying individual proteins that are immunogenic (Akagawa et al., 2007; Huebener et al., 2015; Pastorello et al., 2007; Sotkovsky et al., 2008, 2011) and for assessing the allergenic potential of GM wheat (Lupi et al., 2013, 2014). Published proteome datasets also have proved valuable for estimating the load of CD and FA epitopes in single cultivars (Juhasz et al., 2012). In addition, new mass spectrometry-based methods have been utilized to compare the levels of immunogenic proteins in flour from different sources (Barro et al., 2015; Rogniaux et al., 2015; Uvackova et al., 2013; Van den Broeck et al., 2015).

It is likely that proteomic approaches will continue to play a critical role in expanding knowledge of the many intricacies of the wheat flour proteins. Undoubtedly, rapid improvements in mass spectrometry instrumentation and methodology and the development of gel-free quantitative proteomic methods will offer considerable potential for future studies (Jorrin-Novo et al., 2015) that, in combination with biotechnology and breeding approaches, should contribute to the improvement of one of the world's most important food ingredients.

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Barley Grain Proteomics

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5.1 Introduction

Barley (*Hordeum vulgare*) has been grown as a cereal crop for thousands of years originating from the area of the Middle East known as the Fertile Crescent (Badr et al., 2000). It is a temperate dry land crop with a number of uses including as a food, either as a whole grain (e.g., in porridge) or as barley flour for bread making (e.g., in flatbreads), as animal feed and to make malt for beer, the earliest recorded example being 3000 BC (Iimure and Sato, 2013), or other fermented beverages such as whiskey. Malted barley is obtained when barley is steeped in water and then allowed to partially germinate until the coleoptile emerges. The partially germinated grain is then dried to yield malted barley. This malted barley is then ground to a powder and used to make beer (Goldammer, 2008). The germination to yield malt breaks down the starch into maltose and also begins to breakdown the storage proteins in the seed, hence the protein composition of malted barley grains differs from that of unmalted barley grains. The fermentation of this malt to make beer changes the protein composition such that very little of the barley proteins remain. In this review we will look at proteomic studies of malted and nonmalted barley as well as the barley proteins that remain in beer.

5.1.1 The Structure of the Barley Grain

The barley grain consists of three major components: (1) the aleurone, bran, and hull or husk layers that serve to protect the starchy endosperm; (2) the starchy endosperm that serves as nutrient storage for the growing embryo; and (3) the embryo or germ. These components are easily distinguished from each other in a cross-section of the barley grain as shown in Fig. 5.1.

The layers of cells comprising the hull, bran, and aleurone are quite complex. There are currently both hulled and hull-less varieties of barley grown commercially. The hull-less varieties have a loosely attached hull that is usually removed during harvesting. The hull of the hulled varieties can be removed by a dehulling machine when barley is being used for food as the hull is inedible. The dehulled barley still contains the bran cell layers and these layers are also sometimes removed by “pearling,” which is a technique for abrasive removal of the bran to yield “pearl barley.” The bran consists of multiple layers of dead cells with a

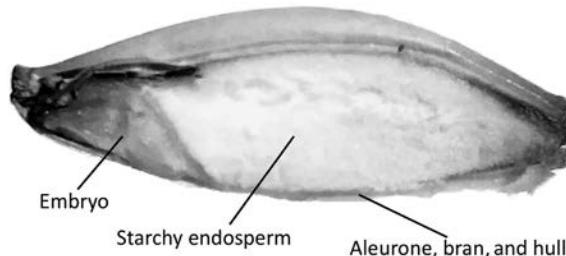


Figure 5.1

Cross-section of barley grain. The barley grain was sectioned longitudinally through the ventral groove of the grain.

thick tissue layer separating these dead cells from the live aleurone cells. The structure of these bran layers is similar in barley and wheat; however, the proteomes of individual barley bran layers have not been reported although this has been reported for wheat (Jerkovic et al., 2010).

The majority of barley grain proteins are found in the endosperm as this represents 80% of the grain by weight, with only 8%–10% of this weight being protein. The first layer of cells surrounding the starchy endosperm is of the aleurone cells, which is usually two to three cell layers thick. This aleurone tissue contains living cells and together with the germ contains the only viable cells in the grain. The aleurone cells are involved in producing hydrolytic enzymes such as α -amylase and various other hydrolytic enzymes to mobilize the food reserves in the endosperm for the developing embryo during germination. The hull and the multiple bran cell layers are present for protection of the endosperm from unfavorable biotic and abiotic environmental stresses.

5.1.2 Historical Background of the Barley Grain Proteome

One of the earliest studies of the protein composition of barley grain and malt was conducted by Osborne in 1895. The aim of the study was to extend Lintner's 1860s fractionation of diastase activity (amylase) from malt, wheat, and barley (cited in Osborne, 1895). Barley and malt proteins were fractionated using selective precipitation and solubilization in water and ethanol solutions. This fractionation approach was aimed at purifying and characterizing the “proteose” and diastase and resulted in a number of water soluble, ethanol soluble, and acidic ethanol insoluble protein fractions. Based on these studies, it is now generally accepted that barley endosperm consists of three main storage protein groups comprising the water soluble albumins and globulins, the alcohol soluble prolamins, known more specifically as hordeins in barley, and the acidic ethanol insoluble glutelins (Ingversen et al., 1973).

The study of proteins found in malted barley dates back even earlier than the work of Osborne as malted barley contains amylases. Amylase and other hydrolytic enzymes are produced by the aleurone and embryo to mobilize the food reserves in the endosperm for the developing



Figure 5.2
Malted barley.

embryo. These hydrolytic enzymes have been of use industrially since the early 19th century (Payen and Persoz, 1833).

5.1.3 Barley Malt and Beer

Malt is obtained by steeping barley in water to achieve a water content of 38%–45% with subsequent germination over 4–6 days. This malted barley is known as “green malt” and has an emerging coleoptile as shown in [Fig. 5.2](#). The malted barley is then dried in a kiln at 80–200°C depending on the type of malt required and is usually ground to a powder, but is sometimes used unground, to make wort for beer production. This dried malt is mixed with water and heated at various temperatures to make the wort, which is then filtered and finally boiled with hops. During these steps most of the proteins from the barley grain are lost, primarily due to precipitation from heat denaturation during the heating steps in wort production (Colgrave et al., 2013). The wort is then cooled and fermented with yeast to make beer (Goldammer, 2008).

5.2 Proteomic Analysis Techniques Used in Analysis of Barley, Barley Malt, and Beer

There have been a number of reviews of barley, barley malt, and/or beer proteomics (Colgrave et al., 2013; Finnie and Svensson, 2009; Iimure and Sato, 2013; Lastovickova and Bobalova, 2012). Many of the earlier studies of the barley proteome mostly relied on protein

identifications based on sequencing using Edman degradation, detection with antibodies, mass spectrometric (MS) analysis using protein sequences derived from expressed sequence tags, or the use of differential extraction coupled with gel electrophoresis to identify classes of proteins.

Gel-based protein separation techniques like sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) have been used for the analysis of barley proteins and more specifically for comparison of barley proteomes since the 1970s. This technique was extended by using a second dimension separation of proteins using isoelectric focusing allowing 2-dimensional gel electrophoresis (2-DE) (see for example [Gorg et al., 1992a,b](#); [Weiss et al., 1991a,b, 1992a,b,c](#)). Many 2-DE maps of barley grain and barley grain tissue fractions have been published providing visualization of changes in protein composition under different experimental conditions or comparative analyses. These types of comparisons have been shown in previous reviews, see [Fig. 5.1](#) in both ([Finnie and Svensson, 2009](#); [Iimure and Sato, 2013](#)) for example, and highlight the large changes in protein composition that occur during malting and in brewing and as shown in [Fig. 5.3](#) ([Perrocheau et al., 2005](#)). These maps have yielded useful information about the changes in the proteome of barley during the malting and beer-making process as well as information about protein isoforms. Gel techniques coupled with immunological detection of proteins have also been useful in studying the stability of barley proteins through the malting and brewing processes ([Iimure and Sato, 2013](#)). Immunological analysis such as western blots and ELISAs have advantages over gel-based proteomic techniques in terms of sensitivity and they have the advantage of

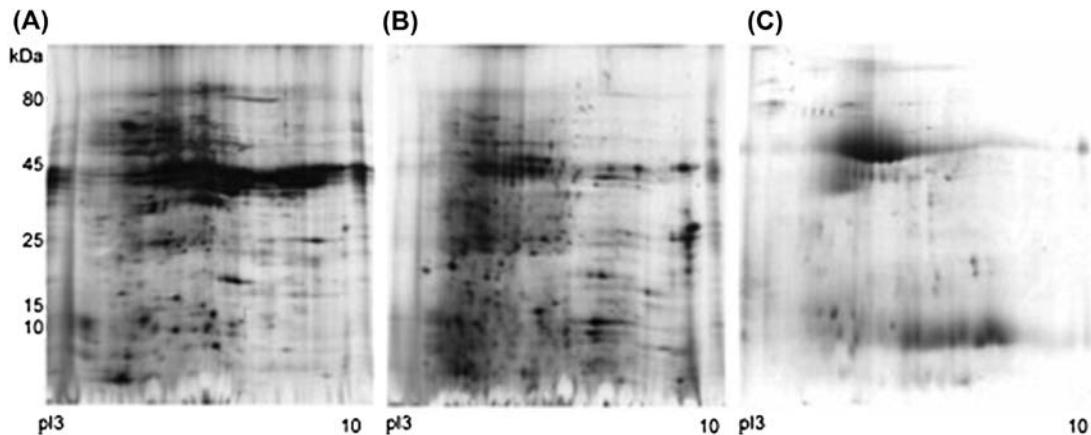


Figure 5.3

2-D gel protein patterns of water-soluble proteins of barley (A), malt (B), and beer (C). Proteins were separated in the first dimension on an IPG strip pI 3-10 and in the second dimension on a 15% acrylamide SDS gel. The proteins were silver stained. *Perrocheau, L., Rogniaux, H., Boivin, P., Marion, D., 2005. Probing heat-stable water-soluble proteins from barley to malt and beer. Proteomics 5, 2849–2858.*

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identifying specific antigens, which survive for example protein degradation, which can occur when barley is malted or during fermentation (Evans et al., 1999; Iimure et al., 2012).

However, if the antigens are modified during fermentation, for example by glycation (see in the following), immunological detection of barley has been shown to be unreliable compared to newer proteomic methods like mass spectroscopy (Tanner et al., 2013a,b).

The completion of the sequencing, mapping, and partial annotation of the 5.1 gigabase genome of the barley cultivar Morex in 2012 (International Barley Genome Sequencing Consortium et al., 2012) has meant that high-quality proteomic analyses using modern MS techniques are now made possible. The rapid improvement in both sensitivity and resolution of MS and sample preparation techniques coupled with the availability of the first sequenced genome for a barley cultivar has seen the various MS methods (e.g., MALDI-MS/MS, LC-MS/MS) becoming the method of choice for barley proteomic analyses (Lastovickova and Bobalova, 2012).

5.3 Barley Grain and Malting Barley Proteome

Many of the first studies of the global barley grain proteome were conducted in the 1990s using differential extraction of proteins from barley followed by 1-DE or 2-DE (Gorg et al., 1992a,b; Weiss et al., 1991a,b, 1992a,b,c). These studies identified the various hordeins, α and β -amylases, and glycosylated proteins and were able to discriminate between different barley varieties (Gorg et al., 1992a; Weiss et al., 1991a,b) or identify changes that occurred during malting (Gorg et al., 1992b; Weiss et al., 1992a,b) based on the pattern of protein bands in 1-DE, or spots in 2-DE. The advent of MS techniques coupled with Edman degradation has allowed identification of many of the protein spots in these 2-DE images. Finnie and Svensson reported in 2009 that 480 protein spots in 2-DE images of the barley grain proteome were identified, representing 300 unique protein sequences.

The molecular and genetic basis for the differing patterns of barley grain proteins from different barley cultivars has been determined using proteomic methods through integration of barley genetic and seed proteome maps (Finnie et al., 2009). This study found that the malting quality of barley cultivars could be more closely correlated with 2-DE maps of endosperm proteins than by the genetic similarity of cultivars deduced using microsatellites. This analysis identified single amino acid changes due to single nucleotide polymorphisms that resulted in changes in spots on the 2-DE map. The implication being that even minor point mutation changes in some proteins could influence the malting quality of barley.

Given the importance of barley for malt production, many of the proteomic studies have concentrated on the changes in the proteome of the embryo, aleurone, and endosperm of barley that occur during steeping and malting (Finnie and Svensson, 2009). Isolation of these separate tissues has proved to be important, especially when using gel-based proteomic methods, because the abundant endosperm proteins can obscure changes that may occur in

these tissues. Specifically, the serine protease inhibitors (serpins) are one of the most abundant protein families found in the endosperm and changes in protein composition occurring in the aleurone or embryo are often masked if whole grain is analyzed. Thus proteomic analysis of aleurone and embryo tissues have been conducted by dissecting out these tissues and analyzing them separately and temporally during malting (Boensager et al., 2007). This study identified that the earliest change in protein composition was the appearance of proteosomal subunit proteins in the aleurone and embryo. The other major early change was the reduction of heat shock proteins and other stress-related proteins in the same tissues (Boensager et al., 2007).

One of the main proteins produced during germination, which is of critical importance during malting, is α -amylase. There are two isoforms of α -amylase, a low pI AMY1 form encoded by four genes and a high pI AMY2 form encoded by six genes (Henrissat and Bairoch, 1996). Spatiotemporal proteomic profiling of these α -amylase proteins during germination detected only one of the four AMY1 gene products and two of the six AMY2 gene products, with the AMY1 appearing less susceptible to proteolytic degradation than the AMY2 forms (Bak-Jensen et al., 2007).

A recent, but interesting application of barley proteomics was the confirmation of the use of barley flour in sourdough bread excavated from a Subeixi cemetery (500–300 BC) in Xinjiang, China (Shevchenko et al., 2014).

Barley, like wheat, contains gluten and approximately 1% of the population worldwide suffers from severe gluten intolerance known as coeliac disease (CD), which is a T-cell-mediated immunological response to gluten. For people with CD, trace quantities of immunogenic proteins or their peptide fragments can elicit an immunogenic response that causes diarrhea, bloating, and other adverse health effects. The gluten proteins found in barley grain are known as the hordeins, comprising four families known as the B-, C-, D-, and γ -hordeins. Targeted MS-based approaches have been developed to identify hordein proteins in beer samples (Colgrave et al., 2012; Picariello et al., 2012) and more recently a very sensitive LC-MS/MS technique, using multiple reaction monitoring (MRM), was developed to detect barley contamination in cereal samples (Colgrave et al., 2016). MRM allows for specific, quantitative identification of multiple peptides in samples using LC-MS/MS. The authors (Colgrave et al., 2016) identified nine barley-specific peptides (four hordein and five nonhordein peptides) that were used to identify trace barley contamination of food samples.

5.3.1 Comparative Proteomics of Barley and Barley Varieties

The protein composition of barley grain varies significantly among feed and malting barley and to date there is only limited comparative genomic data to help differentiate the proteins between barley cultivars. Proteomic analyses using 2-DE and tandem MS on feed barley

cultivar, Yangsimai 3, and malting barley cultivar, Naso Nijo, were used to compare the proteome profile of both cultivars (Guo et al., 2016). Yangsimai 3 is a two-row feed barley variety with high protein content while the Naso Nijo is a two-row malting barley variety with a low protein content. The aim of this study was to identify the protein markers, which influence both the protein composition and to also evaluate the quality of the barley. Three biological replicates, 100 seeds per cultivar were subjected to 2-DE and MS analysis. Overall, 502 protein spots were reproducibly identified, 41 of these were found to be differentially expressed between the two cultivars with 34 of these identified by MS and classified into eight different biological processes including cell signaling, carbohydrate metabolic process, protein degradation, stress, development, and posttranslational modifications. Of these proteins, 13 of them were uniquely expressed in Yangsimai 3 and eight were uniquely expressed in Naso Nijo. A RAB28 GTPase known to be involved in developmental functions in eukaryotes was found only in Yangsimai 3 and two different 14-3-3 proteins involved in signaling were differentially expressed implicating them in regulation of protein expression. Given the observed differential expression of serpin-Z7, serpin-Z4, α -amylase inhibitor BDAI-1, and α -amylase/trypsin inhibitor CMb the authors suggest that quantification of these proteins may be useful in assessing malting quality.

5.3.2 Control of the Barley Grain Proteome Composition

Barley grain is also used as animal feed and one of the limiting factors in terms of nutritional composition of barley, for meat production, has been lysine content. One such high lysine mutant was identified, *lys3*, with the visual appearance of the grain and endosperm unchanged (Ingversen et al., 1973). The *lys3* mutant has a high lysine phenotype due to the drastic changes in amino acid composition and thus the protein composition within the endosperm. Proteome profiling using traditional gel-based methods revealed that the *lys3* mutant causes major changes in the proteome of barley endosperm by affecting the expression of B- and C-hordein, protein Z, β -amylase, and trypsin inhibitor CMe genes (Kreis et al., 1987; Rodriguez-Palenzuela et al., 1989; Sorensen et al., 1989).

In a novel analysis, Munck et al. (2001) compared a *lys3* mutant called *lys3a* and also known as Risø 1508, with the parent by comparing 2-DE of prolamins and albumins/globulins. The significant proteomic changes in these protein fractions were then correlated with near-infrared (NIR) spectroscopy of the whole grains. A synergy interval partial least squares regression model analysis of the NIR spectroscopy could distinguish between mutant and WT, suggesting that spectroscopic methods may be useful in screening for cereal mutants with large proteomic composition changes (Munck et al., 2001).

Barley transgenic lines have been produced, which have the C-hordein gene antisense. These lines have altered amino-acid composition with high lysine content and recent proteomic analysis of these lines reveals that, in addition to having reduced levels of C-hordein as

expected, the lines also have increased levels of proteins involved in metabolism and in the storage globulins that are high in lysine (Schmidt et al., 2016). Similarly, an ultra-low hordein line of barley has been bred from three low hordein lines of barley (Tanner et al., 2016). This ultra-low hordein barley was bred from Risø 1508, the lys3a mutant low in B- and C-hordeins; Risø 56, a B-hordein null mutant lacking the B-hordein genes; and Ethiopian R118, a D-hordein null mutant. This ultra-low hordein line has no detectable B-, C-, or D-hordein and only trace amounts of the γ -hordein detectable by immunological, gel, and MS proteomic methods. The malting and brewing properties of this line was also tested and an acceptable malt and beer was produced (Tanner et al., 2016).

5.3.3 Protein Glycosylation and Glycation in Barley and Barley Malt

Many of the barley proteins found in malt, in bran layers, and from the aleurone are secreted proteins. Most of these secreted proteins are glycoproteins, including the α -amylases (Bak-Jensen et al., 2007), β -glucanases (Doan and Fincher, 1992), the antifungal thaumatin-like protein (Gorjanovic et al., 2007), peroxidases (Laugesen et al., 2007), and aspartic and cysteine proteinases (Barba-Espin et al., 2014; Costa et al., 1997). Recently, isolated barley aleurone layers have been used as a model system for studying protein glycosylation and secretion (Barba-Espin et al., 2014). A total of 65 aleurone layer proteins were identified as being N-glycosylated using glycopeptide enrichment and N-glycosylation analysis. Of these 65 proteins, 29 were uniquely found in the extracellular matrix while 12 were uniquely found intracellularly with the remainder found in both fractions. Most of the glycosylated proteins were enzymes involved in carbohydrate modification, proteolysis, or lipid modification (Barba-Espin et al., 2014).

During malting, proteins can also be glycated, which is different from enzyme-catalyzed glycosylation. Glycation of proteins is a nonenzymatic process in which glucose residues, of mass \sim 162 Da, are covalently attached to proteins and peptides via the Maillard reaction during the kilning of malt commonly modifying Lys residues rather than Asn in the case of N-glycosylation or Ser/Thr in the case of O-glycosylation. It is thought that glycation protects the proteins from proteolytic degradation and can also increase their stability and solubility rendering them resistant to the harsh conditions during the malting and brewing process. The glycated proteins are important in beer foam stability as well as in the flavor, color, and aroma of beer. The majority of barley proteins identified in beer appear to be glycated to some extent (Petry-Podgorska et al., 2010).

5.4 Abiotic and Biotic Stress in Barley

With climate change and extreme weather events likely to decrease barley's range, as well as negatively affecting its yield and quality, it is important to determine how the barley proteome responds to abiotic and biotic stresses, not only to understand these biochemical processes but

also to identify the corresponding proteins, which assist in the development or selection of cultivars that have tolerance to stresses appropriate to the area in which they are to be grown.

In China 13,330 ha of farmland is contaminated with cadmium (Cd), which is a human health threat, so understanding the barley proteome's response to this abiotic stress is an important first step toward safe barley cultivation on this land. In a study by [Sun et al. \(2013\)](#), using 2-DE and MS, the authors compared the grain proteome of a Cd-tolerant and a Cd-susceptible barley cultivar (72.7% higher Cd in grain) after growth in Cd polluted soil. A greater number of significant proteins were seen in the susceptible cultivar, and carbohydrate metabolism, transcription, protein synthesis, and signal transduction were all upregulated in the susceptible in comparison to the tolerant cultivar. Interestingly, although protease inhibition, storage, and stress response proteins were upregulated in both cultivars, the proteins within each group had different functionality between the cultivars. For example, stress-response proteins in the susceptible cultivar were involved in pH control, osmotic stress, and chaperone functions, while proteins from the same functional group in the tolerant cultivar mediated cellular function, calcium signaling, and molecular transport. This latter protein functionality from the tolerant, as well as the greater number and more generalized functions of the proteins from the susceptible cultivar seem to indicate a subtler and more focused stress response to Cd from the tolerant cultivar's proteome.

Like abiotic stress, biotic stress also has a major impact on barley yield and/or quality, with the possibility of introduction of toxins or other compounds that effect animal or human health into the barley grain harvest. To better understand barley grain-microbe interactions, [Sultan et al. \(2016\)](#) examined the proteomes of the barley grain surface proteins and the microbes found on the seed surface. A combination of a 2-DE and an LC-MS/MS proteomics approach found that at the grain surface there was a relatively specific response to microbial colonization, with glucanases, chitinases, and alginate lyases, all being expressed to degrade bacterial, fungal, and algal cell walls. Lower levels of anti-fungal and apoptotic proteins, as well as low-molecular-weight microbial proteolytic-enzyme-inhibitors (which reduce mycelium growth and spore germination) were found, as were a number of proteins involved in a more general stress response such as thioredoxin reductase and a number of stress-related proteins. Microbial proteins were detected at a much lower level, but nonetheless a number of proteins from the bacterial membrane or that had transmembrane domains were detected, as well as others involved in the control of small molecule diffusion (such as antibiotics) and biosynthesis of secondary metabolites and toxins. Defense against the plant antimicrobial response was also seen in a number of microbial ROS scavenging enzymes and chaperones. In contrast, the fungal surface proteome showed a greater potential for plant tissue damage, with proteins involved in plant cell wall and peptide degradation (including xylanases and peptidases), as were proteins necessary to assist fungal growth, primary metabolism, nutrient acquisition, virulence factors, energy metabolism, RNA/DNA synthesis, and proteins involved in steroid and secondary metabolite synthesis.

A similar study, performed on infection of barley grain with the fungus *Fusarium graminearum* at anthesis, examined the grain proteome at different grain ripening stages during infection (Trumper et al., 2016). This fungus is economically important in terms of reducing grain quality, and because fungal mycotoxins can accumulate in products such as beer and effect production, and potentially, human health. 2-DE and MS results from this study showed a number of changes in the grain proteome throughout grain ripening. At early to middle stages of infection protease inhibitors and a putative chitinase were upregulated, as were proteins involved in oxidative burst (flood of ROS leading to programmed cell death), resulting in the neutralization of foreign hydrolytic enzymes, attack against fungal cells, and a physical barrier to foreign attack through cell death. At midinfection stages, upregulation of protein degradation and two thaumatin-like proteins involved in hyphal and spore lysis indicated that the plant cells were shifting toward survival and invader attack. Finally, at the latter stages of infection all proteins afore-mentioned were downregulated, including a notable decrease of those involved in oxidative burst and stress response. Thus, over a number of weeks of infection the barley grain proteome moved from defense, to attack, and finally a downregulated response, possibly because the threat had been managed or cell resources were exhausted.

5.5 Beer Proteomics

The beer proteomics literature has been recently reviewed and covered the proteins found in beer and wort (Colgrave et al., 2013; Iimure and Sato, 2013) and is also the subject matter of Chapter 23 in this book and as such the discussion of proteomic applications to beer will be limited. The concentration of proteins in beer is very low and the beer proteome is simple in terms of the small number of proteins identified (Iimure and Sato, 2013). However, analysis of very low abundance proteins present in beer is potentially more complex as it also includes proteins that originated from yeast and hops. The analysis of the most abundant proteins in beer has been primarily driven by quality issues in beer such as foam stability and chill haze, which impact on consumer purchase decisions (Evans et al., 1999). These types of quality issues usually relate to the concentrations and types of the abundant proteins found in beer. However, analysis and detection of low abundance proteins in beer is also of interest for other reasons such as CD (Colgrave et al., 2012; Picariello et al., 2012).

The two most abundant proteins found in beer, representing around 80% of the protein, are the 40kDa protein Z and the 10kDa lipid transfer protein (LTP) 1 (Iimure and Sato, 2013). These two barley endosperm proteins survive the malting and brewing process. Protein Z is a serpin based on its primary amino acid sequence (Roberts and Hejgaard, 2008) and there are a number of protein Z isoforms that are found in barley and persist in beer. The ability of protein Z isoforms to survive intact through the malting and brewing process may be due to their protease inhibitor properties (Dahl et al., 1996). LTPs are hydrophobic lipid-binding proteins that may escape proteolytic degradation because of their hydrophobic nature (Iimure and Sato, 2013). A combination of SDS-PAGE and ELISA assays have identified both of

these proteins as being important in both foam stability and chill haze (Evans et al., 1999). Chloroform methanol soluble proteins are another protein fraction identified as being important in chill haze and foam stability in beer, with trypsin inhibitor precursor and α -amylase inhibitor being two proteins in this fraction, which are correlated as being important in forming haze (Iimure et al., 2009). Hordeins are the other abundant class of proteins found in beer that have also been implicated in quality traits including chill haze and foam stability, as well as being important in CD (Colgrave et al., 2012; Iimure and Sato, 2013; Picariello et al., 2012).

5.6 Conclusions and Final Remarks

The recent advent of the barley genome sequencing consortium and the release of the first sequenced genome will complement proteomic techniques enabling a deeper probing of the proteins present in barley grain, barley malt, and food products. High-throughput workflows combined with high-resolution, high-sensitivity instrumentation will facilitate rapid proteomic profiling. Multiplexed comparative quantitative proteomics using isobaric labeling strategies such as isobaric tag for relative and absolute quantification (iTRAQ) and tandem mass tags (Bantscheff et al., 2012) will allow more comprehensive comparisons of barley cultivars. These quantitative proteomic methods could also be used in identification of proteins that will influence barley performance and quality in temporal processes like malting and brewing. The isobaric labeling methods appear to have only been applied a few times in barley proteomic analyses (Flodrova et al., 2012, 2015). In these reports the authors used iTRAQ to quantify the changes in the hordein peptide content during malting. Novel label-free data-independent acquisition strategies and targeted MS analyses will facilitate both relative and absolute quantification of a variety of barley proteins spanning a wide dynamic range allowing researchers to understand the interplay between environment and genetics.

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Proteomics of Soybean Plants

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6.1 Introduction

Soybean is a legume crop that has been cultivated for thousands of years in Northern and Southeast Asia and is currently being grown in many regions throughout the world, including the United States, South America, and Europe (Wilcox, 2004). Because soybean contains high protein and oil contents (seeds are composed of approximately 40% protein and 20% fatty acids), and also contains many bioactive components, the global consumption of soybean is projected to continue to increase based on its use in animal feed, vegetable oil, and biodiesel, as well as the human diet (D'Adamo and Sahin, 2014). The wide-ranging health benefits of soybean-based foods include the prevention of cardiovascular diseases, protection from breast cancer, lowered blood cholesterol, and relief from menopausal symptoms (D'Adamo and Sahin, 2014).

Efforts to improve the agronomic traits of soybean have largely contributed to the expansion of the cultivable area and increase in yields. However, even successful examples of genetically improved cultivars are sensitive to drastic changes in climate. In Brazil, which is the largest worldwide producer of soybeans followed by the United States, increased use of monocropping has resulted in lowered production yields (Thoenes, 2006). For example, Brazil's national soybean production in 2011/2012 decreased by 12.9% compared to the previous year because of drought (de Paiva Rolla et al., 2014). Prediction modeling of the impacts of El Niño Southern Oscillation (ENSO) on major crops suggests that the associated drastic changes in rainfall patterns on the Pacific coast of South America will have considerable negative effects on crop yields. Although ENSO positively affected the production of soybean in the United States and Brazil between 1984 and 2004 (Iizumi et al., 2014), soybean yields are predicted to decline by 30% and 50% in the United States and Brazil, respectively, if atmospheric carbon dioxide emissions continue to increase (Iizumi et al., 2013).

Increasing demand for stable supplies of quality protein under the threat of global warming has prompted the genetic modification of many plant species. For soybean, the development of environmental stress-tolerant cultivars is of high priority, but requires extensive molecular investigations of soybean physiology. Complete genome sequencing and functional analyses of genes have provided insights into many fundamental aspects of model legume plants

(O'Rourke et al., 2014). However, despite the importance of soybean as an economic crop, the soybean genome was only completely sequenced and annotated in 2010 (Schmutz et al., 2010). For this reason, proteomic approaches were widely used for the analysis of soybean gene products (Komatsu and Ahsan, 2009). Proteomics is a powerful tool for providing a comprehensive snapshot of protein expression under certain conditions and can also generate data on posttranslational modifications, protein–protein interactions, and selective degradation to obtain a system-level understanding of biological events (Altelaar et al., 2013). In the present review, the physiology of soybean at different developmental stages and under exposure to environmental stresses is reviewed in the first section, and the findings from the proteomic analyses of soybean with respect to food production are described and discussed in the second section.

6.2 Soybean Development and Cultivation

6.2.1 Seed Maturation

Plant seed development comprises a series of morphological, physiological, and biochemical changes and can be divided into three major phases: embryogenesis, including cell division and expansion, seed maturation, and desiccation. The maturation phase is characterized by a significant increase in seed size, formation of storage organelles, including protein storage vacuoles (PSVs) and oil bodies, and storage reserve accumulation of carbohydrates, which are received from the parent plant and are also directly synthesized by the developing seeds (Hajduch et al., 2011). The principal storage proteins in soybean are β -conglycinin and glycinin, which account for 25% and 40%, respectively, of total seed protein (Zarkadas et al., 2007). Accumulation of these storage proteins has been well studied and is influenced by the temporal and tissue-specific synthesis of their subunits (Meinke et al., 1981).

A comprehensive understanding of the physiology of seed maturation has been provided by the proteomic analysis of model plants (Deng et al., 2013; Hajduch et al., 2010; Gallardo et al., 2007). In soybean, multiple forms of a sucrose-binding protein, which was annotated as a putative transporter, showed increased expression during seed development (Fig. 6.1; Hajduch et al., 2005; Agrawal et al., 2008). Evidence suggests that sucrose assimilated from the mother plant is used to construct the cell wall during the early stages of seed development (Rolland et al., 2006) and also promotes the storage of carbohydrates. Comparative proteomic analyses have also revealed diverse pathways that are used for fatty acid synthesis among species (Hajduch et al., 2011). For example, in soybean and castor, specific fatty acid synthesis routes that utilize the malate pathway were detected (Houston et al., 2009; Hajduch et al., 2011). In contrast, the malate pathway does not appear to be active in rapeseed, although this species displays a dramatic increase in the expression of proteins involved in fatty acid synthesis during seed development. Taken together, these results indicate that the

accumulation of storage compounds, which are either in the form of carbohydrates or fatty acids, during seed maturation is regulated by species-specific mechanisms.

In addition to differences among species, cultivar-specific metabolic processes and pathways have been identified using a proteomics approach. The protein and oil contents in soybean cultivars vary between 36%–42% and 18%–22%, respectively and are generally negatively correlated. [Xu et al. \(2015\)](#) conducted a comparative proteomic study of protein- and oil-rich soybean cultivars and identified 40 differentially expressed proteins, including several key enzymes for fatty acid synthesis in the oil-rich cultivar. The identified glycolytic enzymes included triosephosphate isomerase, which was considered to be important because its enzymatic product, glycerone phosphate, plays a pivotal role in glycophospholipid metabolism ([Fig. 6.1](#)). Characterization of the oil composition between the protein- and oil-rich cultivars revealed that the former contained a higher amount of polyunsaturated fatty acids ([Xu et al., 2015](#)).

Because seed maturation is a critical stage of seed development, unfavorable conditions during seed formation could impair the germination of next-generation plants. Although soybean preferentially grows in tropical to temperate climates, excessive temperature and humidity during the maturation period lowers seed vigor ([Egli et al., 2005](#)). Under these conditions, a temperature-sensitive cultivar was adversely affected at early maturation stages and exhibited altered protein expression and deteriorative changes in cell membranes ([Wang et al., 2012](#)). The homeobox protein GmSBH1, which was identified among temperature and humidity stress-regulated proteins of soybean, can improve seed tolerance when overexpressed in *Arabidopsis* ([Shu et al., 2015](#)). Abnormal membranes are indicators of loss of vigor not only during seed maturation, but also during postharvest storage ([Parrish and Leopold, 1978](#)). Target genes for potentially increasing seed vigor are expected to be identified through detailed proteomic analyses of the seed maturation process and may lead to increased germination rates after seed storage.

6.2.2 Germination

Seed germination is a critical phase of the plant reproductive cycle and affects soybean productivity. After dispersion from the mother plant, metabolically quiescent dormant seeds germinate under favorable environmental conditions, including appropriate humidity, light, and temperature. Germination begins with the rapid uptake of water and the initiation of metabolic processes, following by the emergence of the radicle in phase III and establishment of the seedling ([Nonogaki, 2010](#)). The resumption of metabolism proceeds through an almost reverse process to that found during seed filling. To rapidly establish seedlings that can tolerate fluctuations in environmental conditions, the physiological change from seed dormancy to germination is controlled at both the transcriptional and posttranscriptional levels.

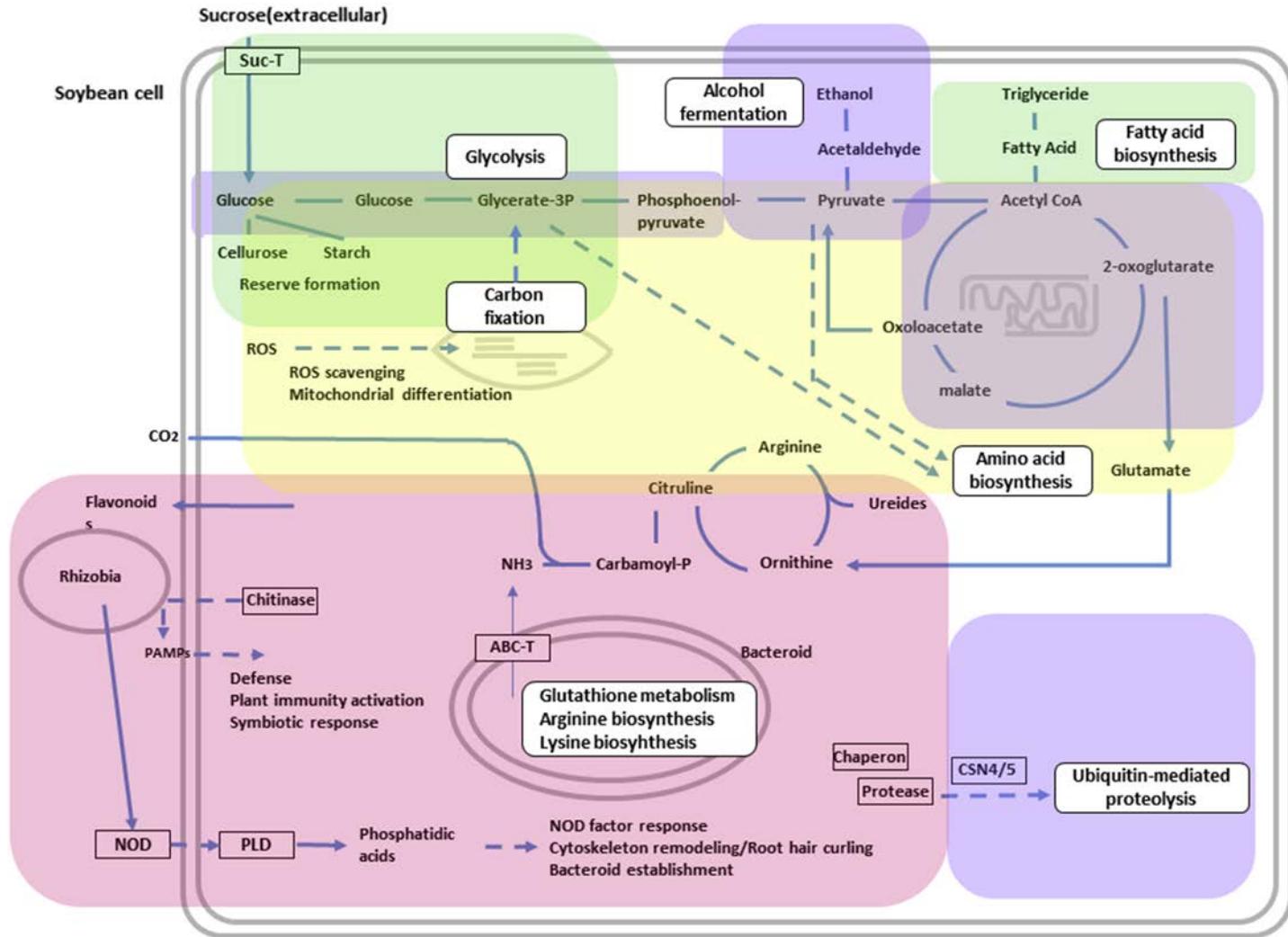


Figure 6.1

Global physiological changes in soybean and rhizobia, as revealed by proteomic analyses. Sucrose intake and reserve formation in the form of both carbohydrates and fatty acids are active during seed maturation (green); cells restore whole biological processes including respiration, photosynthesis, and protein translation during germination (yellow); and interaction with rhizobia induces changes in cellular shape and metabolic networks (red). Environmental stress (flooding) alters energy supply networks and protein quality control (purple).

The enzymes involved in the first step of reserve mobilization are translated from mRNAs that are stored during seed maturation. In rice, proteins that are translated from stored mRNA include metabolic proteins involved in carbohydrate breakdown (Sano et al., 2012). The proteome profiling of germinating soybean confirmed that proteins related to major carbohydrate and lipid metabolic pathways are expressed in the initial stages of germination (Fig. 6.1; Han et al., 2013). Subsequently, the number of PSVs decreases, as can be observed by the gradual mobilization of storage proteins (Kim et al., 2011). The detection of 26S proteasome components is consistent with the increase of total free amino acids (Han et al., 2013). In particular, the presence of methionine synthase, which is one of the most prominent proteins observed during germination and is related to amino acid metabolism, implies that active de novo protein synthesis occurs during germination (Han et al., 2013).

Reactive oxygen species (ROS) are generated during rehydration and respiration and act as signaling molecules for seed dormancy release (Bailly et al., 2008). Because ROS are capable of oxidizing and damaging proteins, ROS scavenging is crucial for coping with oxidative stress. The role of the disulfide reductase thioredoxin in oxidative stress defense was examined in *Medicago truncatula* (Alkhalfioui et al., 2007). The analysis identified several thioredoxin-linked proteins, which performed a wide range of functions, such as metabolism, protein biogenesis and degradation, storage proteins, binding proteins, and response to stress (Alkhalfioui et al., 2007). In addition, several heat shock protein (HSP) family proteins that are oxidized in dry seeds and function to protect other proteins were identified (Alkhalfioui et al., 2007). HSP family proteins are maintained at high levels in soybean seeds and when reduced, contribute to the release of seeds from dormancy (Hajduch et al., 2005). Proteomic analysis of germinating soybean also suggested that ferredoxin-thioredoxin reductase is newly synthesized in the seedlings (Fig. 6.1; Kim et al., 2011). Ferredoxin-thioredoxin reductase is a key enzyme in the photosynthetic redox cascade and is involved in the development of chloroplasts (Wang et al., 2014), which provide energy that supports plant growth following seed germination. The synthesis of ferredoxin-thioredoxin reductase may be involved in the control of soybean germination by linking a respiratory by-product to organelle maturation as a trigger in developing plants.

6.2.3 Symbiosis

Legume plants, including soybean, form symbiotic relationships with rhizobial bacteria, in which the plant provides reduced forms of carbon as an energy source for bacteria, and the bacteria supply the plant with nitrogenous compounds generated through nitrogen fixation (Clarke et al., 2015). The symbiosis between soybean and rhizobia involves complex, multi-level interactions and allows plants to grow in nitrogen-deprived soil. Plant roots secrete numerous compounds that induce root-microbe communication. During the early stages of the interaction, the root exudes flavonoids, a class of plant natural products (Pueppke et al., 1998), which attract and stimulate rhizobia to release signaling molecules called nodulation

(NOD) factors (Jones et al., 2007). Liao et al. (2012) studied the effects of exudate released by 6-day-old soybean under axenic conditions using a proteomic technique and found that most of the identified proteins were related to stress responses. In a comparative study of the legume plants white lupin, cowpea, and soybean, 14 proteins were commonly identified in at least two legume species. Most of the identified proteins were related to defense and included chitinase III-A (Fig. 6.1; Liao et al., 2012). Chitinase-like proteins are thought to function in plant-pathogen interactions by degrading the cell wall of invading organisms to produce pathogen-associated molecular pattern, which induce various plant defense responses. The overexpression of acidic chitinase III in sugarcane improves stress tolerance (Su et al., 2014). The large diversity of chitinase-like proteins among plants indicates that this enzyme family has an important role in plant immunity (Kesari et al., 2015).

Root hairs are typically the target site of infection by rhizobia. Wan et al. (2005) investigated changes in soybean root hair proteins following *Bradyrhizobium japonicum* infection and identified phospholipase D (PLD) and phosphoglucomutase as infection-responsive factors (Fig. 6.1). PLD plays a central role in signal transduction by hydrolyzing phosphatidylcholine to phosphatidic acids and alcohols and is required for NOD factor responses in *Lotus japonicas* (Serna-Sanz et al., 2011). In *Medicago truncatula*, PLD controls early nodulin N5 and promotes efficient nodule formation by restricting root hair curling (Pii et al., 2012). Rhizobial infection of root hairs is followed by bacterial invasion and nodule formation. In soybean nodules, rhizobia differentiate into bacteroids, which are surrounded by both a plant-derived symbiosome membrane and bacterial membrane (Clarke et al., 2015). Several chaperonins and proteases have been identified in the symbiosome membrane, suggesting that protein-processing pathways are activated by symbiosis (Panter et al., 2000). Adenosine triphosphate-binding cassette (ABC) transporter-related proteins have been detected in rhizobial membranes (Fig. 6.1; Sarma and Emerich, 2005; Koch et al., 2010). The importance of ABC transporters was demonstrated in *M. truncatula* infected with *Sinorhizobium meliloti* (Djordjevic, 2004). However, rhizobia appear to employ a host-specific symbiosis strategy, as deletion of the transporter affected symbiosis in siratro, but not in soybean (Koch et al., 2010).

In nodule cells, rhizobial bacteroids adjust their metabolism and are nutritionally dependent on the plant. A comparative proteomic study of free-living and symbiotic *Bradyrhizobium japonicum* revealed that multiple synthetic pathways are activated as indicated by the expression of glutathione synthetase 1, argininosuccinate lyase, aspartokinase, and succinyl-diaminopimelate desuccinylase (Sarma and Emerich, 2006). Efforts to characterize the complete bacteroid metabolic pathways are being undertaken using both proteomic and transcriptomic approaches (Delmotte et al., 2010). Soybean also induces specific metabolic pathways in response to the establishment of symbiosis. Mitochondria in nodules localize at the cell periphery and exhibit distinct specialization that is characterized by extensive cristae folding (Werner and Mörschel, 1978). When compared to mitochondria derived from

uninfected cells, nodule mitochondria contained 28 differentially displayed proteins, including seven specifically induced proteins (Hoa et al., 2004). Phosphoserine aminotransferase and glycine dehydrogenase complex were significantly upregulated among the identified proteins, indicating that amino acid biosynthesis was active in the nodule cells and proceeded using the nitrogen transported from bacteroids (Fig. 6.1; Hoa et al., 2004). As both similarities and differences have been observed between plant responses to symbiotic rhizobia and bacterial pathogens (Serna-Sanz et al., 2011), proteomic investigations of signal transduction networks are expected to provide novel insights into plant interactions with microbes.

6.2.4 Stress Responses

Crop growth and productivity are adversely affected by a number of environmental factors, particularly drought. For this reason, considerable efforts have been put into increasing the drought tolerance of agricultural crops, including soybean. Functional characterization of genes related to drought has led to the development of a number of genetically modified soybean cultivars with increased drought resistance (de Paiva Rolla et al., 2014; Yamaguchi-Shinozaki et al., 2006). Despite these efforts, it is predicted that excessive rainfall and flooding will be more frequent in the coming decades. Soybean is particularly vulnerable to flooding and exhibits impaired growth at all stages of development under flooding conditions. The influence of flooding is most severe during germination and causes significant damage to seedlings that markedly impairs the subsequent growth and maturation of the plant (Wuebker et al., 2001). However, once reaching the vegetative stage, soybean forms secondary aerenchyma under flooding stress to transport atmospheric oxygen to roots (Shimamura et al., 2010). Flooding is a complex phenomenon comprising several adverse environmental factors. Soybean responses to flooding stress include changes in multiple biological pathways at the cellular and whole-plant level. For this reason, elucidating the underlying mechanisms involved in the damage caused by flooding remains challenging.

The response mechanisms of soybean under flooding stress have been characterized using a proteomics approach (Komatsu et al., 2015). These analyses have revealed that glucose degradation and sucrose accumulation are accelerated during flooding, suggesting that energy consumption is shifted under low oxygen condition (Fig. 6.1; Nanjo et al., 2010).

Characterization of the mitochondrial proteome demonstrated that proteins functioning in the tricarboxylic acid cycle are upregulated, even though flooding stress induces disruption of the mitochondrial membrane integrity (Komatsu et al., 2011a). Introduction of alcohol dehydrogenase (ADH), an anaerobic protein that is upregulated in soybean in response to flooding stress, into soybean using a transgenic approach resulted in the reduction of flooding-induced growth inhibition (Fig. 6.1; Komatsu et al., 2011b; Tougou et al., 2012). The level of several proteins related to ROS scavenging are also increased in soybean under flooding conditions; however, the increase in expression is not due to changes in de novo protein synthesis, but is associated with retarded growth, as indicated by impaired root elongation and hypocotyl

pigmentation or modification of the protein degradation cycle (Nishizawa and Komatsu, 2011; Nishizawa et al., 2013).

Changes in protein expression through enhanced protein degradation occur when plants activate endogenous defense systems. Defense-/disease-related proteins are also severely affected in soybean under flooding. One of the most well-known protein degradation mechanisms is the ubiquitin–proteasome (Ub/26S) system, which mediates degradation of misfolded proteins, but is also involved in the maintenance of cellular metabolism (Ulrich, 2002). Yanagawa and Komatsu (2012) reported that flooding induced a decrease in ubiquitinated proteins in soybean root, although the levels recovered after soil drainage. In addition, the accumulation of COP9 signalosome subunits was observed under flooding conditions (Fig. 6.1; Yanagawa and Komatsu, 2012). Ubiquitin–proteasome-mediated proteolysis conceivably controls protein expression and determines the physiological responses of soybean to flooding stress.

Recently, upstream signaling events regulating responses to flooding stress have been studied. For example, the nuclear phosphoproteomic analysis of soybean root tips identified several proteins related to the abscisic acid (ABA) response (Yin and Komatsu, 2015). A number of studies have suggested that ABA has a major role in stress response to flooding. For example, ABA induces the accumulation of ADH in lettuce seedlings under oxygen deprivation (Kato-Noguchi, 2000). The biological relevance of the differentially abundant proteins in the flooding tolerance of soybean should be closely examined. Furthermore, elucidation of the roles of the multiple signaling pathways in stress tolerance and the significance of the cross-talk between these pathways is needed.

6.3 Soybean as a Food Material

6.3.1 Genetic Engineering of Soybean

Although soybean seeds with a high protein content are of high commercial value, several obstacles exist for the utilization of soybean as an economical protein source. One limitation is the presence of considerable amounts of allergenic proteins in soybean. Approximately 0.4% of children are allergic to soybean proteins (Savage et al., 2010). Soybeans are also deficient in sulfur-containing amino acids, such as methionine and cysteine, which are critical for the structure of proteins (Krishnan, 2001). Genetic engineering has the potential to alter soybean protein composition and quality, but may also result in unintended changes in the transgenic organism due to the pleiotropic effects of the targeted gene.

To facilitate genetic modification, variations in the overall allergenic protein abundance between cultivated and wild soybean varieties were described, and substantial variability in allergen abundance was observed (Fig. 6.2A; Natarajan et al., 2007, 2009; Natarajan, 2010; Gomes et al., 2014; Zarkadas et al., 2007). The effect of genetic modification on endogenous allergenicity was determined using an isoxaflutole- and glyphosate-tolerant soybean line,

FG72 (Rouquié et al., 2010). Differences in the accumulation levels of five major allergens between the two lines were detected, but no marked increases in allergenic proteins resulting from genetic modification were found (Fig. 6.2A; Rouquié et al., 2010). However, the allergen levels exhibited fluctuations based on the environmental conditions, indicating that the monitoring of allergenic protein levels is required when designing new soybean varieties with improved traits (Houston et al., 2011; Stevenson et al., 2012). Notably, Graf et al. (2014) stressed the importance of adequate scientific discussion about the utility of proteomic analysis of allergenic proteins in safety assessments of genetically modified crops.

The nutritional quality of soybean could be improved by raising the content of sulfur-containing amino acids in proteins. Attempts to redirect soybean protein synthesis by introducing foreign genes were assessed using proteomic technology (Herman, 2014). Because the seed-filling process is strictly regulated to ensure germination of next-generation plants, the

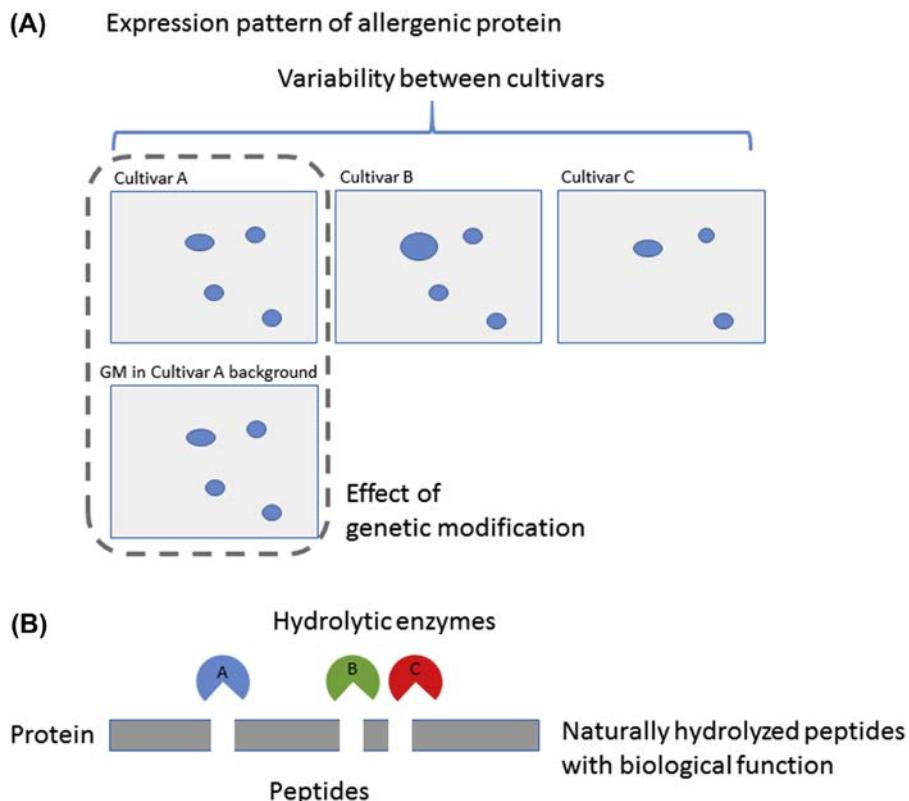


Figure 6.2

Proteomic technique for food evaluation. (A) Proteomic analysis is able to detect variations in target or allergenic/antinutritional proteins among different cultivars. Unintended changes in the proteomic profile caused by genetic modification can also be assessed. (B) Improved sensitivity of proteomic analysis enables the detection and quantification of bioactive peptides contained in soybean-derived foods or plant bodies.

seed protein composition of soybean is relatively stable and is rebalanced in response to genetic modification (Herman, 2014). For example, suppression of the expression of the major seed storage protein β -conglycinin resulted in an increase of the storage protein glycinin and no change in the total protein content (Kinney et al., 2001). Similarly, depletion of both α/β -conglycinin and glycinin by RNA interference (RNAi) was predominantly compensated by an increase in several strictly regulated proteins, such as Kunitz trypsin inhibitor, lectin, and Gly m Bd 30K (Schmidt et al., 2011). Because of this strong rebalancing mechanism, simply introducing heterologous genes in the wild-type soybean background is not expected to result in significant foreign protein accumulation. However, if the transgene is expressed in a background in which major proteins are suppressed, the foreign protein has the potential to accumulate at fourfold higher levels than that found in wild-type background plants (Schmidt and Herman, 2008).

The research group of Kim et al. produced a transgenic soybean line expressing maize δ -zein protein in a β -conglycinin RNAi knockdown background (Kim and Krishnan, 2004; Kim et al., 2014). Maize δ -zein is a sulfur-rich protein that is often used in maize genetic engineering research instead of α -zein, the most prominent storage protein, which is poor in lysine and methionine (Wu and Messing, 2014). The accumulation of δ -zein was increased in the transgenic soybean if adequate sulfur was provided to plants. However, if sulfur supplementation was insufficient, a total methionine in the plant body was limited, and the accumulation of δ -zein was markedly reduced (Kim et al., 2014). Using this approach, proteome profiling can be used to determine the physiological mechanisms that have the potential to interfere with the efficient genetic engineering of soybean. It was thus demonstrated that proteome rebalancing is not the sole obstacle for food fortification and that suitable conditions are needed to produce transgenic plants.

6.3.2 Detection of Soy Protein in Foods

Soybean allergens have the potential to trigger life-threatening reactions in sensitive individuals. Due to the large variety of human and animal foods prepared from soybean proteins, the development of accurate detection methods for soy proteins is essential. Although diverse detection methods have been reported, the enzyme-linked immunosorbent assay (ELISA) is used extensively to detect protease inhibitors, β -conglycinin, glycinins, Gly m Bd 30K, and Gly m 30 (Amnuaycheewa and de Mejia, 2010; Brandon et al., 1991; Ma et al., 2010; Morishita et al., 2008; You et al., 2008). The assessment of multiple detection methods revealed that sandwich ELISA combined with the polymerase chain reaction is a practical option for the detection of allergenic proteins in soybean (Pederson et al., 2008). As 10 ppm is the threshold for an allergenic reaction, the 0.05 ppm detection limit of sandwich ELISA is sufficiently sensitive for practical applications. Because food processing has the potential to change protein confirmation, the use of ELISA antibodies

against both native and modified proteins is highly recommended (Cucu et al., 2011). One ELISA system optimized to detect soy proteins in processed food is commercially available (Sakai et al., 2010). MS-based analysis of complex protein mixtures has been employed to detect soy-derived proteins with an eye on food control because of its high sensitivity. There have been some reports on detection of β -conglycinin by matrix-assisted laser desorption/ionization-MS or glycinin by liquid chromatography-mass spectrometry (LC-MS) (Heick et al., 2011; Cucu et al., 2012). For applications involving food safety assurance, manageable and sensitive detection methods are crucial for quality control. Further studies in soybean, which is one of the most prevalent allergenic foods in the world, are expected to aid in the establishment of more comprehensive and efficient assessment systems for allergenic proteins in food.

6.3.3 Soybean Proteins and Food Processing

Soy proteins are used not only as ingredients in a wide variety of prepared foods, but also as unique soy-derived foods (Fukushima, 1981). Traditional processing methods, such as soaking and fermentation, eliminate or markedly reduce the concentrations of harmful components, including allergenic proteins and antinutritional factors (Natarajan et al., 2006; D'Adamo and Sahin, 2014). Processed soybean proteins tend not to provoke severe allergic reactions compared to raw soybean proteins (Verhoeckx et al., 2015). Moreover, such processing methods may also potentiate the health-enhancing effects of prepared soybean-based foods by promoting the microbial proteolysis of soybean proteins and formation of small peptides that possess biofunctional properties (Fig. 6.2B). The health-enhancing functions of these peptides are often linked to lifestyle-related diseases and include antihypertensive, antiobesity, immunomodulatory, antidiabetic, and anticancer effects (Jang et al., 2008; Lee et al., 2014; McConnel et al., 2015; Shin et al., 2001). These peptides are generally between 3 and 20 amino acids in length, but can be composed of longer amino acid chains.

Lunasin is a 43-amino acid bioactive peptide with anticancer activity. It was initially discovered in soybean, but has subsequently been identified in several other crops, including barley, oats, rye, triticale, and amaranth (Hernández-Ledesma et al., 2009). The mechanistic action underlying the anticancer activity of lunasin is well characterized. After internalization in the murine fibroblast cell line C3H/10T1/2, lunasin binds to and inhibits the acetylation of histones H3 and H4 (Galvez et al., 2001). The acetylation and deacetylation of nuclear histones play a crucial role in the transcriptional control of S phase proteins, as well as cyclin E, by modulating chromatin structure (Csordas, 1990; Marks and Xu, 2009). Lunasin inhibits cell cycle progression at the G1/S phase and induces apoptosis in cells undergoing transformation (Galvez et al., 2001; McConnel et al., 2015). Cell cycle arrest induced by lunasin is related to the altered expression of G1-specific cyclin-dependent kinase complex components, such as p27Kip, phosphorylated Akt, and phosphorylated retinoblastoma protein (McConnell et al., 2015).

Bioactive peptides including lunasin can be artificially enriched (de Mejia et al., 2010). Several peptides of 11–14 amino acids in length with the ability to inhibit fatty acid synthase were isolated from β -conglycinin hydrolysates (Martinez-Villaluenga et al., 2010). Functional characterization of these peptides revealed that they bound and masked the thioesterase domain of fatty acid synthase (Martinez-Villaluenga et al., 2010).

LC-MS technology has greatly contributed to the characterization and quantification of small peptides in foods (Guíjarro-Díez et al., 2014). Future advances in LC-MS are expected to further accelerate studies on small peptides in both foods and plant bodies. In particular, the identification and functional characterization of bioactive soybean peptides is of interest for uncovering the physiological roles of these peptides in the living plant and for determining their therapeutic potential. However, additional research is required to expand the future application of soybean proteins as nutraceuticals.

6.4 Conclusions

Elucidating the mechanisms involved in the control of plant physiological systems and plant–environment interactions is essential to develop crops with desirable agronomic and nutritional traits, including tolerance to environmental stresses. In this review, the proteomic findings that have shed light on the pathway networks for development and growth, symbiosis, and stress responses. Although limitations exist in soybean proteomic analysis, proteomics in combination with metabolomics or transcriptomics can provide valuable data, such as shifts in the biological status of the plant body and circulation of the factors controlling signal transduction. Further improvements in the sensitivity of proteomic techniques will allow food containing soybean protein to be easily evaluated and screened for allergenic protein or bioactive peptides. The ability to analyze soybean for bioactive peptides will not only allow for the advanced utilization of soybean, but may also provide novel insights into soybean physiology.

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Proteomics of Hazelnut (*Corylus avellana*)

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7.1 Introduction

The hazel tree belongs to the birch family of the Betulaceae, Coryloideae subfamily, *Corylus* genus. So far, 11–15 botanic species have been described, the *Corylus avellana* or European hazel is the species most extensively grown worldwide. Avellana originates from Avella, an ancient town in Southern Italy, traditionally known for the production of excellent hazels (Shaw et al., 2014). The kernel of the hazel tree, known also as hazelnut, cobnut, or filbertnut, is edible raw or roasted. Hazelnuts are used widely in the food industry, either as an ingredient in confectionery (chocolate, nougat cookies, pralines, chopped nuts, nut spreads, and breakfast cereals) or in processed formulations (e.g., praline paste and hazelnut oil) (Platteau et al., 2011).

Over the last 10 years, hazelnut production has been largely concentrated in selected areas of United States (Oregon and Washington) and Europe (Italy and Turkey) each with more than 40,500 tons, followed by China, Georgia, and Azerbaijan with 40,000 tons, and then Spain, France, and Greece with less than 25,000 tons (FAOSTAT, 2014; Thompson et al., 1996).

Hazelnuts are storage organs rich in starch, proteins, and lipids. The consumption of 100 g of raw hazelnuts provides roughly 630 kcal, with 14.5 g protein intake, 60 g lipids (mostly represented by high-monounsaturated fats), and 16 g carbohydrates including fibers. Nuts are abundant in vitamin E and C, potassium, phosphorus, magnesium, and calcium (USDA National Nutrient Database for Standard Reference, <https://ndb.nal.usda.gov/>).

Knowledge on the hazelnut is expected to advance in the near future, with contributions from genomic projects aimed at sequencing the hazelnut genome as a model for the Betulaceae. Generally, the number and the type of the proteins expressed in an organism can be estimated based on the numbers of genes predicted from the genome sequences. However, the presence of the gene does not necessarily indicate the synthesis of the related protein. Therefore, proteomic investigation is essential for the identification and the characterization of the protein entities actually expressed in the seed.

7.2 Protein Family Classification and Functional Annotation

The storage proteins serve as a source of nitrogen, carbon, and sulfur during germination and seed growth. Hazelnut proteins can be classified according to their structural and functional characteristics. They represent the most abundant fraction of the seed and can be classified in families: the cupin superfamily, the prolamin superfamily, the pollen-related proteins, and the protective proteins.

7.2.1 The Cupin Superfamily

Globulins are salt-soluble proteins typically deposited in high amounts in the maturing nuts. A broad classification of these proteins is based on the sedimentation coefficient determined by ultracentrifugation (Mills et al., 2004). The legumins have a coefficient ranging between 11S and 13S while the vicilins and albumin have a coefficient of 7/8S. The hazelnut globulins have tertiary structures predominantly consisting of beta sheets (Rigby et al., 2008) as confirmed by circular dichroism (CD) and Fourier transform infrared spectroscopy (FT-IR) of the hazelnut purified proteins.

The 11/12S legumins are hexamers of about 50kDa subunits assembled through noncovalent interactions. These proteins are synthesized as single polypeptides which undergo posttranslational hydrolysis catalyzed by an asparaginyl endopeptidase. The enzyme cleaves specifically the Asn–Gly (NGFEET) bond splitting the protein in two polypeptide chains of 35kDa and pI 5.8 and of 22kDa and pI 9.10. The two chains are linked together via a single intermolecular disulfide bridge. The hazelnut 11S globulin-like protein (Swissprot entry: Q8W1C2) was identified for the first time as an IgE-binding protein by 2-D Western blotting, using sera of hazelnut allergic patients as the source of specific IgE antibodies (Beyer et al., 2002). Two internal amino acid motifs were determined by N-terminal sequencing. Screening of the hazelnut cDNA library prepared with oligonucleotides based on these peptide sequences resulted in the isolation of a novel protein cDNA (Beyer et al., 2002). In 2013 a new isoform of the 11S of 514 amino acid residues was registered (Swissprot entry: A0A0A0P7E3) (Grishina et al., 2013). The two isoforms share 96.5% homology. At least one additional protein of about 55 kDa, which shares high degree of homology with 11S globulins from other seeds, has been identified so far (Nitride et al., 2013). The protein consists of two disulfide-linked subunits as demonstrated by gel electrophoresis analysis. Shotgun proteomics allowed de novo sequencing of six peptides and their assignment to an 11S protein isoform was obtained by BLAST searching the proteome database. The top-scoring proteins were 11S globulin-like proteins from several plant organisms (*Pistacia vera*, *Sesamum indicum*, *Oryza sativa*, *Populus trichocarpa*, *Ricinus communis*) exhibiting significant or high mutual homology. The selected tryptic peptides also shared homology with the “canonical” hazelnut 11S globulin (Nitride et al., 2013).

The 7/8S vicilins are typically trimeric proteins of Mr about 150/190 kDa, with subunits of about 50 kDa. The hazelnut vicilin (Swissprot entry: [Q8S4P9](#)) has an apparent electrophoretic mobility of 48 kDa and its amino acid sequence contains at least two potential glycosylation sites (Asn-X-Thr). MS analysis of the tryptic peptides released from the purified natural protein confirmed the glycosylation at one site (Asn254). The consequent structural characterization of the N-glycans showed that the 74% of the glycans are high mannose type (MMX-type) and the 3% are complex type (MMXF-type) ([Lauer et al., 2004](#)). By MALDI-MS analysis, [Müller et al. \(2000\)](#) characterized the complex glycans as xylose-containing carbohydrate moieties: Man3XylGlcNAc2.

7.2.2 The Prolamin Superfamily

Albumins are water-soluble proteins with sedimentation coefficient 2, typically synthesized by dicotyledonous plants. 2S albumins are heterodimeric proteins with characteristic structural traits including four α -helices and eight cysteines engaged in four disulfide bridges ([Pfeifer et al., 2015](#)). Similar to legumins, the hazelnut 2S albumin (Swissprot entry: D0PWG2) precursor protein is posttranslationally cleaved into a large and a small subunits linked via disulfide bonds ([Garino et al., 2010](#)). [Pfeifer et al. \(2015\)](#) showed the cyclization of the N-terminal glutamine to pyroglutamic acid, describing a typical event that occurs in 2S albumins from various species. The small subunit is variably truncated at the C-terminus, leading to a large degree of micro-heterogeneity also impacting the IgE-binding properties. This heterogeneity does not occur at the N-terminus. The purified protein is thermostable preserving the native protein folding up to 90°C ([Mills et al., 2007](#)). 2S albumin is also very resistant to proteolytic degradation under simulated human gastrointestinal conditions ([Moreno and Clemente, 2008](#)).

The nonspecific lipid transfer proteins (ns-LTP) can be integrated in the huge superfamily of prolamins. The name of the protein derives from the ability to nonspecifically transport lipids within the cellular intermembrane space ([Marion et al., 2004](#)). The ns-LTPs exist in a large number of plant organisms and consist of two congeners, namely ns-LTP1 and ns-LTP2 with MW 9.6 and 7.0 kDa, respectively. In *C. avellana* only ns-LTP1 has been characterized (Swissprot entry: [Q9ATH2](#)). The mature protein is a unique polypeptide chain of 92 amino acid residues with eight strictly conserved cysteines that form four intrachain disulfide bridges. The protein is monomeric with a molecular weight of about 9.5 kDa and alkaline pI of about 9.30 ([Schocker et al., 2004](#)). The secondary structure is composed of four α -helices and a long C-terminal tail that is devoid of defined secondary structure ([Rigby et al., 2008](#)). The 3D structure of purified and crystallized ns-LTP has been recently determined by X-ray diffraction, representing the first high-resolution model of a hazelnut protein ([Offermann et al., 2015](#)). Comparative molecular dynamic simulation has been performed after molecular replacement using the ns-LTP from peach as starting model.

7.2.3 Pollen-Related Proteins

The pathogenesis-related (PR) proteins comprise part of the general defense mechanism of the plant, being overexpressed upon pathogenic attack, stress, and abiotic stimuli (Fernandes et al., 2013). This family includes up to 17 classes of unrelated proteins. The Bet-v1 (*Betula verrucosa*) homolog protein belongs to the PR-10 class with no defined role to date. The hazelnut PR-10, known as Cor a 1 allergen, shares only 63% and 71% identity with the two hazel pollen isoforms. The protein is a monomeric polypeptide chain of about 160 residues with a molecular weight of approximately 17.4 kDa and pI 6.1. Its four proteoforms share 97%–99% sequence identity (Lüttkopf et al., 2002) (Swissprot entries: Q9SWR4, Q9FPK4, Q9FPK3, Q9FPK2). At least three additional isoforms of the PR-10 have been isolated from the cDNA, cloned and characterized (Swissprot entry: Q39453, Q39454, Q08407). Their alignment shows only the 50% identity with the previously described four proteoforms and 60% homology between these isoforms (Breiteneder et al., 1993; Hoffmann-Sommergruber et al., 1997). The secondary structure of the PR-10 is extremely conserved among the different species. The protein appears to be highly stable, rigid, and curved due to the presence of seven-stranded antiparallel β -sheets embracing in a palm-like grip a long C-terminal α -helix (Fernandes et al., 2013).

The profilins are multigene proteins capable of sequestering the actin monomers (G actin) and to inhibit actin filament growth, which involve poly-L-proline (PLP) stretches. The cDNA sequences obtained after RT-PCR from pollen of *C. avellana* reveals the presence of at least 10 encoding genes. The UniProtKB/Swiss-Prot database contains eight isoform sequences for the hazelnut profilins (entry: A4KA40, A4KA44, A4KA45, A4KA43, A4KA41, A4KA39, Q9AXH5, Q9AXH4). The homology of the amino acid sequences varies between 75.7% and 100% (Jimenez-Lopez et al., 2012). The protein length is conserved with 131–133 amino acid residues giving rise to a molecular weight of 14 kDa and pI 4.9. The secondary structure changes among isoforms but is mainly characterized by six α -helices and one β -sheet. Little is known about the 3D structure of hazelnut profilins. It may be mainly stabilized by hydrogen bonds and electrostatic interactions. The disulfide bridge for the pair Cys¹³ and Cys¹¹⁷ seems to be essential in preserving the stability of the PLP interacting surface (Jimenez-Lopez et al., 2013).

7.2.4 Regulatory Proteins

Low molecular weight, or small, heat-shock proteins (sHSPs) are generally accumulated in seed plants in response to high-temperature stress. These proteins can be also expressed in the absence of stress, during the developing of the seed, and might play a role in dehydration tolerance, dormancy, or germination (Wehmeyer et al., 1996). The cDNA sequence suggests that a putative hazelnut HSP has a sequence of 150 amino acids (Swissprot entry: Q9ZPQ0). The characteristic hydrophobic domain of the HSP, GVLTV is located within the (64–135) conserved C-terminal domain (Rigola et al., 1998).

Luminal binding protein, also known also as immunoglobulin binding protein (BiP), is located in the endoplasmic reticulum (ER) where it interacts with polypeptide folding intermediates transiting the secretory compartment. BiP is a member of the HSP-70-related family, mutually sharing 70%–75% of sequence identity. This protein is expressed during seed maturation and is associated with conditions of stress. The hazelnut BiP is a soluble nonglycosylated protein with apparent molecular weight 70 kDa (Swissprot entry: [Q9FSY7](#)). cDNA analysis revealed that the mature chain was 639 amino acid in length with a theoretical molecular weight of 70.6 kDa and acidic pI of 4.92. The expressed protein was purified from hazelnut pollen and characterized. Two-dimensional Western blot analysis showed that BiP consists of at least two acidic polymorphic forms at pI 5.7 and 6.1. The difference between calculated and actual pI were ascribed to posttranslational phosphorylation of the proteins. Indeed, at least 14 potential phosphorylation sites for different kinases have been detected in the sequence ([Fontesm et al., 1991](#)).

Plant lipoxygenases (LOXs) are enzymes which display diverse functions in several physiological processes such as growth, development, and response to biotic and abiotic stresses. These enzymes catalyze the addition of oxygen to polyunsaturated fatty acids. The development of off-flavors in foods is a direct consequence of lipid oxidation, with development of volatile aldehydes such as hexanal and nonanal. The enzymatic reaction generally occurs at the C-9 or C-13 residue of mono- or polyunsaturated fatty acids, therefore the enzymes are referred to as 9-LOXs or 13-LOXs, respectively ([Santino et al., 2003](#)). The cDNA deduced amino acid sequence of the hazelnut LOX (Swissprot entry: [Q93YI8](#)) showed a high homology with the previously characterized almond counterpart sharing 79.5% homology. The protein consists of two domains: PLAT domain of 126 and the enzymatic domain of 697 amino acidic residues. The hazelnut LOXs show dual product specificity forming a mixture of 9- and 13-hydroperoxides in an approximate 2:1 ratio ([Santino et al., 2003](#)).

The thaumatin-like proteins (TLP) can be divided into three classes responding to pathogen attacks (PR-5), osmotic stress (osmotins), and antifungal proteins. These polypeptides have molecular weights ranging 20–30 kDa. TLP are cysteine-rich with eight disulfide bridges and very stable 3D structural organization rendering the protein resistant against proteolysis, pH, and heat denaturation ([Breiteneder, 2004](#)). Hazelnut TLP has been not structurally characterized yet, but the ability to elicit immunoreaction has been described. Ten percent of peach allergic patients from seven Spanish geographical areas, with different pollen profiles, were found cross-sensitive to purified TLP from hazelnut ([Palacín et al., 2012](#)). A discrepancy has been noted for the annotation of hazelnut TLP in this latter paper, because the UniprotKB protein entry ascribed to hazelnut TLP actually corresponds to apple TLP 1b (*Malus domestica*). To the best of our knowledge, no protein entry in protein databases corresponds to hazelnut TLP.

7.2.5 *Lipophilic Proteins*

The storage triacylglycerols, accumulated in plant seeds and other oil-storing tissues, are packed in distinct particles called lipid bodies which consist of a lipid core surrounded by a monolayer of phospholipids contributing to keep the size and the stability (Vance and Huang, 1987). The oleosins are interpolated within the monolayer interacting with lipids and phospholipids and contribute to stabilize lipid bodies during desiccation of seeds in order to prevent coalescence of the oil bodies (Akkerdaas et al., 2006). Oleosins are alkaline proteins ($\text{pI} \sim 10$) with molecular weight ranging between 15 and 25 kDa. Oleosin primary structure can be divided into three domains. The three structural domains appear to be formed by a conserved central hydrophobic domain (70 amino acid residues, rich in lysine and prolines), containing the highly preserved sequence PLFIIFSPVLVP distinguishing the oleosin family (Akkerdaas et al., 2006), flanked by more variable N- and C-terminal domains. The C-terminal domain forms an amphipathic α -helix located on the oil body surface while the N-terminal domain may contain a mixture of structures and be located in the cytoplasm. In contrast, the β -sheet-rich hydrophobic domain is thought to be buried within the triacylglycerol matrix (Napier et al., 2001). Two isoforms of hazelnut oleosins have been identified so far, based on the cDNA library screening. Probably due to the alkaline pH and to their hydrophobic nature, oleosin isoforms escape 2-DE detection. Thus, oleosin have been characterized at the protein level using a targeted approach based on 1D SDS-PAGE, Western blotting, and mass spectrometry (Zuidmeer-Jongejan et al., 2014). The two proteins have an electrophoretic MW of 14 and 17 kDa with 32% sequence homology, probably differing in their synthesis during development or in their tissue specificity. One isoform has 159 amino acidic residues (Swissprot entry: Q84T21), while the second is shorter with 140 AA owing to a gap of 16 AA (starting from the 13th residue) and the lack of the last three amino acids (Swissprot entry: Q84T91).

7.3 *Molecular Characterization and Proteomics*

Extraction is a key concern in the workflow for the analysis of proteins from tree nuts. In order to define the optimal conditions to maximize extraction yields and to limit loss of specific components, the effects of several factors, including types of buffer, extraction time, and use of defatting processes, on protein extractability have been recently investigated (L'Hocine and Pitre, 2016). High temperature ($>50^\circ\text{C}$) and high buffer-to-protein ratio (1:250, w:v) significantly increased protein recovery, while longer extraction time did not improve yields. The roasting and the defatting processes considerably reduced the protein recovery. The effect of the extraction conditions on the IgE-binding capacity has been also studied. Roasting does not drastically affect the IgE recognition, whereas the defatting decreases the IgE-binding of specific components. In particular, vicilin, the fat-soluble/associated protein oleosin and lipid transfer proteins may be lost during the defatting process.

Once proteins have been efficiently extracted, the univocal identification of the hazelnut proteins still remains challenging, in particular when using a “classical” two-dimensional electrophoresis (2-DE)-omic approach (Schocker and Becker, 2001; Hird et al., 2000). One and 2-DE analysis of raw hazelnut proteins (Fig. 7.1, data not published) show the high complexity of the proteome, due to the presence of multiple components with close molecular weight and pIs. This heterogeneity displayed alludes to the existence of several proteoforms, made up of multiple glycoforms, and posttranslationally protease-cleaved subunits. Only the major proteins were identifiable using a bottom-up strategy based on in-gel trypsin digestion and mass spectrometry (MS) analysis. Due to its intrinsic pitfalls, the classical 2-DE-based proteomic approach does not facilitate identification of low level proteins or those with extreme MW and/or pIs. Furthermore, many protein components escape identification owing to their absence in protein databases either due to incomplete genome sequencing efforts or lack of annotation.

To improve the proteome coverage, 2-DE analysis has been integrated with chromatography-based methods and MS characterization. To this end, crude hazelnut phosphate-buffer saline (PBS) extract was separated by RP-HPLC coupled to ESI-MS and in parallel eluted fractions were scrutinized by either native or reducing SDS-PAGE (Fig. 7.2, data not published). Protein identification was carried out by MALDI-TOF and LC-ESI-MS/MS peptide mass

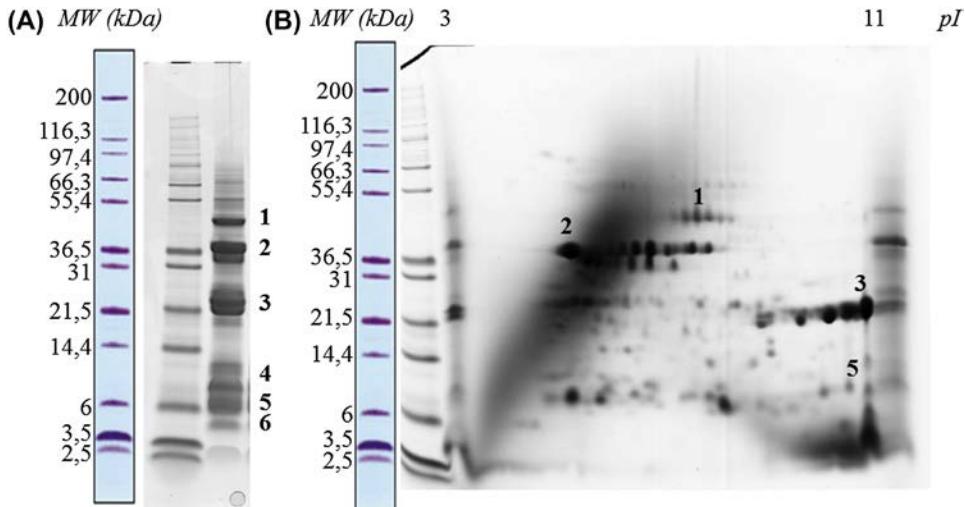


Figure 7.1

1D gel electrophoresis (panel A) and 2D IEF-SDS PAGE (panel B) of crude protein extract from raw hazelnut (in 0.1 M PBS, 1:10 w/v). The 4% to 12% gradient gel was stained with Coomassie. The in-gel trypsin digestion of the excised protein bands followed by mass spectrometry analysis combined with database interrogation allowed the localization of (1) 7S 48 kDa glycoprotein, (2) acidic subunit 11S globulin-like protein, (3) basic subunit 11S globulin-like protein, (4) 2S albumin, (5) ns-lipid transfer protein, and (6) heat shock protein.

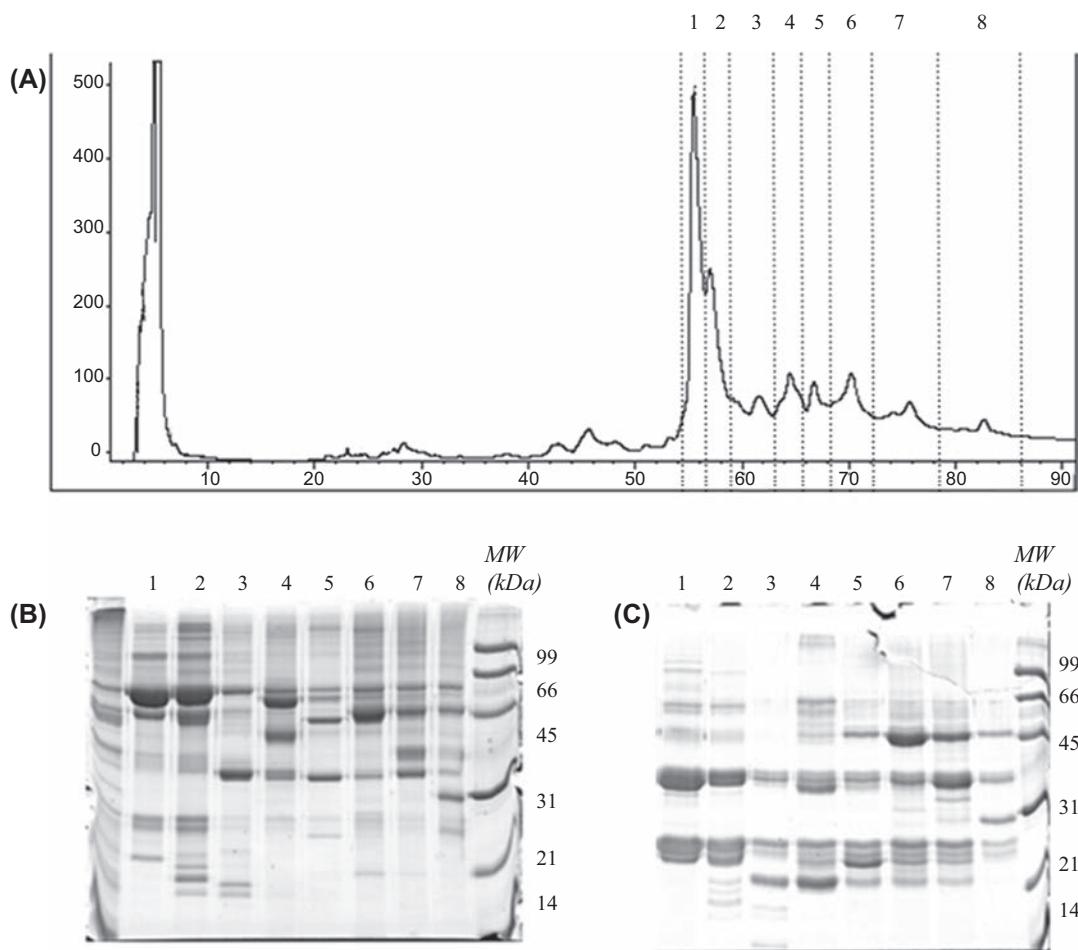


Figure 7.2

PBS soluble proteins were fractionated by reversed phase (RP)-HPLC using an HP 1100 Agilent Technology modular system (Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C8 column (208TP52, 5 μ m, 2.1 \times 250 mm). After 10 min of isocratic elution using 25% solvent B (0.1% TFA in acetonitrile, v/v), a fractionated step was applied: 25%–30% B for 5 min, 30%–35% B for 30 min, 35%–50% B for 60 min, 50%–55% B for 15 min at a flow rate of 0.200 mL/min. Solvent A was 0.1% TFA in water (v/v). For each analysis, approximately 200 μ g of hazelnut proteins, dissolved in 0.1% TFA, were injected. Column effluents were monitored by UV detection at 220 and 280 nm. Protein fractions were manually collected and used for both native (panel B) and reducing 1D 12% SDS-PAGE (panel C).

fingerprinting following trypsinolysis. The main chromatographic peak (peak 1, panel A) corresponded to an intense single band of about 60 kDa in the native gel (panel C) identified as the 11S globulin-like protein, which was split in two bands of 35 and 25 kDa upon reduction of disulfide bonds. The minor unresolved HPLC peaks numbered 6–8 in Fig. 7.2A corresponded to 7S vicilin, whose glycoforms were dispersed over a relatively large retention

window. Native electrophoretic analysis of the HPLC peaks (panel B) showed the presence of additional minor proteins.

Plants of the tree nut family share extensive genomic homology, such that protein identification can be performed by homology with gene products (protein orthologs) indexed for other seeds. In this way, proteins can be identified through automated database searching of tandem MS (MS/MS) data and also allows de novo peptide sequencing. In the shotgun proteomic approach, the whole proteome or a protein subset is proteolytically digested and resulting peptides are analyzed by LC-MS/MS. Protein identity is inferred by bioinformatics-assisted association of MS/MS-sequenced peptides to the parent proteins. The list of hazelnut protein entries identified by homology with shotgun proteomics is reported in [Table 7.1](#) (data not published). Recently, a similar approach applied to investigate the proteome of the *Corylus heterophylla* discovered 303 proteins, among which 183 (73.8%) have catalytic activity (e.g., oxidoreductase, kinase, peptidase), and 170 (68.5%) carrier or binding activity ([Chunlei et al., 2015](#)).

Table 7.1: Summary of Mascot search results from shotgun proteomic analysis of total hazelnut extracted proteins.

| DB | Accession | Score | Mass (Da) | Matches (Unique) | Description |
|--------|--------------|-------|-----------|------------------|--|
| NCBInr | gi 557792009 | 802 | 59,200 | 52(36) | Cor a 9 <i>Corylus avellana</i> |
| NCBInr | gi 18479082 | 676 | 59,605 | 46(30) | 11S globulin-like protein <i>C. avellana</i> |
| NCBInr | gi 19338630 | 270 | 51,110 | 18(11) | 48 kDa glycoprotein precursor <i>C. avellana</i> |
| NCBInr | gi 743909002 | 118 | 32,928 | 2(1) | Predicted: putative lactoylglutathione lyase <i>Populus euphratica</i> |
| NCBInr | gi 22135427 | 110 | 62,814 | 3(2) | Castanin <i>Castanea crenata</i> |
| NCBInr | gi 719977520 | 103 | 36,529 | 3(0) | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic-like <i>Nelumbo nucifera</i> |
| NCBInr | gi 604298903 | 96 | 51,680 | 3(1) | Hypothetical protein MIMGU_mgv1a024892 mg <i>Erythranthe guttata</i> |
| NCBInr | gi 157384600 | 94 | 96,650 | 2(2) | 7S vicilin <i>Carya illinoensis</i> |
| NCBInr | gi 388514675 | 90 | 36,480 | 3(0) | Unknown <i>Lotus japonicus</i> |
| NCBInr | gi 697124150 | 82 | 36,829 | 3(0) | Predicted: glyceraldehyde-3-phosphate dehydrogenase, cytosolic-like <i>Nicotiana tomentosiformis</i> |
| NCBInr | gi 7484767 | 82 | 57,185 | 2(0) | Probable major protein body membrane protein MP27/major protein body protein MP32 precursor—cucurbit |
| NCBInr | gi 6580762 | 82 | 70,802 | 2(1) | Vicilin-like protein precursor <i>Juglans regia</i> |
| NCBInr | gi 568214447 | 81 | 36,815 | 3(0) | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic-like <i>Solanum tuberosum</i> |
| NCBInr | gi 976921531 | 81 | 31,505 | 3(0) | Glyceraldehyde 3-phosphate dehydrogenase, catalytic domain-containing protein <i>Cynara cardunculus</i> var. <i>scolymus</i> |

Continued

Table 7.1: Summary of Mascot search results from shotgun proteomic analysis of total hazelnut extracted proteins.—cont'd

| DB | Accession | Score | Mass (Da) | Matches (Unique) | Description |
|-----------|---------------|-------|-----------|------------------|---|
| NCBInr | gi 13507262 | 81 | 12,368 | 2(0) | Lipid transfer protein precursor <i>C. avellana</i> |
| NCBInr | gi 720070447 | 75 | 36,844 | 2(1) | Predicted: malate dehydrogenase, mitochondrial <i>N. nucifera</i> |
| NCBInr | gi 1026084716 | 73 | 36,147 | 2(1) | Predicted: malate dehydrogenase, mitochondrial <i>Capsicum annuum</i> |
| NCBInr | gi 449454752 | 71 | 33,501 | 1(1) | Predicted: putative lactoylglutathione lyase <i>Cucumis sativus</i> |
| NCBInr | gi 703128551 | 70 | 56,447 | 2(0) | Vicilin-like antimicrobial peptides 2-2 <i>Morus notabilis</i> |
| NCBInr | gi 11762102 | 69 | 17,614 | 3(0) | Major allergen variant Cor a 1,0402 <i>C. avellana</i> |
| NCBInr | gi 502091513 | 67 | 36,641 | 2(0) | Predicted: glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic <i>Cicer arietinum</i> |
| NCBInr | gi 590669895 | 65 | 36,785 | 2(0) | Glyceraldehyde-3-phosphate dehydrogenase C subunit 1 <i>Theobroma cacao</i> |
| NCBInr | gi 262317107 | 63 | 13,226 | 2(0) | Glyceraldehyde-3-phosphate dehydrogenase <i>Manihot esculenta</i> |
| NCBInr | gi 223502611 | 58 | 21,854 | 1(1) | Hypothetical protein RCOM_2131030 <i>Ricinus communis</i> |
| NCBInr | gi 629093630 | 54 | 25,738 | 1(1) | Hypothetical protein EUGRSUZ_H02364 <i>Eucalyptus grandis</i> |
| NCBInr | gi 600108 | 53 | 54,977 | 1(1) | Legumin A precursor <i>Vicia narbonensis</i> |
| SwissProt | Q9C7X7 | 52 | 68,656 | 1(1) | Heat shock 70 kDa <i>Arabidopsis thaliana</i> |
| NCBInr | gi 326506132 | 52 | 71,476 | 1(0) | Predicted protein <i>Hordeum vulgare</i> subsp. <i>vulgare</i> |

Picariello, G., Mamone, G., Nitride, C., Addeo, F., Camarca, A., Vocca, I., Gianfrani, C., Ferranti, P., 2012. Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides. *J. Proteomics* 75, 5872–5882.

A rapid expansion of the hazelnut proteome annotation is expected in the near future, associated with the genomic sequencing efforts and the complementary application of high-resolution MS instruments.

7.4 Hazelnut Allergens

The consumption of nuts is associated with several interesting health benefits, for example, contributing to prevent coronary diseases and to control cholesterol serum levels (Crespo et al., 2006). On the other hand, tree nuts including hazelnuts contain both class I (complete) and class II (incomplete) food allergens. Allergic reaction to nuts can elicit even serious and

**Table 7.2: Hazelnut allergens—nomenclature: species
“*corylus avellana*—cor a.”**

| Allergen | Function/Type |
|------------|--|
| Cor a 1.04 | Major allergen variant Cor a 1.0401 |
| Cor a 2 | Profilin |
| Cor a 8 | Nonspecific lipid transfer protein |
| Cor a 9 | 11S—seed storage globulin (legumin) |
| Cor a 10 | Luminal binding protein, hazelnut pollen |
| Cor a 11 | 7S—48 kDa glycoprotein (vicilin) |
| Cor a 12 | Oleosin |
| Cor a 13 | Oleosin |
| Cor a 14 | 2S albumin |

Besler, M., Koppelman, S., Pumphrey, R., 2001. Allergen data collection—update: hazelnut (*Corylus avellana*). *Internet Symp. Food Allergens* 3, 1–22.

life-threatening symptoms in sensitive subjects. Seventy percent of allergic reactions to nuts occur after the first exposure, suggesting a basal sensitization over the general population.

The prevalence in European countries of hazelnut allergy ranges between 0.1% and 0.5% (Koppelman et al., 1999) while the global prevalence is estimated to be between 0.1% and 4% (Zuidmeer et al., 2008). Unfortunately, an exact evaluation of the prevalence of hazelnut allergy is hindered at least in part by the lack of availability of standardized allergens for clinical use (Andersson et al., 2007). Over the years, at least nine proteins have been reported as potential hazelnut allergens (Table 7.2).

The Europrevall (Datema et al., 2015) population-based survey, including allergic subjects from 12 European cities (85% adults), strongly supported the concept of the geographically predominance of specific hazelnut allergens (Pastorello et al., 2002), as well as the dependence on the age of the allergic subjects (Verweij et al., 2012). The IgE levels of reaction against the Cor a 1 were generally 5–10 times higher than those against other hazelnut allergens. Birch pollen exposure has a dominant role in the occurrence of hazelnut allergy. Geographical regions where the exposure to birch is reduced (Athens, Madrid, Sofia) have lower frequency of hazelnut-related allergenic reactions. Nevertheless, severe life-threatening allergies to the Cor a 8, Cor a 9, and Cor a 11 are mainly concentrated in the Mediterranean regions (Blanc et al., 2015). The Europrevall survey indicated Cor a 1 as the predominant allergenic protein in Europe, with the exception of Athens and Madrid. Pollen-related hazelnut allergy is normally restricted to mild oral allergy syndromes (OAS). Cor a 1 is a thermolabile allergen and easily undergoes pepsinolysis during digestion (Ćirković Veličković and Garović-Jankulović, 2014). The IgE-binding capacity of the protein significantly decrease by a factor of 100 after heat treatment and the activity is almost completely absent after roasting (140°C), as a direct consequence of the destruction

of conformational epitopes within the three-dimensional structure of the native protein (Worm et al., 2009; Hansen et al., 2003; Gieras et al., 2011). All the patients affected by OAS, independently of age, were sensitized to Bet v 1 and to homologs Cor a 1.04 and Cor a 1.0101 (De Knop et al., 2011).

The clinical symptoms of nonpollen-related hazelnut food allergy can be severe, systemic, and are frequently associated with the presence of linear epitopes. The sensitization to Cor a 8 dominates in Athens, while Cor a 9 and/or Cor a 14 sensitization is more common in Prague, Reykjavik, Utrecht, Manchester, and Madrid. Significant sensitization to Cor a 11 is found only in Prague (Datema et al., 2015). The ns-LTPs are the causative agents of a well-documented cross-reactive allergy to a large spectrum of botanically unrelated plant-derived foods (Wijesinha-Bettoni et al., 2010). The characteristic structure (typically containing 8 Cys and rich in α -helices) of the prolamin superfamily renders these proteins resistant to both heat treatment and proteolytic digestion. This seems to be the key structural feature relating their allergenicity to severe anaphylactic reactions (Schocker et al., 2004).

Clinical trials based on immunoCAP assays demonstrated the extensive sensitization to seed storage proteins in children (7–9 years old) recruited in a birch endemic region to the native (n)Cor a 9 and/or Cor a 14, besides a faint immune recognition for the expected recombinant (r)Cor a 1 and (r)Cor a 8 (Verweij et al., 2011; Datema et al., 2015). In contrast, adults reacted at a higher rate to the Cor a 1, birch-related aeroallergen Bet v 1 from *B. verrucosa*, as well as to Cor a 2, related to the birch pollen profilin Bet v 2 (Ebner et al., 1995; Datema et al., 2015). This phenomenon justifies the lower incidence of severe and systemic reactions to hazelnut in adulthood (De Knop et al., 2011).

The main nonpollen-associated allergen in the United States appears to be the acidic chain of the 11S globulin-like protein (Beyer et al., 2002). 11S globulin displays from ~8 to 15 linear epitope-bearing domains that are scattered throughout the length of the acidic and basic subunits (Robotham et al., 2009). The conformational analysis of the surface exposed IgE-binding epitopes showed that Cor a 9 exhibits some homology with other tree nut allergens, such as the cashew Ana o 2, the peanut Ara h 2 and the soybean glycinin (Barre et al., 2008). Recently, a protein not previously characterized, most likely a divergent Cor a 9 isoform, was described as the major IgE immunoreactive protein in Southern Italian children (Nitride et al., 2013). The 2S albumins (Cor a 14) can be cross-reactive with corresponding major allergens in walnut, Brazil nuts, oilseed rape, castor beans, and mustard seed (Moreno and Clemente, 2008).

An immunoCAP-based study showed an additional possible immunoreactivity to the 48 kDa glycoprotein (Cor a 11) in young subjects (Verweij et al., 2012). By comparing the immunoreactivity of glycosylated and chemically deglycosylated Cor a 11, it was demonstrated that only the protein backbone and not the carbohydrate moieties was able to cross-link specific IgE-antibodies from hazelnut allergenic subjects. Due to steric hindrance N-glycans

obstructed the access of digestive proteases to cleavage sites, thereby indirectly affecting the allergenic potential (Nishiyama et al., 2000).

Two oleosin isoforms (Cor a 12 and Cor a 13) were also identified as allergens in hazelnut (Akkerdaas et al., 2006) that were associated with systemic reactions and a negative skin prick test (Zuidmeer-Jongejan et al., 2014). According to a recent estimation, 12%–25% of the overall European population is sensitized to the Cor a 12 (Datema et al., 2015). It is clear from earlier that the pattern of IgE reactivity is complex and may result in poor diagnostic and prognostic accuracy (Akkerdaas et al., 2003a, 2003b).

7.5 *Detection of “Hidden” Hazelnut Allergens: Proteomic-Based Methods*

Protein-based determination of low levels of hazelnut contamination is generally carried out by immunochemical and MS detection. The earlier developed methods were enzyme-linked immunosorbent assays (ELISA). These assays are based on polyclonal or monoclonal immune-purified antibodies developed in rabbits. ELISA quantification provides a suitable LOD, which can range between 0.03 and 0.4 mg/kg and recovery of about 67%–132% depending on the food matrices. However, this technique only provides a static and indicative snapshot of the allergens, since frequently the epitopes used in the kits are nonspecific or can be masked by chemical or physical changes during food processing (Koppelman et al., 1999; Ben Rejeb et al., 2003). To this end, the use of antibodies developed toward process-modified allergens has been proposed as a more reliable approach for ELISA determination of allergens (Cucu et al., 2012a).

The preincubation of the ELISA system with a diluted model blank chocolate seems to help by reducing the matrix effect on binding efficiency. This evidence was provided in a study by Costa et al. (2014) wherein an in-house noncompetitive assay based on rabbit polyclonal and mouse monoclonal antibodies was developed with an LOD and LOQ of 1 and 50 mg/kg, respectively. Since hazelnut belongs to the same botanical order of walnut, pumpkin, cashew, almond, and pecan, cross-reactivity is expected to occur at high percentage with possible false-positive bias. Raw or roasted hazelnuts are widely used in food products containing significant amounts of sugars and fats. During food processing (roasting, mixing, cooking), hazelnut proteins might be glycated by coupling with reducing sugars (Maillard reaction) or oxidized by interaction with lipids and carbonylation. These events might decrease allergenicity or, conversely, generate neo-epitopes through cross-linking, conjugation, and deamidation (Picariello et al., 2011). The 11S globulins are highly thermostable and may unfold at temperature above 94°C, while the 7S have their thermal transition at 70–75°C. Since structural modifications are much more extensive when the content of water is high, typical heat treatments of dry nuts, such as roasting, do not significantly affect protein allergenicity (Hansen et al., 2003). López et al. (2012) described a high-pressure treatment with autoclaving that appears to reduce allergenicity and IgE-reactivity of hazelnut. However, it is probable

that the observed effects are dependent on a reduced extractability of proteins, which as aggregates will undergo gastrointestinal digestion releasing practically the same epitopes than their native counterparts. Thus, the reduction of allergenicity upon high-pressure processing remains to be demonstrated *in vivo*, for example by food oral challenges.

MS-based techniques are antibody-independent and directly target the allergen or derived peptide signatures. Indeed, the high sensitivity, specificity, and broad dynamic range of MS are best suited to detecting peptides rather than large-sized proteins. The 11S and the 7S, being the most abundant proteins in hazelnut, are appropriate candidates for the selection of peptide probes to determine allergens in complex food matrices. Interestingly, MS can be used in a targeted fashion or alternatively provide structural information about the nature of modifications which are not known in advance. The great degree of homology within nut proteins makes difficult the selection of unique peptides to increase the selectivity and the specificity of the method. However, the production of multiple peptides from a given protein generally offers a variety of possible choices for opportune monitoring. MS has the additional advantage of multiplexing for the assessment of multiple allergens in a single experiment. [Table 7.3](#) lists the selected marker peptides, the food matrices, and the analytical approaches which have been developed so far. The parallel validation in cereals and biscuits of LC–MS/MS and LC–SRM methods reached LOD and LOQ of 30 and 90 mg/kg and 35 and 110 mg/kg, respectively ([Bignardi et al., 2010](#)). Lower LOD and LOQ were obtained in chocolate matrices ([Costa et al., 2014](#)) and bread ([Heick et al., 2011](#)). For 11S globulin-like protein, representing one of the most abundant proteins, the estimated LOQ was less than 5 mg/kg and the LOD was 1 mg/kg. With the exception of bread, which was incurred prior to heat treatment, the LC–MS methods were developed on spiked materials. This may underestimate the effects of the food processing on extraction and digestion of the proteins. To overcome the limitations due to chemical–physical modification of the protein/peptide, it is fundamental to identify stable allergen-derived peptides that will be monitored in complex matrixes. [Cucu et al. \(2012b\)](#) described a list of peptides released from 11S globulins and the 7S vicilins that were very stable to Maillard reaction after incubation in a glycation model system. Similarly, [Sealey-Voysner et al. \(2016\)](#) selected the most intense peptide ions as candidate markers for the detection of hazelnut in processed food matrixes, based on their ability of surviving thermal processing.

7.6 Conclusions

Over the years, the advanced “omics” technologies have played a key role improving the volume of data related to the hazelnut proteome. Despite the encouraging developments, further and focused studies are needed to cover the remaining gaps in genomic, proteomic, and metabolomics knowledge. Hazelnut is among the most relevant allergen sources, responsible for severe reactions. As clinical symptoms of hazelnut allergy seem to be associated with the nature of the allergens, the component-resolved “molecular approach” aimed at the

Table 7.3: Studies using biomarker peptides for the detection of hazelnut proteins at trace levels.

| Protein | Peptide | Method of Analysis | Food Matrix | References |
|---------|--|---------------------------|--|------------------------------|
| 11S | LNALEPTNR ^b INTVNSNLPVLR ^b WLQLSAER ^a | LC-QTOF-MS | Raw and roasted nuts/commercial products | Sealey-Voysner et al. (2016) |
| 7S | LLSGIENFR ^b | | | |
| 11S | INTVNSNLPVLR ALPDDVLANAFQISR ^b VQVDDNGNTVFDDEL ^b QGQQQFGQ ^b | MALDI-TOF-MS | Raw/proteins incubated with glucose and lipids | Cucu et al. (2012b) |
| 7S | GNIVNEFER ^b | | | |
| 11S | ADIYTEQVGR ^b INTVNSNLPVLR QGQLTIPQNF ^b AVAK ALPDDVLANAFQISR | SRM-QTrap 4000 LC-MS/MS | Ingredient incurred bread | Heick et al. (2011) |
| 11S | QEWER ^a ADIYTEQVGR | LTQ XL linear ion trap | Biscuits/breakfast cereals | Bignardi et al. (2010) |
| 11S | WLQLSAER ALPDDVLANAFQISR TNDNAQISPLAGR ^a INTVNSNLPVLR RAESEGFEWVAFK ^a SRADIYTEQVGR ^b | MALDI-TOF-MS | Extra virgin olive oil | De Ceglie et al. (2014) |
| 11S iso | ITSLNSLNLPILR ^b | | | |
| 11S | ALPDDVLANAFQISR QGQLTIPQNF ^b AVAK INTVNSNLPVLR WLQLSAER | SRM - QTrap 4000 LC-MS/MS | Raw nuts | Ansari et al. (2012) |
| 7S | AFSWEVLEAALK ^a LLSGIENFR ELAFNLPSR ^b | | | |
| ns-LTP | GIAGLNPNLAAAGLPGK ^b | | | |
| 11S | ALPDDVLANAFQISR QGQLTIPQNF ^b AVAK INTVNSNLPVLR | SRM-QTrap 4000 LC-MS/MS | Chocolate | Costa et al. (2014) |
| 7S | WLQLSAER AFSWEVLEAALK LLSGIENFR ELAFNLPSR | | | |
| ns-LTP | GIAGLNPNLAAAGLPGK | | | |

^aCommon to other nuts.^bUnique.

Ansari, P., Stoppacher, N., Baumgartner, S., 2012. Marker peptide selection for the determination of hazelnut by LC-MS/MS and occurrence in other nuts. *Anal. Bioanal. Chem.* 402, 2607–2615; De Ceglie, C., Calvano, C.D., Zambonin, C.G., 2014. Determination of hidden hazelnut oil proteins in extra virgin olive oil by cold acetone precipitation followed by in-solution tryptic digestion and MALDI-TOF-MS analysis. *J. Agric. Food Chem.* 62, 9401–9409.

precise identification of allergenic determinant(s) is expected to greatly improve both the diagnosis and treatment of food allergies. The development of specific protein/peptide-based methods capable of the detection and quantification of hazelnut proteins at low levels in processed foodstuffs is essential to ensure consumers' health and well-being.

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Proteomic as a Tool to Study Fruit Ripening

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8.1 Introduction

Fruits are among the most consumed products worldwide, with tomato (*Solanum lycopersicum*), grape (*Vitis vinifera*), and apple (*Malus × domestica* Borkh) production exceeding 100 million metric tons per annum (FAOSTAT, 2015). Although fruits are in demand because of their nutritional value, quality traits such as color, acidity, juiciness, and size are important determinants of consumer perception. These are dependent on the physiological processes that take place during development and ripening (Marondedze, 2011; Molassiotis et al., 2013). Physiological changes that occur during the ripening phase are critical and have an impact on fruit quality, shelf-life, consumer acceptance, and profit/loss for farmers.

Fruit development and ripening are well-coordinated, genetically programmed irreversible processes. They are observed both at the molecular and physiological levels and have valuable economic implications for the agricultural industry (Bonghi and Manganaris, 2012; Wong et al., 2016). Physiological changes include softening of hypanthium brought about by changes in cell wall metabolism affected by the partial solubilization of pectin and cellulose (Payasi et al., 2009; Marondedze and Thomas, 2012); accumulation of simple sugars following degradation of starch may also contribute to changes in texture (Carrillo-Lopez et al., 2003; Li et al., 2012) and increased production of volatile compounds (Etienne et al., 2013; Rambla et al., 2014). Fruit ripening is accompanied by phenotypic changes that include skin and hypanthium color changes resulting from different pigmentation content of degrading chlorophyll compounds (Grassi et al., 2013). The latter has been well characterized in tomato where loss of chlorophyll has been accompanied by an increase in carotenoid synthesis, a stage characterized by the transformation of chloroplast into chromoplast (Giovannoni, 2001; Pareek, 2016). These distinct variations are regulated by hormones and developmental genes, as well as environmental factors such as light and heat (Matas et al., 2009). Consequently, fruits have different ripening mechanisms that are classically grouped into two categories namely, climacteric and nonclimacteric. Climacteric fruits are characterized by an increase in respiration and a concomitant burst of ethylene at the onset of ripening, while nonclimacteric fruits do not show increased respiration although a small amount of ethylene is detected. Therefore, dissecting and understanding these physiological processes regulating ripening are of paramount importance.

The introduction of proteomics as a complement to classical genomics and transcriptomics technologies in fruit biology has hastened our understanding of fruit development and ripening. Quantitative proteomics is a useful tool to gain insights on the molecular events occurring during fruit ripening and offers the potential to find biotechnological strategies to improve horticultural fruit quality traits (Marondedze et al., 2014). Thus far, differential proteomic analyses of the different ripening stages, ranging from immature to mature fruits, have been performed to gain evidence on the molecular events that occur during ripening and acquire an informative dynamic depiction of the transformation of fruits (Palma et al., 2011). Analysis of proteins has also been key in understanding expression levels and turnover rate of proteins involved in food allergy as well as the nutritional composition of fruits. In this chapter, we focus on the use of proteomics to better understand the ripening process in fruits. We also describe briefly ripening-responsive proteins that could be of interest for further characterization, particularly to unveil their potential role to prolong postharvest shelf-life or to improve fruit quality.

8.2 Proteomics and Ripening of Climacteric Fruits

In climacteric fruits, the onset of fruit ripening is characterized by a peak in respiration and a concurrent burst of the gaseous phytohormone ethylene biosynthesis. Thus, climacteric events are primarily regulated by ethylene that also partakes in the decrease in fruit flesh firmness, a typical phenomenon in economically relevant climacteric crops such as banana (*Musa* spp.), pear (*Pyrus communis*), plum (*Prunus armeniaca*), apple, peach (*Prunus persica*), papaya (*Carica papaya*), tomato, and melon (*Cucumis melo*). In addition, an increase in the abundance of ethylene biosynthesis-related proteins has been described (Hiwasa et al., 2003; Nilo et al., 2012; Zheng et al., 2013) and further shown to tremendously influence fruit shelf-life through increasing fruit softening (Good et al., 1994; Marondedze, 2011; Marondedze and Thomas, 2012; Li et al., 2015). The key ethylene biosynthesis proteins observed to increase during fruit ripening include S-adenosylmethionine decarboxylase, 1-aminocyclopropane-1-carboxylic acid synthase (ACS), and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Oeller et al., 1991; Zhang et al., 2011; Lokkamalue and Huehne, 2013; Pan et al., 2014). In apples, gene-silencing studies have shown that when ACS or ACO genes are silenced, transgenic plants produce fruits of firmer texture and increased shelf-life without significant changes in sugar and acid accumulation (Flores et al., 2001; Dandekar et al., 2004). In papaya, delayed ripening has been observed following co-suppression of ACO (Lopez-Gomez et al., 2009). Furthermore, a study on “Charentais” melons demonstrated that both ethylene-dependent and ethylene-independent pathways co-exist in climacteric fruits (Hadfield et al., 2000). Here, in addition to characterizing cDNAs corresponding to fluctuations in mRNAs abundance during ripening, a set of mRNAs were observed in the transgenic fruits to decrease but could be induced by ethylene treatment. This indicated that these genes were directly regulated by ethylene, while another group of genes

remained either significantly unaltered or lowly expressed after ethylene treatment, rendering themselves ethylene independent (Hadfield et al., 2000).

Proteomics has been applied on climacteric fruits to understand their development and ripening processes. In tomato, one of the most globally consumed “vegetables” (botanically tomatoes are classified as fruit, but publically perceived as vegetables) and model system for fleshy fruits, proteins extracted from unripe, medium ripe, and fully ripe fruits were analyzed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). This study led to the identification of two proteins, a 34 kDa and 44 kDa protein, corresponding to pectin esterase and heterotrimeric GTP-binding protein fragment, respectively (Schuch et al., 1989). These proteins were observed as upregulated during fruit ripening and were associated with cell wall softening. They were proposed as candidate ripening specific markers in tomato, as their expression levels increased during tomato ripening (Schuch et al., 1989).

Another study looking at the proteome changes associated with cherry tomato pericarp maturation and using two-dimensional gel electrophoresis (2-DE) revealed distinct protein abundance patterns among six different developmental stages (Faurobert et al., 2007). Ninety responsive protein spots were identified using either MALDI-TOF peptide mass fingerprinting (PMF) or liquid chromatography-MS sequencing and expressed sequence tag database searching (Faurobert et al., 2007). In young fruits, amino acid metabolism or protein synthesis-associated proteins were dominant but their expression decreased as the fruit matured. During the cell expansion stage, protein spots associated with photosynthesis and cell wall formation transiently increased. Spots linked to proteins involved in carbohydrate metabolism or oxidative processes increased in abundance during fruit development, indicating active metabolism associated with fruit growth and development (Faurobert et al., 2007).

Rocco et al. (2006) carried out a proteomic analysis from two tomato ecotypes (regional and commercial) during ripening. About 57% gel overlapping coordinates were detected, showing that a relatively large number of proteins were ecotype specific. Proteins identified during maturation processes were mainly associated with physiological processes such as defense, stress, redox control, carbon metabolism, energy metabolism, and cell signaling (Rocco et al., 2006). Thus far, further studies of different ecotypes are critical to identify if the changes depicted are consistent, and this will be important for defining specific molecular markers for economically valuable traits.

Organelle-targeted proteomics has also been performed on fruits such as tomatoes to dissect organelle-specific changes occurring during ripening. A comprehensive analysis of the nuclear proteome in tomato fruits was also reported (Wang et al., 2014). Using isobaric tags for relative and absolute quantification (iTRAQ) proteins induced during ripening were identified and the most represented classes were cell signaling and transcriptional regulation.

Similar to other tomato proteomics studies performed on different ripening stages, proteins involved in cell defense and protein folding as well as protein degradation and metabolism were differentially expressed. Authors further evaluated nuclear proteome changes in the ripening-deficient mutant, ripening inhibitor, and observed an overlap of differentially expressed proteins against the wild-type tomato (Wang et al., 2014).

Another proteomic study on tomato focusing on chilling injury during ripening was performed using 2-DE. Chilling injury is considered a stress agent that tomatoes are susceptible to and generally display physiological disorders characterized by uneven fruit ripening and color development, pitting, and decay (Vega-García et al., 2010). This study showed that the fruit proteome is affected by cold particularly proteins involved in carbon metabolism, photosynthesis, protein processing and degradation, and oxidative and cold stress.

Proteomic changes during ripening have also been characterized in apricot (*Prunus armeniaca* L.) (D'Ambrosio et al., 2013). Proteins from fruits of cultivar Vesuviana harvested at three different ripening stages were extracted and resolved by 1-DE and 2-DE.

Comparative analysis of the 2-DE maps was carried out and identification of differentially expressed spots was done by MALDI-TOF-based PMF and tandem mass spectrometry (MS/MS). A number of proteins involved in biochemical processes such as carbohydrates and energy metabolism, cell wall restructuring, stress response, and ethylene biosynthesis were identified as part of the proteins influencing metabolic and/or structural changes occurring during maturation (D'Ambrosio et al., 2013). Although similar biochemical and physiological processes were also observed in peach, most proteomic studies were performed at postharvest (Lara et al., 2009; Zhang et al., 2011). Prinsi et al. (2011) conducted a proteomic analysis on peach mesocarp of two cultivars at two ripening stages to determine proteins involved in the transition from preclimacteric to climacteric phase. In this study, about 50 proteins were identified with roles including carbohydrate metabolism, ethylene biosynthesis, and stress response, which are typical of fruit maturation.

In apples, proteomic analysis has also been carried out to characterize fruit ripening. Mitochondrial protein expression changes during fruit ripening in cultivar Fuji were comparatively examined on fruits treated with 2% and 100% oxygen that alleviate and induce oxidative stress respectively (Qin et al., 2009a,b). Induction of oxidative stress and acceleration of fruit senescence resulted in differential expression of enzymes involved in the citric acid cycle, electron transport chain, and membrane carriers. Some of the proteins identified included the mitochondrial manganese superoxide dismutase, an antioxidant scavenging superoxide in the mitochondria, whose expression decreased after high oxygen exposure. This also increased oxidative protein carbonylation, suggesting that reactive oxygen species may have a role in modulating fruit ripening and senescence particularly by altering expression levels of specific mitochondrial proteins and furthermore impairing their biological function (Qin et al., 2009a,b).

Comparative proteomic analysis of the banana pulp at preclimacteric and climacteric stages was performed using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) (Toledo et al., 2012). Fifty proteins were identified and chitinases were the most abundant in the preclimacteric stage, while heat shock proteins and isoflavone reductase were the most abundant at the climacteric stage. Expression of proteins associated with ethylene biosynthesis increased during climacteric stage. In addition, proteins associated with defense, regulation of gene expression, protein folding, flavor and fruit texture were identified as responsive to ripening (Toledo et al., 2012).

Another highly perishable fruit and yet of notable economic significance is mango (*Mangifera indica* L.). Similarly to the banana, mango fruit proteome changes during ripening were characterized by comparing preclimacteric and climacteric mango fruits from cultivar Keitt (Andrade et al., 2012). The majority of proteins responsive to ripening were associated with carbohydrate metabolism, energy metabolism, and stress responses. Similar to apple and other characterized fruits, antioxidant enzymes were observed to fluctuate during ripening (for review see Molassiotis et al., 2013). Proteins identified included chitinases that are involved in flesh softening (also identified in banana as described earlier) and fibrillin that is involved in color development and ripening-associated heat shock protein (Andrade et al., 2012).

Proteomics analyses have also been performed to assess the effect of plant elicitors on fruit ripening. For example, in the muskmelon, an economically important crop in China (Bi et al., 2007), proteomics provided new insights for elucidating the regulatory mechanism of muskmelon ripening particularly in response to benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). BTH is capable of inducing disease resistance in crops and has been shown to increase respiration rate, ethylene production, and delay in the decline of fruit firmness in the muskmelon (Li et al., 2015). Comparative proteomics led to positive identification of 52 BTH-responsive protein spots resolved by 2-DE and identified by MALDI-TOF/TOF. Functional categorization revealed that proteins identified were involved in energy metabolism, defense and stress responses, protein synthesis, destination and storage, cell structure, signal transduction and transporters, an indication that major physiological and biochemical changes occur and effectively have an influence on the proteome of muskmelon fruit during ripening (Li et al., 2015).

8.3 Proteomics and Ripening of Nonclimacteric Fruits

Nonclimacteric fruits ripen independently of ethylene levels, that is, fruits do not display a drastic increase in ethylene biosynthesis and do not show any increase in respiration (Adams-Phillips et al., 2004). As a matter of fact, nonclimacteric fruits do show a decline in ethylene synthesis and respiration rate throughout the ripening process. Examples of fruits that display these characteristics include orange (*Citrus sinensis*), lemon (*Citrus limon*), strawberry

(*Fragaria ananassa*), grape (*Vitis vinifera*), and pepper (*Capsicum annuum* L.). Besides the differences in ethylene levels between climacteric and nonclimacteric crops at the onset of ripening, metabolic events and physiological traits such as flavor, juiciness, texture, and color are undistinguishable (Giovannoni, 2001). Of importance to note is that climacteric and nonclimacteric physiological behavior is not phylogeny specific as evidenced by fruits of the same family such as tomato and pepper that display contrasting ripening behavior (Palma et al., 2011; Aizat et al., 2013a,b). Interestingly, in the nonclimacteric fruits, a “climacteric syndrome” has been observed although it is not directly related to the ripening onset process. For example, in strawberries, ethylene synthesis begins to increase during the red ripe stage (Iannetta et al., 2006) and in grapes, it occurs at the véraison stage (Chervin et al., 2004).

Proteomic studies on nonclimacteric fruits during ripening have been performed on different fruit tissues yielding tissue-specific proteome changes that occur during development and ripening. Examples of these studies include proteomics on the whole fruit (Aizat et al., 2013a,b), the flesh (Muccilli et al., 2009; Martinez-Esteso et al., 2011; Marondedze et al., 2014), and skin (Deytieux et al., 2007; Martinez-Esteso et al., 2011).

In grapes, proteomic analyses were performed to investigate differential proteome changes at different fruit development and ripening stages from fruit set to ripening stage. Using DIGE and MS/MS, changes in expression of proteins linked to grape development and ripening were detected (Martinez-Esteso et al., 2011). In this study, the profiles of enzymes involved in carbohydrate metabolism were observed to be consistent with a net conversion of sucrose to malate during grape fruit development, particularly expression of sucrose synthase decreased during fruit development while soluble acid invertase increased. Sucrose synthase together with UDP-glucose pyrophosphorylase, which also decreases with fruit development, are enzymes involved in the conversion of sucrose into glycolysis substrates. This switch has been reported to correlate with the fate of sucrose conversion into malate during green fruit development and eventually into glucose and fructose from véraison to full grape ripening (Ruffner and Hawker, 1977; Martinez-Esteso et al., 2011). In addition, several proteins involved in various biological processes have also been reported to change during grape berry ripening initiation using quantitative iTRAQ labeling technique (Lucker et al., 2009) (Table 8.1).

In date palm (*Phoenix dactylifera*), MS-based 2-DE comparative proteomics revealed that abundance of about 200 proteins fluctuates during date fruit development and ripening (Marondedze et al., 2014). In this study, 29 proteins, not previously reported to be linked to fruit ripening, were detected. Of importance to note is the unexpected decrease in abundance of enzymes involved in ethylene biosynthesis, namely S-adenosylmethionine synthetase (SAMS) and S-adenosyl-L-homocysteine hydrolase, while abundance of other ethylene biosynthesis-associated enzymes remained unchanged. This observation suggests that the cultivar, “Barhi,” used in this study may follow an ethylene-independent ripening pathway unlike other date cultivars that are considered, to date, climacteric (Marondedze et al., 2014).

Table 8.1: Major functional categories of fruit development and ripening-responsive proteins in climacteric and nonclimacteric fruits.

| Functional classification | Species | Reference(s) |
|---|--|---|
| Anthocyanin biosynthesis | Strawberry, date palm, sweet orange | Bianco et al. (2009), Muccilli et al. (2009), Marondedze et al. (2014) |
| Amino acid metabolism | Citrus, banana | Katz et al. (2011), Toledo et al. (2012) |
| Carbohydrate metabolism | Peach, grape, apple, tomato, sweet orange, banana | Deytieux et al. (2007), Muccilli et al. (2009), Nilo et al. (2012), Toledo et al. (2012), Zheng et al. (2013), Kambiranda et al. (2014), Pan et al. (2014), Shi et al. (2014) |
| Cell structure | Apricot, apple, banana, date palm | Toledo et al. (2012), D'Ambrosio et al. (2013), Marondedze et al. (2014), Shi et al. (2014) |
| Cell wall metabolism | Papaya, tomato | Nogueira et al. (2012), Pan et al. (2014) |
| Cellular signaling | Apple, tomato, banana, date palm | Rocco et al. (2006), Toledo et al. (2012), Marondedze et al. (2014), Shi et al. (2014) |
| Defense response | Apple, tomato, banana, date palm | Rocco et al. (2006), Toledo et al. (2012), Zheng et al. (2013), Marondedze et al. (2014), Shi et al. (2014) |
| Ethylene biosynthesis | Peach, papaya, apple, banana | Prinsi et al. (2011), Nogueira et al. (2012), Toledo et al. (2012), Zheng et al. (2013) |
| Energy metabolism | Apple, tomato, banana, strawberry, date palm | Rocco et al. (2006), Toledo et al. (2012), Li et al. (2013), Zheng et al. (2013), Marondedze et al. (2014), Shi et al. (2014) |
| Oxidative stress | Grape, apple, sweet orange, tomato | Rocco et al. (2006), Deytieux et al. (2007), Muccilli et al. (2009), Zheng et al. (2013) |
| Pathogen defense | Grape, mango | Deytieux et al. (2007), Andrade et al. (2012) |
| Photosynthesis | Grape, tomato | Deytieux et al. (2007), Pan et al. (2014) |
| Protein synthesis, folding and processing | Apple, tomato, banana, strawberry, date palm | Toledo et al. (2012), Li et al. (2013), Marondedze et al. (2014), Pan et al. (2014), Shi et al. (2014) |
| Primary and secondary metabolism | Peach, apricot, apple, sweet orange, banana, strawberry, date palm, papaya | Muccilli et al. (2009), Prinsi et al. (2011), Nogueira et al. (2012), Toledo et al. (2012), D'Ambrosio et al. (2013), Li et al. (2013), Marondedze et al. (2014), Shi et al. (2014), Song et al. (2015) |
| Stress response | Peach, papaya, grape, mango, apple, tomato, sweet orange, banana, strawberry | Rocco et al. (2006), Deytieux et al. (2007), Muccilli et al. (2009), Bianco et al. (2009), Prinsi et al. (2011), Andrade et al. (2012), Nogueira et al. (2012), Toledo et al. (2012), Li et al. (2013), Shi et al. (2014) |
| Storage Transporters | Strawberry, banana Banana, tomato, grape, apple, peach, date palm | Bianco et al. (2009), Toledo et al. (2012) Faurobert et al. (2007), Zhang et al. (2008), Qin et al. (2009a,b), Nilo et al. (2010), Toledo et al. (2012), Marondedze et al. (2014) |

Continued

As previously reported, SAMS is also involved in the biosynthesis of polyamines that are necessary for cell growth and division (Liu et al., 2006; Bianco et al., 2013) and this could explain the increased accumulation of SAMS during early fruit development (Marondedze et al., 2014).

In pepper, a 2-DE proteomic approach was employed to identify differentially expressed proteins at various stages of ripening. In this study, seven protein spots were detected as selectively present either in green or breaker red 1 stage. Identified proteins implicate diverse biochemical processes occurring during fruit development and ripening such as carbon metabolism, protein synthesis and chaperones, and biosynthesis of amino acids and polyamines and ethylene-associated proteins (Aizat et al., 2013a,b). Unlike in the date cultivar “Barhi,” expression of one of ethylene biosynthesis enzymes, ACO increased at the onset of ripening and decreased sharply at later ripening stages. In addition to protein levels increasing at breaker red 1 stage, a transient upregulation of *ACO* transcripts was also observed (Aizat et al., 2013a,b). A transient increase of *ACO* transcripts in nonclimacteric fruits was also reported in previous studies, for example in grapes during the véraison stage (Chervin et al., 2004; Sun et al., 2010), at the beginning of ripening in strawberry (Trainotti et al., 2005), in prickly pear (Collazo-Siques et al., 2003), and pineapple (Cazzonelli et al., 1998).

Furthermore, a combination of 2-DE and MS/MS approaches has also been employed to identify differentially expressed proteins between cultivars “Moro” and “Cadenera” of blood sweet orange at the stage of ripening (Muccilli et al., 2009). Here, 55 differentially expressed proteins were identified and distinct overrepresentation of proteins involved in secondary metabolism and oxidative stress was noted in the cultivar “Moro,” pinpointing a key role of the anthocyanin pathway during the ripening stage of “Moro” (Muccilli et al., 2009).

On the other hand, studies looking at different organs revealed that it is equally important to understand the biological mechanisms and changes occurring during fruit ripening in the skin as it is in the flesh. In grapes, for example, characterizing proteins isolated from the skin tissue sheds light to identify key compounds for wine quality. It is however not an easy task to extract proteins from skins for 2-DE analysis (for review see Deytieux et al., 2007). Deytieux et al. (2007) analyzed quantitatively proteomic profiles of the grape skin during grape fruit ripening. Just like in the flesh, soluble proteins were also detected to change during ripening. Stage-specific signatures were noted, e.g., proteins involved in photosynthesis, carbohydrate metabolism, and stress response were highly abundant at the onset of ripening or véraison depicted by the beginning of color change. The full ripe stage that is normally the end of color change was characterized by an increased abundance of proteins involved in anthocyanin biosynthesis. It has also been shown that abscisic acid mediates the expression of genes involved in anthocyanin biosynthesis pathway (Hiratsuka et al., 2001) and this hormone has been observed to accumulate in the skin during ripening (Gagne et al., 2006).

8.4 Commonly Regulated Proteins During Fruit Ripening

Protein changes occurring during ripening of fruit are specific and vary considerably with the stage of fruit development. As noted in climacteric fruits, the increase in respiration during ripening is consequently followed or associated with changes in the redox homeostasis, stress-/defense-associated responses, protein denaturation, and metabolism changes that ultimately lead to the degradation state functional to seed release (Qin et al., 2009a,b; D'Ambrosio et al., 2013). Besides, some enzymatic changes that occur during ripening are common between climacteric and nonclimacteric fruits. An example is the change in expression levels of the antioxidant enzymes, cytosolic [Cu-Zn]-superoxide dismutase and L-ascorbate peroxidase. Expression of the former generally increased during fruit ripening in apricots (D'Ambrosio et al., 2013), peach mesocarp (Prinsi et al., 2011), papaya mesocarp (Huerta-Ocampo et al., 2012), mango mesocarp (Andrade et al., 2012), tomato (Rocco et al., 2006), and grape tissues (Martinez-Esteso et al., 2011). However, in some cases the enzyme activity was reported to increase during maturation and then decline thereafter (Jimenez et al., 2002) or remain stable during ripening (Camejo et al., 2010). L-ascorbate peroxidase, one of the main enzymes responsible for hydrogen peroxide removal in subcellular compartments, also increases during fruit ripening in apricots (D'Ambrosio et al., 2013), tomato (Rocco et al., 2006; Faurobert et al., 2007), grape (Giribaldi et al., 2007), and strawberry (Bianco et al., 2009) but insignificant changes were observed in the peach mesocarp during softening (Nilo et al., 2010, 2012).

Proteins involved in secondary metabolism have also been observed to respond to fruit ripening and play a critical role in adaptive responses (D'Ambrosio et al., 2013). Examples of proteins detected in both climacteric and nonclimacteric fruits include isoflavone reductase and polyphenol oxidase that catalyzes the oxidation of monophenols and *o*-diphenols to *o*-quinones, which are involved in fruit browning reactions (Mayer and Harel, 1991). In apricot (D'Ambrosio et al., 2013), peach (Nilo et al., 2010, 2012), strawberry (Bianco et al., 2009), and grape (Giribaldi et al., 2007; Negri et al., 2008; Martinez-Esteso et al., 2011), both enzymes showed abundance changes, with polyphenol oxidase decreasing while the isoflavone reductase increasing during maturation. In addition, expression of quinone oxidoreductase-like protein, a protein associated with the production of furaneol, a main component of fruit flavors (Raab et al., 2006), increased during maturation in apricot (D'Ambrosio et al., 2013), peach mesocarp (Nilo et al., 2012), and strawberry (Bianco et al., 2009).

Another important biological process represented in proteomics studies of fruit development is primary metabolism. Some of the proteins in this category include NAD-dependent malate dehydrogenase (MDH), which increased with ripening. In fruits, cytosolic MDH together with phosphoenolpyruvate carboxylase are key enzymes of malic acid biosynthesis while mitochondria MDH catalyzes malate oxidation (Etienne et al., 2002; Sweetman et al., 2009).

In apricot, the abundance of MDH and NADP-dependent malic enzyme were observed to increase during ripening and the latter is believed to be important in reducing the acidity of the fruit as it metabolizes malic acid (D'Ambrosio et al., 2013). This has also been observed in other fruits including tomato, papaya mesocarp, grape skin, and peach (Rocco et al., 2006; Negri et al., 2008; Qin et al., 2009a,b; Martinez-Esteso et al., 2011; Nogueira et al., 2012), while with some fruits there is poor or negative correlation (Giribaldi et al., 2007; Nilo et al., 2010; Andrade et al., 2012; Marondedze et al., 2014). Another protein worth noting is alcohol dehydrogenase (ADH), which has been detected to increase in abundance with fruit ripening. This increase when the rate of glycolysis surpasses that of respiration causes generation of excess cytosolic pyruvate and production of ethanol, a fermentation product (D'Ambrosio et al., 2013). Expression of ADH has been observed to increase in grape (Giribaldi et al., 2007), strawberry (Bianco et al., 2009), mango (Andrade et al., 2012), apricot (D'Ambrosio et al., 2013), apple (Zheng et al., 2013), and melon (Li et al., 2015). ADH is involved in fermentation and is capable of interconverting aldehydes and alcohols. It is hypothesized to play a role in the regulation of fruit maturation (Page et al., 2010) and volatile compounds formation that ultimately determine fruit taste and aroma (Yun et al., 2010; Di Carli et al., 2011; D'Ambrosio et al., 2013). In addition to diverse changes in metabolic processes, organelles particularly mitochondria and chloroplasts increasingly become damaged with ripening. As such, chloroplasts are transformed into chromoplasts and are eventually rendered functionless at the full ripe stage (Giovannoni, 2004; Rocco et al., 2006; Muccilli et al., 2009; Barsan et al., 2010; Egea et al., 2010). This eventuality has been associated with cell energy impairment during final maturation stage and has been supported by the downregulation of proteins such as mitochondrial ATP synthase 24 kDa and d subunits, dihydrolipoyl dehydrogenase, chloroplast ATP subunit β , oxygen-evolving enhancer protein 1 in many fruits (Rocco et al., 2006; Faurobert et al., 2007; Giribaldi et al., 2007; Negri et al., 2008; Qin et al., 2009a,b; Martinez-Esteso et al., 2011; Andrade et al., 2012; Nilo et al., 2012; D'Ambrosio et al., 2013).

Overall, proteins that have been shown to markedly increase during ripening in different fruits have been classified into various functional categories including carbohydrate metabolism, cell wall metabolism, stress/defense response, ethylene biosynthesis, and carbon and nitrogen metabolism (Table 8.1). All these diverse processes suggest that fruit development and ripening is a metabolically active process, not only at the molecular level but also at the sensory and phenotypic levels.

8.5 Conclusions and Future Perspectives

In summary, comparative proteomics provides insights and inference into physiological processes occurring during fruit maturation at the systems level. It also offers a reference protein atlas for the study of regulatory mechanisms informative toward application of molecular and biotechnological approaches critical to further improve horticultural traits such as yield or

firmness. Although proteomics is an important tool to understand diverse mechanism occurring within cells and has potential biotechnological implications, combining this approach with posttranslational modifications, genomics, transcriptomics, metabolomics, and physiological analysis is key to comprehend an overall global picture of cellular functions. Together, these approaches offer the opportunity to equip the scientific community with better tools for molecular breeding intervention and even genetic modification for creating better varieties that are stress and disease tolerant together with high yield and long shelf-life. Although there are technical challenges that scientists and breeders still face, technological advances and availability of big data promises to ease the tension and provides prospects for better understanding of fruit development and ripening and the aptitude to accurately model and predict cultivars and seedlings for cropping as well as environmental effects during postharvest storage.

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SECTION 2

Application to Farm Animals – Meats, Dairy, and Eggs

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From Farm to Fork: Proteomics in Farm Animal Care and Food Production

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9.1 Introduction

Farm animal products (meat, milk, eggs, and their processed products) are important protein sources for human nutrition and are consumed by the majority of the population as part of a balanced diet. Quality testing and a high standard for obtained products are thus key for ensuring consumer health and safety. This can only be achieved with the “farm to fork perspective,” i.e., by caring about the welfare of the live animals, keeping them clinically healthy and with minimal stress. As animal breeding and food production are large industries, besides veterinary and consumer aspects, economic considerations are also taken into account and the efforts to supply food for a growing world population. For all three aspects mentioned, monitoring animal health, optimizing production, and securing food quality, proteomics has proven to be a reliable and excellent analytical tool (Fig. 9.1). The first two aspects together with a brief introduction to proteomics in the field of farm animals will be the focus of the present chapter, whereas food-related issues are dealt with in the following dedicated chapters.

9.2 General Aspects

Farm animal proteomics has gained importance only recently, as an excellent analytical tool to support the study of biological and veterinary questions, including aspects related to animal-derived products (Eckersall and Whitfield, 2011; Almeida et al., 2015).

9.2.1 Methods for Proteomic Investigations

As in many other fields, proteomics in animal-related and food science started with application of two-dimensional electrophoresis (2-DE), separating proteins according to isoelectric point in the first and according to molecular mass in SDS-PAGE in the second dimension

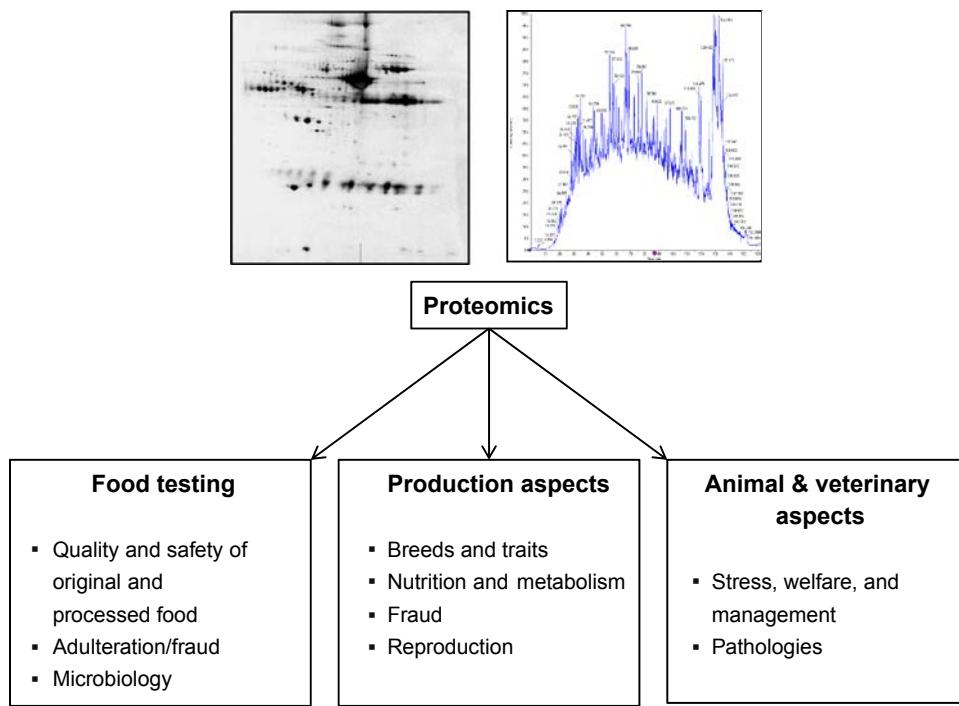


Figure 9.1
Aspects in farm animal-related food proteomics.

(Miller, 2011). The most modern form is fluorescence difference in gel electrophoresis (DIGE), wherein samples are preelectrophoretically labeled with up to three well-matched fluorophores. After the separation step, the spot patterns of applied samples are detected in parallel and compared to each other or to an internal standard included in the sample set (see example in Fig. 9.2). This approach minimizes gel to gel variation and allows detection of small concentration differences (Timms and Cramer, 2008). Spots of interest, usually differentially regulated ones, are excised from the gels and proteins identified by tandem mass spectrometric (MS/MS) analysis based on their peptide fragmentation patterns. Besides gel-based approaches, gel-free separation strategies were developed for peptide mixtures derived from enzymatic digestion of the original protein mixtures. Mass spectrometry (MS) separation and analysis is most often performed in combination with high-resolution multidimensional chromatography (Soares et al., 2012). Recent developments in equipment allow detection and quantification of hundreds of proteins per sample, based on the use of isotopic or chemical labels (previously introduced into the samples) or, more recently, in a label-free mode. Also recently, whole protein MS methods have been successfully applied, being able to distinguish between intact and modified proteoforms (Labas et al., 2015b).

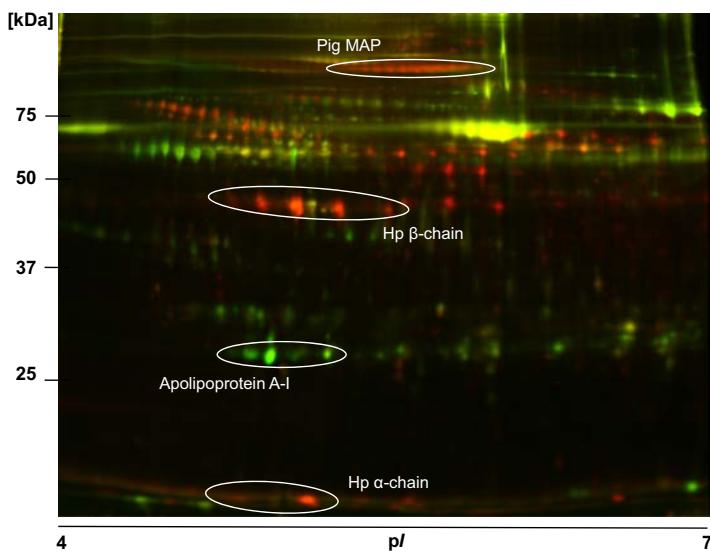


Figure 9.2

Influence of inflammation of the serum proteome. Difference in gel electrophoresis image of serum samples of a healthy pig (green spots; sample labeled with Cy3) and an animal with PCV2 infection resulting in massive inflammation (red spots; sample labeled with Cy5); spots in common and of similar concentration are displayed in yellow. Acute phase proteins are highlighted. *Adapted from Marco-Ramell, A., Miller, I., Nöbauer, K., Möglinger, U., Segalés, J., Razzazi-Fazeli, E., Kolarich, D., Bassols, A., 2014. Proteomics on porcine haptoglobin and IgG/IgA show protein species distribution and glycosylation pattern to remain similar in PCV2-SD infection. J. Proteomics 101, 205–216.*

All of the described proteomic methods share the commonality that investigation starts as a holistic approach, looking for differences between two or more sample sets. The study sets out to identify differentially regulated proteins, which are further characterized and whose functions are related to specific pathways. Newly developed targeted proteomics [selected reaction monitoring (SRM)] aims at quantifying larger sets of known proteins, based on their unique protein-specific peptides and shows promise especially for future clinical use. Up to now, only a few applications in the veterinary/animal field have been presented. These include SRM-based methods for absolute quantification of a panel of acute phase proteins (APPs) in bovine body fluids and tissues (Bislev et al., 2012b).

9.2.2 Sample Types

For health assessment in live animals, monitoring of success of treatment, or progression of disease, body fluids are commonly employed. This includes mainly serum (Cairolí et al., 2006; Ceciliani et al., 2012), saliva (Lamy and Mau, 2012; Gutiérrez et al., 2014), and milk

(Hogarth et al., 2004), but also seminal or other reproductive tract fluids (Rodríguez-Martínez et al., 2011; Souza et al., 2012). Preferable are fluids obtained by minimally to noninvasive methods, e.g., saliva, which minimize animal stress, allowing multiple sampling and even short intervals in time course experiments as well as simpler sample collection. Body fluids contain proteins in a soluble form, but proteins span a wide dynamic range. To avoid the highly abundant proteins that tend to obscure the overall patterns, sample pretreatment strategies have been developed, e.g., depletion of the most abundant proteins or enrichment of minor proteins (Soler et al., 2016b; Marco-Ramell and Bassols, 2010). Suitable approaches in the animal field rely on protein properties (e.g., isoelectric point, size, solubility, affinity) (Miller, 2011), which may be species specific; thus, commercial reagents developed for human samples need to be carefully tested. Additionally, such sample pretreatment bears the risk of unintentionally removing potentially useful marker proteins and has been critically reviewed (Gianazza et al., 2016).

For research experiments that aim to study the effect of diverse influences from feeding, metabolism, or different diseases, tissues and different organs are often sampled, e.g., organs (mainly liver), muscles, and different parts of the intestine. Depending of the rigidity of their structure they need specific types of homogenization, such as mechanical disruption or grinding in liquid nitrogen, and extraction with lysing buffers compatible with downstream analysis. Sample pretreatment, for instance subcellular fraction or precipitation (e.g., with trichloroacetic acid and/or acetone) may help to enrich specific subproteomes, remove contaminating compounds, or increase protein concentration. Specific approaches for food or animal-derived products will be dealt with in the respective chapters.

9.2.3 Databases

Proteomics is a data intensive research field, and interpreting, storing, and sharing proteome data has remained a challenge for translating proteome knowledge across laboratories and studies and for making transitions from fundamental biological research to application in veterinary health and in food production.

While initial 2-DE approaches aimed at setting up maps identifying proteins in all spots visible in the spot patterns, nowadays the aim is at targeting only spots differentially regulated in comparisons between at least two different groups. The most widely used publicly accessible protein databases are UniProtKB (<http://www.uniprot.org/uniprot/>) and the NCBI database (<http://www.ncbi.nlm.nih.gov/guide/proteins/>) that in addition to offering protein sequence information also contain direct links to numerous other protein information sites and tools. The latest assemblies of species-specific genomes can be downloaded from these sites, for use as reference sequences in a wide variety of search engines that are needed to analyze MS proteome data. UniProtKB contains both manually annotated and reviewed

entries (Swiss-Prot) and unreviewed automatically annotated data (TrEMBL), and consensus sequences and variants of the same gene are collapsed in single entries. This is not the case for the RefSeq databases at NCBI, which present only a single canonical entry for every known gene and protein species. UniProt and NCBI cover more than 5000 organisms, also including complete farm animal genomes for pig, cow, and chicken, while cross-species identification of conserved proteins is still a common practice for farm animal species where the genome sequences are incomplete or unannotated, for example, in goats and sheep.

With the recent expansion of MS-based proteomics, a wealth of data has emerged, which can be easily stored, transformed, and explored for global proteome information. The bewildering selection of current proteome data repositories highlights the fact that no single repository is ideally suited to fully cover the many different types of users and their individual needs for access to proteome data. The most developed and global repositories include PRIDE (Vizcaino et al., 2009), the Global Proteome Machine Database (Craig et al., 2004), and PeptideAtlas (Deutsch et al., 2008), and these play a fundamental role in successful development of new methods and progress in all branches of biological sciences. Most of these open repositories mainly cover human proteomes and those of common model organisms, while farm animals are still widely underrepresented. We want to highlight here the versatility of the PeptideAtlas, which is by far the main provider of farm animal proteome data, encompassing the cow (Bislev et al., 2012a) and pig (Hesselager et al., 2016) atlas projects. The latter publication presents the contents of the pig peptide atlas and delivers a tutorial for how to access knowledge about APPs in pig tissues and body fluids.

Table 9.1 gives a short overview on a few of the most comprehensive data repositories and highlights some of special interest for research in food and farm animal proteomics. For more information please refer to recent reviews (Perez-Riverol et al., 2015; Colangelo et al., 2015; Vizcaino et al., 2010).

9.3 Animal/Veterinary Aspects

9.3.1 Stress, Welfare, and Management

Animal welfare is prerequisite for guaranteeing both food quality and safety. Welfare is a complex issue that involves actual measures of housing location, hygienic conditions, and objective numbers about crowding, availability of food and water, temperature, humidity, and many other factors. Presently, it also involves subjective conditions of the individual, which should be able to express their own natural behavior (Manteca et al., 2009). Welfare problems are important for ethical reasons and public opinion, but also from the economic point of view since individuals under stress or low welfare are prone to suffer diseases and may lead to undesirable consequences in meat quality. Indeed, any cause of preslaughter stress affects meat quality and may lead to a pale, soft, and exudative or to dark, firm, and dry appearance of meat, as detailed in further chapters.

Table 9.1: Selected proteome data repositories containing resources relevant for farm animal and food proteomics.

| Repository | Contents and Features |
|--|--|
| UniProtKB | www.uniprot.org |
| NCBI and RefSeq | |
| www.ncbi.nlm.nih.gov | |
| GPM | http://thegpm.org |
| PeptideAtlas | |
| www.peptideatlas.org | |
| PRIDE | |
| www.ebi.ac.uk/pride/archive/ | |

Stress may occur at many points in the life of animals and can be acute or chronic. Acute stress is related, for example, to weaning, reallocation, mixing, transport, and slaughter, whereas chronic stress is associated with management conditions: space allowance, environmental enrichment, etc. There have been initiatives to establish internationally accepted protocols to measure welfare. For example, the Welfare Quality protocol is an attempt to organize an assessment system to control every aspect of animal welfare (Manteca et al., 2009). Nevertheless, behavioral tests are time consuming and have an inherent subjective character. Laboratory measures for stress involve most notably the stress hormones: adrenaline, cortisol, and corticosterone, but these hormones have several drawbacks: adrenaline has a very short half-life; and cortisol has a very high interindividual variability. Both hormones are secreted under acute stress conditions, but are not adequate markers for chronic stress. Other hormones and markers have been proposed as markers for stress, in particular, APPs (Ceciliani et al., 2012). Nevertheless, they are also primarily markers of inflammation and thus lack specificity. Objective laboratorial criteria to evaluate animal stress and welfare are still lacking and proteomics may be a useful approach to achieve this goal. Serum has been the most used sample to perform these studies (Fig. 9.2), but saliva has also been used as a convenient, noninvasive specimen to assess acute stress (Fuentes-Rubio et al., 2014).

Pigs are easily stressed by many conditions. High stocking density is one of the most common conditions that detrimentally affects growing and finishing pigs in commercial farms. Minimum space requirements have been stipulated (Council Directive, 2008/120/EC) but they may fall below the current limits during the latter part of the finishing period, when pigs attain their heaviest weight. The serum proteome of piglets subjected to periods of high and low stocking density was compared by DIGE, and cytoplasmatic β -actin was found to be increased in serum after high density periods (Marco-Ramell et al., 2011). Individual confinement of pregnant sows has been analyzed by using DIGE and isobaric tags for relative and absolute quantitation (iTRAQ) and differentially abundant proteins comprised APPs, antioxidant enzymes, apolipoproteins, heat shock proteins, metabolic enzymes, and structural cytoplasmic proteins (Marco-Ramell et al., 2016). Both studies point out to cell or tissue damage as a source of protein biomarkers.

Proteomics has been used to study chronic stress and the influence of living conditions in the adaptive physiological response of the organism also in cattle. The serum proteome of cows maintained in winter under different living conditions was analyzed by DIGE and the antioxidant enzymes paraoxonase-1 and glutathione peroxidase 3 (GPx3), proteins related to the complement system and APPs were identified indicating that a harsh environment provokes oxidative stress and a response of the immune system (Marco-Ramell et al., 2012).

One of the problems in the search of stress biomarkers is the nonspecificity of the response. As mentioned above, an immune response, oxidative stress, metabolic changes,

and/or tissue damage are quite common findings. Many of these reactions may also be found in pathologic conditions. Nevertheless, proteomics is helping to ascertain the specificities of each condition. Thus, a study designed to differentiate stress (weaning, transportation, and social reorganization) and viral infection (bovine respiratory disease) in young calves allowed to differentiate between both conditions by analyzing proteomic, metabolomic, and elemental profiles of serum samples with multivariate statistics (Aich et al., 2007).

9.3.2 Pathologies

A detailed report on the contribution of proteomics to diagnose farm animal pathologies is out of scope for this chapter, since the European Union has strict regulation of the hygienic rules and the official veterinary inspection at the slaughterhouse in order to avoid the entrance of dangerous products of animal origin into the food chain [Regulations (EC) No 853/2004 and 854/2004].

Viral and bacterial infections and parasitic diseases are the main sanitary problems in livestock production. Several proteomic studies have highlighted useful information for diagnosis, pathogenesis, prevention, and treatment of many common veterinary diseases. Indeed, the use of proteomics for microbial infections (Almeida et al., 2015; Eckersall and Whitfield, 2011; Soares et al., 2012; Di Girolamo et al., 2014; Bassols et al., 2014) and parasitic diseases (Marcelino et al., 2012) has been recently reviewed. Gut health in pigs and metabolic diseases in cattle are also important issues in animal farming (Danielsen et al., 2011; Kuhla et al., 2009). Special attention has been given to the characterization by proteomic techniques of APPs as main markers of infection and inflammation (Fig. 9.2) and the structural modifications caused by infectious diseases (Cairolí et al., 2006; Ceciliani et al., 2012; Marco-Ramell et al., 2014, 2015). On the other side, foodborne pathogens as *Salmonella* spp, *Campylobacter* spp, *Listeria*, and *Escherichia coli* have also been characterized by using proteomic technologies (Bassols et al., 2014). As detailed elsewhere, the use of alternative sample types as milk or saliva to assess the health status of the animal has also been a matter of study from the proteomic perspective (Hogarth et al., 2004; Lamy and Mau, 2012; Yang et al., 2012).

9.4 Production Aspects

9.4.1 Breeds and Traits

The biology of farm animals is strongly influenced by more than 10,000 years of genetic selection aimed at improving the yield and quality of meat, milk, and eggs. In the postgenomic era, the selection for improved productivity traits has been further accelerated by efficient genotyping and by correlation of genotypes and traits enabling strategies like GWAS (Gene Wide Association Study) and QTL (Quantitative Trait Loci) mapping (Goddard and Hayes, 2009). Selective breeding has been very successful for improving productivity, e.g., in pigs where average feed conversion rates declined from 3.5% to 2.4% and average litter size increased from 7 to 14 in the period

of 1975–2005 (<http://www.thepigsite.com>). Likewise, line breeding of cows has resulted in current milk breeds (e.g., Holstein breed) that produce more than 50L of milk/day, while meat breeds (e.g., Belgian Blue) can grow more than 5kg muscle mass/day. But this progress comes with a cost, namely the dramatic increase in animal mortality rates, which raises important ethical and animal welfare concerns, and reminds us that biological traits and genetic factors are connected in complex pathways, that we still are far from fully understanding.

Selective breeding to improve farm animal health is currently gaining much interest, but is far more challenging than breeding for productivity (e.g., growth rate and milk yield). Unlike productivity traits, readily measured in liters and kilograms, it is not yet straightforward to measure traits like pathogen resistance, because objective health measures are more demanding to develop and have not yet been widely developed for any farm animal species. The result is that current breeding and research strategies have proven inadequate to solve the growing problems with high animal mortality rates. Moreover, unlike productivity traits that are often dominated by single or a few genes, traits like “health” (e.g., pathogen resistance) depend on complex interactions of both animal genetics and environmental factors (e.g., feed and gut bacteria), and research opportunities and appropriate animal models needed to describe such complex interactions are still immature and challenging. For example, well-controlled and genotyped animal models that could reveal correlations between genetic and feeding factors are rarely studied at the molecular level. We therefore lack an understanding of, for example, which pig breeds and gene variants can benefit from a specific feeding regime or vaccine. Proteome research and in particular SRM-based quantification of host response proteins could deliver more accurate health measures for farm animals, which are essential for improved genetic selection for health. A few recent examples in this field are given at the end of the next section.

9.4.2 Nutrition and Metabolism

The early lactation period of dairy cows is normally characterized by a status of negative energy balance because the ingestion of nutrients and energy are insufficient to meet the high energy demands of milk production. The consequent hypoglycemia causes mobilization of fat and muscle reserves and synthesis of ketone bodies in the liver, leading to ketosis and metabolic acidosis. Furthermore, oxidative stress increases and the immune function is hampered (Kuhla et al., 2007). Subclinical ketosis is a severe problem in dairy farms since it affects nearly 40% of cows in a herd although the incidence could be higher. Proteomics has helped to understand this syndrome: plasma proteomics showed that ketosis is associated to inflammation, since it revealed an increase in APP, complement and coagulation pathways, and an increase of antioxidant GPx as compensatory mechanisms (Yang et al., 2012). By using 2-DE and iTRAQ, proteomic analysis of the liver showed deregulation of metabolic pathways involved in energy production, metabolism, and oxidative stress (Moyes et al., 2013; Xu and Wang, 2008).

Due to lipid mobilization to the liver, there may be an exaggerated deposit of lipids, giving rise to the so-called fatty liver. This syndrome has been studied in a series of studies in nonpregnant Holstein cows subjected to nutrient deprivation. The proteomic analysis of the liver showed that the enzymes of β -oxidation were downregulated suggesting a diminished degradation of fatty acids, which might increase the liver fat content (Kuhla et al., 2009). 2-DE analysis of the skeletal muscle showed a decrease in proteins related to glycogen synthesis and the Krebs cycle and an increase in proteins related to glycolysis, fatty acid degradation, and lactate production, thus providing the Cori cycle with substrates for hepatic gluconeogenesis (Kuhla et al., 2011).

Proteomic technologies have also helped to understand the consequences of this metabolic switch in other tissues such as the hypothalamus and pituitary, which control appetite (Kuhla et al., 2007), the mammary gland, which increases its energy requirements during lactation (Daniels et al., 2006; Rawson et al., 2012), and adipose tissue since it is a central regulator of metabolism (Zachut, 2015). In conclusion, the characterization of high-yielding dairy cows better adapted to peripartum metabolic stress may be facilitated by the proteomic identification of metabolic status-related biomarkers.

Half way between welfare and nutrition is seasonal weight loss (SWL), one of the main problems in animal production, especially in tropical climates characterized by an uneven distribution of rain in two seasons with consequences on pasture availability. The establishment of biomarkers of tolerance to SWL could be accomplished by studying the metabolic effects of feed restriction or by comparing SWL tolerant to sensitive breeds. Gel-based and label-free proteomic approaches have been used to identify proteins differentially abundant in the muscle and mammary gland in goats under the influence of SWL (Almeida et al., 2016; Hernández-Castellano et al., 2016).

Heat stress is another important aspect in animal production, especially in poultry. The effects of heat stress on the liver proteome of different susceptibility duck breeds was studied with a 2-DE approach, revealing differences in the regulation of the glycolytic pathway, antigen processing, and apoptosis (Zeng et al., 2013). Heat stress also causes a decrease of fertility in roosters and two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) analysis of chicken testes revealed that acute heat stress impairs the processes of translation, protein folding, and protein degradation, thus resulting in apoptosis and interfering with spermatogenesis (Wang et al., 2014). These findings may have implications for breeding chickens that can tolerate more extreme conditions.

Another important aspect in pig production is feed efficiency. Improving feed efficiency is a relevant strategy to reduce feed cost and environmental waste in livestock production.

Residual feed intake (RFI) is the difference between observed feed intake and expected feed intake based on the production measures of average daily gain and backfat. Using pigs from low-RFI (efficient) and high-RFI (inefficient) selected lines, 2D-DIGE was used to identify

differentially abundant proteins in muscle (Vincent et al., 2015) and serum (Grubbs et al., 2016). These proteins may be suitable biomarkers for feed efficiency selection.

Finally, some attempts have been made by using proteomics to identify the effects of natural additives used to increase growth performance and muscular metabolism (Zhang et al., 2015) or as immunomodulators against microbial infections (Park et al., 2011).

9.4.3 Fraud: Steroid Treatment, Growth Promoters

Despite the ban by the EU (Council Directive 96/23/EC), growth-promoting agents (GPAs) like anabolic and sexual steroids (mainly androgens and estrogens) and β 2-agonists are still illicitly used in cattle and other food-producing species to increase feed conversion and lean meat production. Due to the very short breeding cycle, veal calves are mostly subjected to illicit treatments. Besides the adverse effect that these molecules may exert on the animal, the use of GPAs is of concern because the eventual accumulation of residues may be harmful for the consumer. At present, the extensive control programs run by EU member states consist in the analytical identification of a number of banned molecules by physicochemical and immunochemical methods, but control of illegal use of drugs is difficult to achieve since they are used in combination at very low dosages, have a short half-life in serum and urine, may be hormone precursors or very similar to endogenous hormones, or have unknown chemical structure. To enable an efficient control of food safety, indirect detection techniques based on the biological effects of the molecules, rather than solely on their chemical structures are being developed. Omics technologies and, in particular, proteomics offer the global approach needed for this intention. Plasma and serum due to their easy availability, and skeletal muscle after slaughter, since this is where the growth-promoting action is mainly exerted, are the most used samples.

In plasma and serum, despite the diversity of results, data suggest a linkage between administration of anabolic steroids and/or hormones and apolipoprotein pattern. In one of these studies, two synthetic anabolic steroids, boldenone and boldione, were tested in calves and, using a 2-DE and LC-MS/MS proteomic approach, upregulation of a specific truncated form of apolipoprotein A1 (ApoA1) was found. ApoA1 is a major constituent of high-density lipoproteins (HDL) and is associated with cholesterol metabolism and transport (Draisci et al., 2007). Another study in calves treated with a combination of estrogens, anabolic steroids, and an insulin-like synthetic agent, focused on the low molecular proteome profile ranging between 3 and 20 kDa using LC-MS/MS, and a fragment of β 2GP-I (apolipoprotein H) was identified (Della Donna et al., 2009). This specific MW range gives information on proteolytic maturation and posttranslational modifications. The modulation of the apolipoproteins upon administration of anabolic steroids has already been established in athletes (Hartgens and Kuipers, 2004).

Other studies have found a complex response to the use of growth promoters. After estradiol/nortestosterone (E/NT, day 15) and dexamethasone (day 39) administration in calves and

using a 2D-DIGE approach, different proteins were identified in plasma during the E/NT phase or after dexamethasone (McGrath et al., 2013). Other studies in cattle have identified potential plasma biomarkers (Kinkead et al., 2015). Unfortunately, these studies have not reached a common unique protein marker. These results illustrate one of the main problems about the identification of new markers, i.e., changes depend on the type and duration of the illegal activity and the elimination kinetics of administered compounds. Likewise, several physiological mechanisms are altered in response to GPA treatment; amongst the proteins identified in the earlier mentioned studies are serotransferrin and fetuin-A, which are reflective of altered hepatic metabolism and acute-phase reaction activity; sex-hormone binding globulin (SHBG) and inhibin, which probably reflect the response to sexual hormones; and osteocalcin and the procollagen-propeptides, which reflect the action on the musculoskeletal system. The nature of such proteins suggests that several protein markers of hepatic and nonhepatic origin and varying biological function should be employed in conjunction.

Given that these anabolic molecules are known to enhance myogenesis and muscle growth, muscle tissue has been also used to identify an altered protein pattern following drug administration. For example, small biopsies of the *biceps brachii* skeletal muscle were collected at the slaughterhouse after dexamethasone administration at subtherapeutic dosage. The study was extended to muscle samples collected at random under field conditions. Several proteins were found in common to both studies including muscle structural proteins and muscle enzymes (Stella et al., 2014). The same group analyzed changes in the proteome of bulls treated using a commercially available ear implant containing trenbolone acetate and β -estradiol that is approved in the United States in bovine breeding. Shotgun proteomics employing tandem mass tags (TMT) defined a set of candidate protein markers that displayed a significantly altered abundance induced by steroid treatment that were able to discriminate the control and treated groups by multivariate analysis. They were proteins involved in muscle structure organization, muscle fiber contraction, cell metabolism and cell growth, consistent with the previous findings after treatment with dexamethasone (Stella et al., 2015).

The applicability of these findings will also depend on the development of targeted quantitative techniques. In this regard, immunobased surface plasmon resonance assay systems (McGrath et al., 2013) and SRM (Stella et al., 2015) are being developed.

9.4.4 Reproduction

Success of fertilization, number, and growth of (healthy) offspring are important points in animal breeding. Proteomic investigations have been involved in the study of all different aspects of conception, both of females and males: in the study of sperm maturation and search for markers of fertility (Rodríguez-Martínez et al., 2011; Belleanné et al., 2011; Kwon et al., 2015), the uterus, implantation, and embryo development (Arnold and Fröhlich, 2011; Jalali et al., 2015; Faulkner et al., 2013). Follicular fluid was investigated in less fertile cows (Zachut et al., 2016), and MS

methods were applied to predict sperm fertility in chickens (Soler et al., 2016a; Labas et al., 2015a), just to mention a few very recent findings. MS-based investigation of neuropeptide patterns proved helpful to study the hypothalamic–pituitary–gonadal axis and their influence on reproduction (Colgrave et al., 2011; Le et al., 2013). Additionally, proteomic studies were performed to monitor the (optimal) development of the newborns, for instance to follow the composition of colostrum proteins from uptake to digestion in different parts of the intestine in piglets (Danielsen et al., 2011). Gut health is an important issue during animal growth and development of the immune system (Soler and Miller, 2015).

9.5 Outlook

Thanks to intensive research efforts and new developments in instrumentation and workflows over recent years, great advances in our knowledge of the protein compositions of tissues and body fluids in different organisms and their changes under diverse conditions related to farm animals have been made possible. Connecting and deepening these findings by further improving the proteomic technologies and integrating data from other fields (genomics/transcriptomics, metabolomics, biomedical information, and production parameters) with the help of bioinformatics is the aim of systems biology (Hollung et al., 2014). Proteomics may help in the search of biomarkers and provide more accurate health measures for farm animals, which are essential for improving genetic selection for health, whether it be disease resistance, stress tolerance, or stress management. The growing use of proteomics in the field of farm animal production is expected to bring considerable benefit to both the animal and the consumer.

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Proteomics of Color in Fresh Muscle Foods

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10.1 Introduction

Visual appearance is an important criterion by which consumers judge the quality of fresh muscle foods, including red meats, poultry, and seafood. Fresh muscle foods undergo discoloration during retail display, which can have an adverse effect on the consumer perception of quality. Consumers often associate meat discoloration with spoilage, ultimately leading to product rejection and economic loss. In the United States, meat industry incurs an estimated annual revenue loss of more than \$1 billion due to discoloration-induced price discounts (Smith et al., 2000).

Myoglobin, the sarcoplasmic heme protein in muscle, is the major pigment responsible for meat color. In post-mortem skeletal muscles, myoglobin exists mainly in three redox forms (i.e., deoxymyoglobin, oxymyoglobin, and metmyoglobin), and the equilibrium between these redox forms play critical role in determining meat color (Mancini and Hunt, 2005). The redox state of myoglobin is influenced by its interactions with cell organelles and biomolecules in the post-mortem skeletal muscles (Faustman et al., 2010; Suman and Joseph, 2013; Richards, 2013; Suman et al., 2014). The intrinsic and extrinsic factors influencing myoglobin chemistry and meat color have been reviewed extensively and will not be addressed in this chapter (Mancini and Hunt, 2005; Suman and Joseph, 2014a; Suman et al., 2014).

10.2 Proteomics Applied to Meat Color

Novel tools in proteomics have been utilized to characterize the interactions between proteins and other biomolecules at molecular and cellular levels. Although proteomic techniques have been extensively used in agricultural and life sciences, their application in meat science is relatively new. In the past couple of decades, proteomic tools have been utilized to address the biochemical basis of pre- and post-harvest aspects of meat quality, such as muscle to meat conversion (Lametsch et al., 2002; Jia et al., 2006, 2009),

tenderness (Huff-Lonergan et al., 2011; Laville et al., 2009; Lametsch et al., 2011), and meat color (Suman et al., 2007; Joseph et al., 2012a; Nair et al., 2016). Several recent review articles have summarized the proteomic approaches in elucidating the fundamental mechanisms governing meat quality (Joseph et al., 2015; Picard et al., 2015; Almeida et al., 2015; Gobert et al., 2014; Suman and Joseph, 2014b; Suman, 2012; Bendixen, 2005; Hollung et al., 2007). Furthermore, proteomic tools are also utilized to examine the quality of processed meats (as discussed in Chapter 12), such as cooked ham (Pioselli et al., 2011), Italian Parma ham (Paredi et al., 2013), dry cured-ham (Di Luccia et al., 2005), and Spanish dry-cured hams (Sentandreu et al., 2007; Mora et al., 2009a,b; Escudero et al., 2013).

The focus of this chapter is the recent advances in the proteomic approaches for elucidating the molecular and biochemical basis of color of meat/muscle foods harvested from livestock, poultry, and fish. Earlier proteomic investigations of meat color focused primarily on myoglobin chemistry, and these studies examined the interactions between myoglobin and lipid oxidation products employing mass spectrometry. On the other hand, recent investigations focused on the proteome profile of skeletal muscles and its relation to meat color.

10.3 Myoglobin Chemistry

In living skeletal muscles, myoglobin functions both as an oxygen-delivery and oxygen-storage protein. However, in post-mortem skeletal muscles, through its oxygen-binding property, myoglobin serves as the major pigment responsible for the color of fresh muscle foods.

Myoglobin is composed of a globin polypeptide chain and a heme group in the center. The iron atom, located centrally in the heme group, can exist in reduced ferrous (Fe^{2+}) or oxidized ferric (Fe^{3+}) state and coordinates six different bonds. Four of these bonds are with pyrrole nitrogen atoms, while the fifth one coordinates the proximal histidine (position 93) of globin chain. The sixth coordination site is available to bind with different ligands such as oxygen, carbon monoxide, and water. The valence state of iron in heme and ligand present at the sixth coordinate dictates the redox state of myoglobin (Mancini and Hunt, 2005; Suman and Joseph, 2013, 2014a).

In fresh muscle foods, myoglobin can exist in three redox forms, i.e., deoxymyoglobin, oxymyoglobin, and metmyoglobin. Deoxymyoglobin occurs when no ligand is present in the sixth coordination site and the heme iron is in ferrous state. It is purplish-red in color and is associated with color of freshly cut and vacuum-packaged meat. On exposure to air, the sixth coordination site is occupied by an oxygen molecule resulting in formation of oxymyoglobin, which gives fresh meat the consumer-preferred cherry-red color. Oxidation of both deoxymyoglobin and oxymyoglobin yields ferric metmyoglobin, which is brown in color. In this redox form, a water molecule occupies the sixth coordination site. Muscle foods with a high proportion of metmyoglobin have an undesirable brownish-red color.

10.4 Lipid Oxidation-Induced Myoglobin Oxidation

Lipid oxidation happening in post-mortem muscles generates a variety of secondary reactive products, such as aldehydes and ketones (carbonyls), which are responsible for the off-flavors and off-odors associated with rancidity in muscle foods. The processes of lipid oxidation and myoglobin oxidation promote each other (Baron and Andersen, 2002; Faustman et al., 2010; Monahan et al., 1994; Yin and Faustman, 1993) and accelerate meat quality deterioration.

This principle in turn is applied in preharvest aspects of meat production to improve color and oxidative stability of fresh meats. Supranutritional supplementation of vitamin E minimizes lipid oxidation and improves color stability in fresh beef (Faustman et al., 1989), and the color-stabilizing effect of vitamin E is through its antioxidant property on muscle lipids (Lynch et al., 1998). Several studies were undertaken using mass spectrometry and proteomic tools to characterize the biochemical basis of lipid oxidation-induced oxidation in myoglobins from livestock and poultry. Hydroxynonenal (HNE) is an α,β -unsaturated aldehyde, generated by the oxidation of n-6 polyunsaturated fatty acids in the biological membranes (Schneider et al., 2001), and HNE has been used as a model aldehyde to investigate lipid oxidation-induced myoglobin oxidation and subsequent meat discoloration.

Faustman et al. (1999) utilized electrospray ionization mass spectrometry (ESI-MS) to examine the effect of lipid oxidation products (i.e., HNE) on equine oxymyoglobin. The results indicated that HNE exerted a prooxidant effect on myoglobin and formed mono-, di-, and tri-adducts with the heme protein through Michael addition. These results indicated that HNE can accelerate equine myoglobin oxidation by forming covalent adducts. Subsequently, extensive studies on lipid oxidation-induced oxidation in beef myoglobin were conducted by Alderton et al. (2003). These authors utilized liquid chromatography-mass spectrometry to characterize HNE adduction to myoglobin and reported that the histidine residues in myoglobin are covalently adducted by HNE. Six histidines (at positions 24, 64, 93, 116, 119, and 152) were modified by HNE, including the proximal (93) and distal (64) histidine residues. The proximal and distal histidines play a critical role in redox stability of myoglobin through spatial interaction with the hydrophobic heme pocket, and HNE adduction at these residues compromises the stability of myoglobin. Further studies examined lipid oxidation-induced redox instability in tuna (Lee et al., 2003a) and pork (Lee et al., 2003b) myoglobins, wherein HNE adducts with histidines were identified using ESI-MS.

Later studies examined the molecular bases of species-specific effect of vitamin E on the color of beef and pork. While meat discoloration and lipid oxidation were minimized by vitamin E supplementation in beef (Faustman et al., 1989; Chan et al., 1996), pork color was not improved by vitamin E, although the antioxidant minimized lipid oxidation in pork (Houben et al., 1998; Phillips et al., 2001). Suman et al. (2006) compared HNE adduction in pork and beef myoglobins at pH 5.6 and 4°C (meat conditions). Both mono- and di-adducts were detected in beef myoglobin, whereas only monoadducts were present in pork

myoglobin. While tandem mass spectrometry revealed four histidine adduction sites (36, 81, 88, and 152) in beef myoglobin, only two histidines (24 and 36) were found to be adducted in pork myoglobin. These results indicated that the effect of lipid oxidation on myoglobin redox stability and meat color stability are species specific. Further studies (Suman et al., 2007) examined the kinetics of preferential HNE adduction in pork and beef myoglobin and revealed that histidine 36 was preferentially adducted in pork myoglobin, whereas histidine 81 and 88 were the major sites of early HNE adduction in beef myoglobin. These authors also concluded that the preferential adduction of HNE at proximal histidine (93) observed exclusively in beef myoglobin was responsible for increased lipid oxidation–induced oxidation in beef myoglobin compared to pork myoglobin. Beef myoglobin was observed to be more susceptible to nucleophilic attack and subsequent adduction by HNE than pork myoglobin. Together the aforementioned studies (Suman et al., 2006, 2007) explained why vitamin E is effective in stabilizing color in beef, but not in pork.

Lipid oxidation–induced oxidation in poultry myoglobins were also investigated by several researchers. Maheswarappa et al. (2009) reported that the redox stability of chicken and turkey myoglobins was similar when challenged with lipid oxidation products (HNE). On further investigation, Naveena et al. (2010) determined that the proximal and distal histidines, critical to maintaining the redox stability, were adducted by HNE in chicken myoglobin. The molecular basis of lipid oxidation–induced oxidation in ratite myoglobins was investigated utilizing mass spectrometric tools (Nair et al., 2014). Although emu and ostrich myoglobins share 95% sequence similarity, HNE-induced oxidation was greater in ostrich myoglobin compared with emu myoglobin. Mass spectrometric data revealed that HNE adducted histidine 36 in ostrich myoglobin, whereas histidines 34 and 36 were adducted in emu myoglobin. The results indicated that the minor variations in the primary sequence of myoglobins could have a significant impact on the redox stability, particularly in the presence of prooxidants.

10.5 Primary Structure of Myoglobins

Proteomic and mass spectrometric tools have been widely utilized to characterize the primary structure and determine the exact molecular mass of myoglobins to detect meat adulteration and for meat species identification (Taylor et al., 1993). In an attempt to investigate darker color of meat from water buffalo compared to beef, Dosi et al. (2006) characterized water buffalo myoglobin and reported that its molecular mass is 86 Da heavier than beef myoglobin. The primary structure determined using a combination of Edman degradation and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS revealed that the observed difference in molecular mass was caused by three amino acid substitutions in buffalo myoglobin, which could affect the heme protein’s stability in post-mortem skeletal muscles. Similarly, sequencing of goat myoglobin using Edman degradation and MS revealed that goat and sheep myoglobins share 98.7% sequence similarity although their color attributes are different (Suman et al., 2009), which indicated that minor variations in amino acid sequence of myoglobin can affect the heme protein’s redox stability and meat

color. Investigations by Joseph et al. (2010a) reported that bison and beef myoglobins share 100% sequence similarity and exhibited similar physicochemical properties (thermal stability, autoxidation, and lipid oxidation–induced oxidation).

Turkey myoglobin exhibits greater thermal stability than its beef counterpart (Trout, 1989). The incomplete thermal denaturation of turkey myoglobin is considered as the underlying reason for the occurrence of pink color defect in turkey, wherein precooked, uncured turkey appears pink leading to consumer rejection (Holownia et al., 2003). In an attempt to explain the biochemical basis of the pink color defect, Joseph et al. (2010b) determined the molecular mass of turkey myoglobin using MALDI-TOF MS and reported that it is 300–350 Da heavier in molecular mass compared with red meat myoglobins. Further studies reported that turkey myoglobin and chicken myoglobin share 100% sequence similarity (Joseph et al., 2011) and their sequence is significantly different from those of the red meat myoglobins. This difference in the primary structure coupled with a greater pH in turkey meat can cause a greater thermal stability in turkey myoglobin, leading to the pink color defect in precooked turkey products.

Mass spectrometric tools also have been extensively used to characterize the molecular mass and primary structure of myoglobins from other meat-producing species such as emu (Suman et al., 2010), white-tailed deer (Joseph et al., 2012b), ostrich (Dosi et al., 2012), donkey (Dosi et al., 2012), Japanese quail (Goodson et al., 2015), and northern bobwhite (Goodson et al., 2015). All of these studies elaborated the understanding of myoglobin functionality and highlighted that myoglobin chemistry and meat color stability are species dependent.

10.6 Role of Muscle Proteome in Fresh Meat Color

The sarcoplasmic proteome, which comprises about 30% of total muscle proteins, plays a critical role in meat color, especially through its interactions with myoglobin. The changes in the skeletal muscle proteome continue post-mortem (Hollung et al., 2007) and critically influence meat color. Proteomic tools have allowed the evaluation of the dynamic muscle proteome and its relation to meat color. Although the muscle proteome could be analyzed using both gel-free and gel-based approaches, two-dimensional electrophoresis (2-DE) has been the method of choice for protein profiling in meat research. Usually performed under reducing and denaturing conditions, protein–protein interferences are minimized in 2-DE. Another advantage of 2-DE is that it allows simultaneous separation and analyses of thousands of proteins based on charge and molecular weight. Proteomic investigations in fresh meat color focused on traditional muscle food commodities, such as beef, pork, chicken, and fish.

10.6.1 Beef

Beef muscles demonstrate muscle specificity in color stability, which affect their retail shelf-life (McKenna et al., 2005). Beef muscles have been categorized into color-stable and color-labile (O’Keeffe and Hood, 1982). Joseph et al. (2012a) investigated the effect of

differential abundance of sarcoplasmic proteins on color stability of longissimus lumborum (LL; color-stable muscle) and psoas major (PM; color-labile muscle) in beef hind quarters using proteomic tools. These researchers isolated sarcoplasmic proteome from LL and PM, and correlated the differentially abundant proteins with meat color traits. Color-stable LL demonstrated greater abundance of metabolic enzymes (β -enolase and triose phosphate isomerase), antioxidant proteins (thioredoxin, peroxiredoxin-2, dihydropteridine reductase, aldose reductase, and peptide methionine sulfoxide reductase), and chaperones (heat shock protein-27 kDa, heat shock protein-1 B-70 kDa, and stress-induced phosphoprotein-1) compared with color-labile PM. Moreover, the proteins overabundant in LL exhibited a positive correlation with redness (aldose reductase, creatine kinase, and β -enolase) and surface color stability (peroxiredoxin-2, dihydropteridine reductase, and heat shock protein-27 kDa), whereas a protein overabundant in PM (mitochondrial aconitase) exhibited negative correlation with redness. The high color stability of LL was attributed to the greater abundance of antioxidant and chaperone proteins (peroxiredoxin-2, dihydropteridine reductase, and heat shock protein-27 kDa) compared with PM. The antioxidant proteins can minimize oxidation of lipids and myoglobin, resulting in improved color stability of LL. A similar study was conducted by [Wu et al. \(2015\)](#) to examine the relationship between color and sarcoplasmic proteome profile of LL and PM in Chinese Luxi yellow cattle. These authors also reported several glycolytic and antioxidant proteins differentially expressed between LL and PM, which could be utilized as biomarkers for color stability in Chinese Luxi yellow cattle.

Large muscles in beef hind quarters exhibit intramuscular differences in color stability. Beef semimembranosus is one such muscle that could be separated into color-stable outside (OSM) and color-labile inside (ISM) regions. The variations in temperature decline and pH drop during carcass chilling are considered to be partly responsible for this intramuscular color difference. [Nair et al. \(2016\)](#) investigated the proteome basis of the color difference between OSM and ISM steaks. OSM steaks demonstrated greater redness, color stability, and metmyoglobin reducing activity after 2 and 4 days of display. The proteome analysis revealed that ISM steaks had greater abundance of glycolytic enzymes (fructose-bisphosphate aldolase A, phosphoglycerate mutase 2, and β -enolase) than their OSM counterparts. A combination of rapid pH decline (due to possible rapid glycolysis as a result of increased enzyme levels) and the high temperature (due to the location within the carcass) in ISM during the immediate post-mortem period could have an adverse effect on myoglobin redox stability ([Suman and Joseph, 2013](#); [Faustman et al., 2010](#); [Mancini and Hunt, 2005](#)), thus compromising the meat color stability.

Animal-to-animal variations in color stability of beef LL has been documented previously ([King et al., 2010, 2011](#)), and [Canto et al. \(2015\)](#) examined the biochemical basis of this variation using proteomic tools. The metabolic enzymes (phosphoglucomutase-1, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase M2) differentially abundant in color-stable and color-labile LL steaks and overabundant in color-stable steaks exhibited a positive correlation with color

stability. Further, these authors reported the possibility of posttranslational modifications in myoglobin. Four protein spots (with similar molecular weight, but different isoelectric points) were identified as myoglobin suggesting possible posttranslational modification via phosphorylation.

[Marino et al. \(2014\)](#) utilized proteomic tools to evaluate the effect of breed (Romagnola \times Podolian, Podolian, and Friesian) and aging time (1, 7, 14, and 21 days) on color and sarcoplasmic proteome profile of beef longissimus muscles. Lightness (L^* value) of all the three breeds increased during aging, whereas the redness (a^* value) varied according to the breed. Steaks from the Podolian and Romagnola \times Podolian bulls exhibited an increase in redness during aging, whereas steaks from Friesian young bulls were not affected by aging. 2-DE investigations demonstrated extensive changes in the sarcoplasmic proteins among breeds and with aging. The results indicated that the abundance of proteins such as β -enolase, creatine kinase M-type, fructose-bisphosphate aldolase B, glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, glutathione S-transferase P, and protein DJ-1 decreased during aging. Breed also influenced the soluble proteome profile; proteins such as phosphoglycerate kinase 1, β -enolase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase B, creatine kinase M-type, adenylate kinase isoenzyme 1, peroxiredoxin-6, peroxiredoxin-2, superoxide dismutase, and histidine triad nucleotide-binding protein exhibited differential abundance based on the breed. The observed relationship between proteome changes and variations in beef quality traits suggested the potential to use these proteins as biomarkers.

[Clerens et al. \(2016\)](#) performed proteomic and peptidomic analysis of four muscles (semitendinosus, longissimus thoracis et lumborum, psoas major, and infraspinatus) from New Zealand-raised Angus steers. Although the muscles exhibited similar 2-DE profile, there was significant intensity difference between many proteins, including hemoglobin subunit beta, carbonic anhydrase 3, triosephosphate isomerase, phosphoglycerate mutase 2, serum albumin, and β -enolase. Several of these proteins have been previously found to be correlated to meat color ([Sayd et al., 2006](#); [Joseph et al., 2012a](#); [Canto et al., 2015](#)), and therefore, could be utilized as muscle-specific biomarkers for beef color.

10.6.2 Pork

Investigations on pork color utilizing proteomic tools focused on the relationship between proteome and paleness/lightness of fresh meat. [Sayd et al. \(2006\)](#) utilized 2-DE and tandem mass spectrometry to characterize sarcoplasmic proteome of pale and dark pork meat from semimembranosus muscle. Twenty-two proteins were differentially expressed between the light and dark groups. Proteins related to oxidative metabolism (HSP27, α B-crystallin, and glucose-regulated protein 58 kDa) were more abundant in darker meat, whereas glutathione S-transferase and glycolytic enzymes were overexpressed in lighter meat. The authors

suggested that the predominant oxidative metabolism in dark muscles delays post-mortem pH fall and offers protection against protein denaturation. Similarly, [Hwang et al. \(2005\)](#) examined the rate of post-mortem proteolysis and L^* value (lightness) during aging in pig longissimus muscle and identified several proteins (alpha actin, myosin light chain 1, cofilin 2, and troponin T) that were correlated to L^* value. [Laville et al. \(2005\)](#) examined proteome of pale, soft, and exudative (PSE) zones in deep regions of ham (semimembranosus muscle) and reported that creatine kinase fragments were more prominent in PSE zones, indicating a more pronounced proteolysis. Moreover, the authors observed that the protein changes in PSE zones resembled those induced by an acceleration of the post-mortem glycogenolysis.

10.6.3 Chicken

Compared to red meats, limited research has been undertaken on the proteomic aspects of color and appearance in fresh poultry. PSE meat is a major quality defect of broiler breast. PSE occurs when carcasses at high temperature experience acidic conditions within their muscles during rigor mortis ([Solomon et al., 1998](#)). The combination of elevated muscle temperature and acidic pH leads to rapid breakdown of muscle proteins, resulting in PSE meat. The increased reflectance of light on the meat surface, due to denaturation of myoglobin or its adsorption onto myofibrillar proteins ([Kauffman and Marsh, 1987](#)), increases the paleness, which negatively impacts the product quality and consumer acceptance ([Owens et al., 2009](#)). PSE meat tissue has poor protein functionality and is dry and tough after cooking ([Van Laack et al., 2000](#)), leading to economic loss for the poultry industry.

[Desai et al. \(2016\)](#) examined the whole muscle proteome of normal and PSE broiler breast meat and identified 15 differentially abundant proteins. Actin alpha, myosin heavy chain, phosphoglycerate kinase, creatine kinase M type, β -enolase, carbonic anhydrase 2, proteasome subunit alpha, pyruvate kinase, and malate dehydrogenase were overabundant in PSE broiler breast, whereas phosphoglycerate mutase-1, α -enolase, ATP-dependent 6-phosphofructokinase and fructose 1, 6-bisphosphatase were overabundant in normal meat. These results indicated that the overabundance of proteins involved in glycolytic pathways, muscle contraction, proteolysis, ATP regeneration, and energy metabolism in PSE breast could be related to the quality differences between normal and PSE meat.

10.6.4 Fish

Fish fillet color plays an important role in a consumer's purchase decision ([Maciel et al., 2014](#)), and catfish fillets that deviate from the typical white flesh color are less marketable ([Kin et al., 2010; Lovell, 1984](#)). Exposure to stress during aquaculture practices, environmental conditions, capture, transport, and harvest can adversely affect fish fillet quality and color. Red color defect is one such quality defect observed in channel catfish. Myofibrillar and sarcoplasmic proteomes in normal and reddish catfish fillets were examined to understand the

biochemical basis of this quality defect (Desai et al., 2014). The only protein that was differentially expressed between normal and reddish fillets was hemoglobin, which had a 22-fold greater abundance in red fillets. These results concluded that the red color defect in catfish fillets is primarily due to the overabundance of hemoglobin.

The effects of specific ante-mortem stressors such as oxygen concentration, temperature, and handling on muscle proteome and instrumental quality characteristics of channel catfish fillets were evaluated recently (Ciaramella et al., 2016). In general, the stressors resulted in a decrease in fillet redness and yellowness. Proteomic results indicated that ante-mortem stressors induced changes in structural proteins and those involved in protein regulation and energy metabolism. The muscle metabolism shifted to ketogenic pathways when fish was reared under hypoxic conditions. Proteomic data suggested that the downregulation of structural proteins due to stress factors could be responsible for the alterations in color and texture. These results indicated that environmental stressors and harvest practices can affect the channel catfish muscle proteome and ultimately the fillet color quality.

10.7 Conclusions

Fresh meat color is a complex quality trait influenced by a multitude of endogenous factors, including the muscle proteome profile, myoglobin chemistry, and lipid oxidation. The interactions of these factors affect myoglobin structure and redox state, and ultimately influence the color stability of fresh meats. Proteomic investigations have expanded the understanding of the cellular and biochemical mechanisms governing color of fresh muscle foods, and the results of these studies will aid the food industry's efforts to engineer novel processing strategies to improve the color of muscle foods.

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Proteomic Investigations of Beef Tenderness

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11.1 Introduction

Tenderness, juiciness, and flavor are important attributes of beef eating quality (Shackelford et al., 2001). Among them, tenderness is internationally the single, most important attribute affecting consumer's acceptability and their decision to purchase (Miller et al., 2001). The wide inconsistency of tenderness is the main reason for consumer's dissatisfaction leading to a reduction in beef consumption and has also been identified as a major problem for the beef industry (Brooks et al., 2000; Lorenzen et al., 1999). For decades, researchers have focused on the mechanisms behind this important attribute. Muscle structure (connective tissue) and biochemical properties (proteolysis of myofibrillar and cytoskeletal proteins) are the primary factors influencing beef tenderness variability (for review: Listrat et al., 2016). However, the fine mechanisms controlling tenderness are still unclear (Ouali et al., 2013). It is well documented that genetic and rearing factors, slaughter conditions, and post-mortem aging and processing have significant effects on beef tenderization (Przybyski and Hopkins, 2015). All these factors are at the origin of the inconsistency of beef tenderness and explain the difficulty to control this quality attribute.

Tenderness can be evaluated by instrumental or sensory tools which are time-consuming and expensive (Platter et al., 2005). Moreover, these methods can be applied only after the slaughter of the animal. There is still a need to find a universal method to evaluate the tenderness phenotype from paddock to plate. Among the omics tools developed over the last decade, proteomics appears to hold promise as a method to analyze protein modifications during post-mortem aging and to reveal biomarkers of tenderness (for review: Picard et al., 2013, 2015; Cassar-Malek and Picard, 2016). Meat aging and processing involve many biochemical steps inducing changes in protein abundance and structure, consequently the use of proteomics appears to be relevant for understanding the post-mortem conversion of muscle into meat (Gagaoua et al., 2015a,b). The importance of proteomics in the field of meat science is illustrated by a large number of papers and reviews concerning its applications to characterize the cellular and molecular mechanisms behind meat quality, skeletal muscle in the context of livestock production, or biological traits and diseases in farm animals (Bendixen, 2005;

Picard et al., 2010; D’Allessando and Zolla, 2013; Franco et al., 2015). The aim of this chapter is to summarize the main results obtained on beef tenderness investigations using proteomic tools. We will focus on the most relevant proteins related to beef tenderness identified through these approaches and discuss their biological roles on the process of muscle to meat conversion.

11.2 Proteomics in the Field of Meat Science and Investigations on Beef Tenderness

Most studies of skeletal proteome changes related to beef tenderness have been conducted by comparing extreme groups: tender versus tough for one muscle (Fig. 11.1) using different

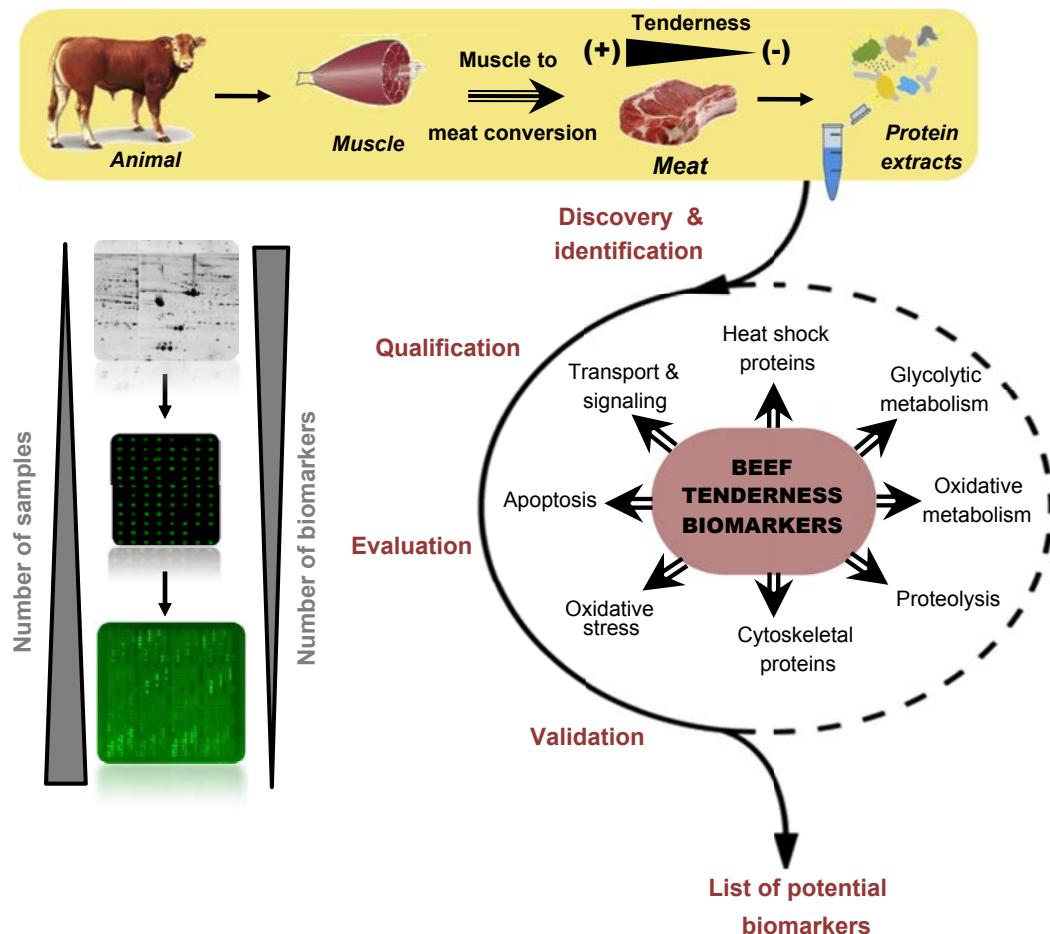


Figure 11.1

A workflow of the proteomic strategy adopted for the discovery of protein biomarkers of beef tenderness.

proteomic methods (Table 11.1). Most of the studies have been performed on the *Longissimus thoracis* muscle, which is a valuable cut for steak. The innovations in bioinformatics with the development of databases, bioinformatic tools, and web services offer large possibilities of data-mining and interpretation of proteomic results. For example, the bioinformatic analysis of [Guillemin et al. \(2011\)](#) allowed the construction of a network of beef tenderness which revealed the main biological functions implicated and permitted the detection of differences between

Table 11.1: A summary of gel-based and gel-free techniques used for quantitative beef proteomic studies.

| Approach | Objective | References |
|-----------|--|--|
| 1-DE | Relationship between myofibrillar 1D protein patterns of <i>Longissimus</i> muscle with tenderness | Sawdy et al. (2004) |
| | Beef tenderness prediction using separated myofibrillar 1D protein bands | Zapata et al. (2009) |
| DIGE | Assess the changes in the myosin light chains of <i>Longissimus</i> myofibril proteins during <i>p-m</i> aging | Muroya et al. (2007) |
| | Identification of protein markers of meat tenderness in <i>Longissimus</i> muscle | Jia et al. (2009) |
| iTRAQ | Explore the basis of meat variation in tenderness by studying the changes in protein abundances | Anderson et al. (2012) |
| | Identification of the differentially expressed proteins involved in intramuscular fat deposition | Bjarnadottir et al. (2012) |
| MALDI-TOF | Mapping of bovine <i>Semitendinosus</i> muscle of Charolais cattle | Mao et al. (2016) |
| | Changes of meat quality in <i>Longissimus</i> muscle following water bath and ohmic cooking | Bouley et al. (2004a) |
| LC-ESI-MS | Identification of early predictors of meat eating qualities of the Blonde d'Aquitaine bulls | Tian et al. (2016) |
| | Separation and identification of the potential proteins explaining the variability of beef tenderness | Morzel et al. (2008) |
| LC-MS/MS | Relationship between changes in <i>p-m</i> degradation of myofibrillar proteins and tenderness development | Baldassini et al. (2015) |
| | Phosphorylation of sarcoplasmic and myofibrillar proteins in response to <i>p-m</i> electrical stimulation | Marino et al. (2015) |
| nLC-MS/MS | Explore the proteome changes in the <i>Longissimus</i> muscle in response to preslaughter stress | Li et al. (2015) |
| | Explore the effect of high pressure on skeletal muscle proteome and the induced changes | Franco et al. (2015) |
| LESA-MS | Occurrence of low MW peptides in the <i>Pectoralis</i> muscle during aging and cooking | Marcos and Mullen (2014) |
| | Analysis of skeletal muscle proteins derived from a mixture of standard proteins and raw meat | Bauchart et al. (2006) |
| | | Montowska et al. (2014) |

1-DE, one-dimensional electrophoresis; DIGE, difference Gel Electrophoresis; iTRAQ, isobaric Tag for Relative and Absolute Quantification; LC-ESI-MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; LESA-MS, liquid Extraction Surface Analysis Mass Spectrometry; MALDI-TOF, matrix-Assisted Laser Desorption/Ionization-time-of-flight; nLC-MS/MS, nanoliquid chromatography-tandem mass spectrometry.

muscle types (*Longissimus thoracis*: LT vs. *Semitendinosus*: ST) identifying key proteins with central roles in the network such as *HspB1* coding for HSP27. This bioinformatic analysis proposed a number of proteins inside our dataset, but not revealed by the comparative proteomic analysis, as potential biomarkers of tenderness. For example, as depicted in Fig. 11.2, [Guillemin et al. \(2011\)](#) showed in their network that three proteins H2AFX, SUMO4, and TP53 were situated at crossroads between groups of proteins involved in tenderness, as metabolism, structure, and stress proteins. For example, H2AFX a histone involved in the cellular response to DNA damage, notably during oxidative stress, was confirmed by a proteomic study to be related with beef tenderness ([D'Allessando and Zolla, 2013](#)).

One limit of the electrophoretic separation used is the difficulty to extract proteins from extracellular matrices which are not solubilized by the buffer used. Hence, the proteins of connective tissue known to be important for meat tenderness are not considered in these studies. However, one benefit of these methods is the possibility to study posttranslational modifications (PTMs) such as phosphorylation, glycosylation/glycation, oxidation, and

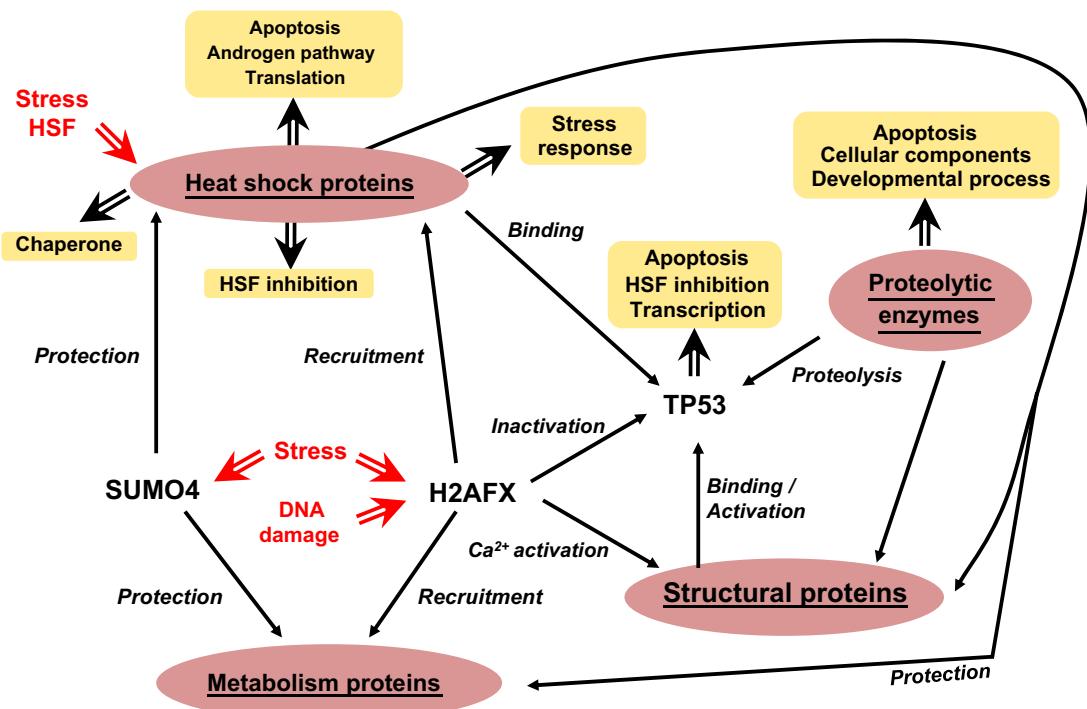


Figure 11.2

Functional network of tenderness in post-mortem muscle. Functional interactions between proteins are symbolized with arrow, with a brief description. Heavy arrows indicate cellular functions assured by the proteins. HSF, heat shock factor. Adapted from [Guillemin, N., Bonnet, M., Jurie, C., Picard, B., 2011. Functional analysis of beef tenderness. J. Proteomics 75, 352–365.](#)

ubiquitination/sumoylation. These PTMs are described to play major roles in the post-mortem process in meat science (Huang et al., 2011; D'Alessandro and Zolla, 2013). For example, phosphorylation is a reversible protein modification that is known to affect the protein structure and activity of many enzymes in vivo, and hence have a potential role in the tenderization period through regulation of the activities of glycolytic enzymes. For example, myosin light chain (MyLC2) was doubly phosphorylated during rigor mortis installation in bovine *Longissimus* muscle (Muroya et al., 2007).

Comparative proteomic analyses using the approaches summarized in Table 11.1 allowed the identification of potential biomarkers of beef tenderness (Table 11.2) that belong to numerous biological pathways, namely the heat shock protein (HSP) family, glycolytic or oxidative metabolism, oxidative stress, muscle structure and contraction, and proteolysis (Fig. 11.1 and Table 11.2). The strategy depicted in Fig. 11.1 was to comprehensively identify proteins and evaluate and validate their relation with tenderness on large number of samples. The validated

Table 11.2: Selected protein markers identified by proteomics approach associated with the variability of beef tenderness.

| Protein biomarkers (gene name) | Tenderness trait ^a | Direction ^b | References |
|--|-------------------------------|------------------------|-----------------------------|
| Heat shock proteins (HSP) | | | |
| HSP20 (<i>HspB6</i>) | WBSF | + | Zapata et al. (2009) |
| | | + | Guillemin et al. (2011) |
| | | * | Polati et al. (2012) |
| | | - | D'Alessandro et al. (2012b) |
| | | +/- | D'Alessandro et al. (2012a) |
| HSP27 (<i>HspB1</i>) | WBSF | + | Kim et al. (2008) |
| | WBSF | * | Polati et al. (2012) |
| | WBSF | - | Carvalho et al. (2014) |
| | WBSF | -/** | D'Alessandro et al. (2012b) |
| α B-crystallin (<i>Cryab</i>) | Tenderness (S) | +/- | Morzel et al. (2008) |
| | | + | Zapata et al. (2009) |
| | | - | Guillemin et al. (2011) |
| | | * | Polati et al. (2012) |
| | | ** | D'Alessandro et al. (2012b) |
| HSP40 (<i>Dnaj</i>) | Tenderness (S) | +/- | Morzel et al. (2008) |
| | | - | Bernard et al. (2007) |
| HSP71 (<i>HspA8</i>) | WBSF | - | D'Alessandro et al. (2012b) |
| HSP70-1A (<i>HspA1A</i>) | WBSF | + | Bjarnadottir et al. (2012) |
| | | + | Carvalho et al. (2014) |
| | | - | D'Alessandro et al. (2012b) |
| HSP70-1B (<i>HspA1B</i>) | WBSF | + | Baldassini et al. (2015) |
| | | - | D'Alessandro et al. (2012b) |
| HSPP70-GRP75 (<i>HSPA9</i>) | WBSF | + | Jia et al. (2009) |
| | | - | Guillemin et al. (2011) |
| | | - | Grabez et al. (2015) |

Continued

Table 11.2: Selected protein markers identified by proteomics approach associated with the variability of beef tenderness.—cont'd

| Protein biomarkers (<i>gene name</i>) | Tenderness trait ^a | Direction ^b | References |
|---|-------------------------------|------------------------|-----------------------------|
| Energy metabolism | | | |
| (A) Glycolytic metabolism | | | |
| Enolase 1 (<i>ENO1</i>) | WBSF | * | Polati et al. (2012) |
| | | - | D'Alessandro et al. (2012b) |
| Enolase 3 (<i>ENO3</i>) | WBSF | - | Bjarnadottir et al. (2012) |
| | | + | Guillemin et al. (2011) |
| | | * | Polati et al. (2012) |
| | | +/- | Marino et al. (2014) |
| Malate dehydrogenase 1 (<i>MDH1</i>) | WBSF | - | Jia et al. (2009) |
| Triosephosphate isomerase 1 (<i>TPI1</i>) | WBSF | - | D'Alessandro et al. (2012b) |
| | | - | Grabez et al. (2015) |
| Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>) | WBSF | ** | Polati et al. (2012) |
| | | + | Laville et al. (2009) |
| | | - | D'Alessandro et al. (2012a) |
| | | ** | Marino et al. (2014) |
| Lactate dehydrogenase A (<i>LDHA</i>) | WBSF | * | Polati et al. (2012) |
| | | +/- | Baldassini et al. (2015) |
| Pyruvate kinase (<i>PKM</i>) | WBSF | + | Baldassini et al. (2015) |
| | | - | Laville et al. (2009) |
| Phosphoglucomutase 1 (<i>PGM1</i>) | WBSF | - | D'Alessandro et al. (2012b) |
| (B) Oxidative metabolism | | | |
| Aldehyde dehydrogenase (<i>ALDH2</i>) | WBSF | - | Grabez et al. (2015) |
| | | */** | Jia et al. (2007) |
| Creatine kinase M type (<i>CKM</i>) | WBSF | - | Zapata et al. (2009) |
| | | - | Bjarnadottir et al. (2012) |
| | | */** | Polati et al. (2012) |
| | | - | Laville et al. (2009) |
| | | - | D'Alessandro et al. (2012a) |
| Succinate dehydrogenase (<i>SDH</i>) | Tenderness (S) | + | Morzel et al. (2008) |
| Pyruvate dehydrogenase (<i>PDHB</i>) | WBSF | - | Grabez et al. (2015) |
| Oxidative stress | | | |
| DJ-1 (<i>PARK7</i>) | WBSF | + | Jia et al. (2009) |
| | | * | Jia et al. (2007) |
| | | * | Polati et al. (2012) |
| Peroxiredoxin 1 (<i>PRDX1</i>) | WBSF | * | Polati et al. (2012) |
| Peroxiredoxin 2 (<i>PRDX2</i>) | WBSF | * | Jia et al. (2007) |
| Peroxiredoxin 3 (<i>PRDX3</i>) | WBSF | * | Jia et al. (2007) |
| Peroxiredoxin 6 (<i>PRDX6</i>) | WBSF | - | Jia et al. (2009) |
| | | + | Guillemin et al. (2011) |
| | | * | Marino et al. (2014) |
| Superoxide dismutase (<i>SOD</i>) | WBSF | * | Marino et al. (2014) |
| | | - | Grabez et al. (2015) |
| Carbonic anhydrase (CA3) | WBSF | ** | Polati et al. (2012) |

Table 11.2: Selected protein markers identified by proteomics approach associated with the variability of beef tenderness.—cont'd

| Protein biomarkers (<i>gene name</i>) | Tenderness trait ^a | Direction ^b | References |
|---|-------------------------------|------------------------|----------------------------|
| Structure | | | |
| Myosin light chain 1 (<i>MLC1</i>) | WBSF | + | Bjarnadottir et al. (2012) |
| | | - | Jia et al. (2009) |
| | | +/- | Polati et al. (2012) |
| Myosin light chain 2 (<i>MLC2</i>) | WBSF | + | Zapata et al. (2009) |
| | | - | Bjarnadottir et al. (2012) |
| | | +/- | Polati et al. (2012) |
| Troponin C | WBSF | + | Zapata et al. (2009) |
| Troponin T | WBSF | + | Polati et al. (2012) |
| Myosin heavy chain 1 (<i>MHC1</i>) | WBSF | - | Zapata et al. (2009) |
| | | - | Guillemin et al. (2011) |
| | | +/- | Polati et al. (2012) |
| Myosin heavy chain 2 (<i>MHC2</i>) | WBSF | - | Zapata et al. (2009) |
| Myosin heavy chain 7 (<i>MHC7</i>) | WBSF | - | Zapata et al. (2009) |
| | | + | Guillemin et al. (2011) |
| Desmin | WBSF | - | Zapata et al. (2009) |
| | | - | Guillemin et al. (2011) |
| Tubulin β | WBSF | - | Zapata et al. (2009) |
| CapZ- β | | */** | Bjarnadottir et al. (2010) |
| α -actin | WBSF | - | Zapata et al. (2009) |
| | WBSF | + | Bjarnadottir et al. (2012) |
| | WBSF | +/- | Polati et al. (2012) |
| Myosin binding protein-H (<i>MBP-H</i>) | Tenderness (S) | + | Morzel et al. (2008) |
| | WBSF | + | Guillemin et al. (2011) |
| | Tenderness (S) | + | Morzel et al. (2008) |
| Proteolysis | | | |
| μ -calpain | Tenderness (S) | - | Guillemin et al. (2011) |
| | WBSF | +/- | Picard et al. (2014) |
| <i>m</i> -calpain | Tenderness (S) | - | Guillemin et al. (2011) |
| Transport and signalling | | | |
| Serum albumin | WBSF | - | Laville et al. (2009) |
| Serotransferrin | WBSF | + | Bjarnadottir et al. (2012) |
| Parvalbumin | Tenderness (S) | + | Bouley et al. (2004b) |
| Myoglobin | WBSF | +/- | Polati et al. (2012) |
| Fatty acid-binding protein (<i>FABP</i>) | WBSF | +/- | Polati et al. (2012) |
| Apoptosis ^c | | | |
| Annexin A6 | WBSF | + | Bjarnadottir et al. (2012) |
| Galectin-1 | WBSF | + | Bjarnadottir et al. (2012) |
| Inositol 1,4,5-triphosphate receptor type 1 (IP3R1) | WBSF | + | Kim et al. (2008) |

^aTenderness was evaluated by instrumental Warner-Bratzler Shear Force (WBSF) or by a sensory panel (S).

^b(+) positively related; (-) negatively related; (+/-) positively and/or negatively related depending on the protein isoform; (*) increase of the biomarker was reported; (**) decrease of the biomarker was reported.

^cAmong the HSP, transport and signaling, and oxidative stress proteins, many of these proteins are also involved in the apoptotic pathway.

proteins are then used for tenderness prediction (Picard et al., 2014). The final objective is to apply prediction equations in a paddock tool for the beef sector (Cassar-Malek and Picard, 2016).

11.3 Protein Biomarkers of Meat Tenderness

The main biomarkers identified in relation to mechanical or sensory beef tenderness are summarized in Table 11.2. The analyses of these biomarkers and the biological functions in which they are involved produced new insight about tenderness.

11.3.1 Heat Shock Proteins

In the majority of proteomic beef tenderness investigations, HSPs have consistently been reported to differ between tender and tough beef samples and were also found to be correlated with instrumental and sensory tenderness (Bernard et al., 2007; Morzel et al., 2008; D'Alessandro et al., 2012b; Bjarnadottir et al., 2012; Carvalho et al., 2014). HSPs are ubiquitous and evolutionarily conserved proteins classified into five subfamilies on the basis of their molecular weight (MW), e.g., HSP60, HSP70, HSP90, and HSP100 and the small HSPs (MW 12–43 kDa). In the field of meat science, Ouali et al. (2006) were the first who hypothesized the role of HSPs in meat tenderness due to their antiapoptotic functions. Consequently, we speculated that HSPs are variably recruited to hinder the onset of apoptosis to prevent cells from damage, thus playing a great role in post-mortem muscle changes. As depicted in Figs. 11.2 and 11.3, HSPs have essential roles in post-mortem skeletal muscle since they contribute to proper conformation of proteins and preservation of their biological functions. They act as molecular chaperones during protein assembly, protein folding and unfolding, and in the refolding of damaged proteins, thus maintaining cell homeostasis (Lomiwes et al., 2014).

Skeletal muscle proteome studies have identified several HSPs that are changed in post-mortem bovine muscle (Pulford et al., 2008; Jia et al., 2009). Their increased abundance soon after slaughter may be due to their response to cell stress signals. Thus, we recently hypothesized that the upregulation of the large HSP70 may be useful for maintaining muscle cell integrity and repairing denatured proteins (Picard et al., 2014). In accordance, the abundance of large HSP70 proteins was found to be associated with meat tenderness (Jia et al., 2009; D'Alessandro et al., 2012a; Bjarnadottir et al., 2012; Carvalho et al., 2014; Baldassini et al., 2015). A negative relationship between tenderness and HSP40, a protein that helps to retard cell death in muscle tissue was reported Bernard et al. (2007). Likewise, HSP27 was down-regulated in animals that gave lower tenderness values of *Longissimus* muscle (Morzel et al., 2008). Furthermore, small HSPs are recruited to prevent structural damage and degradation of proteins due to apoptosis in muscle cells and protect actin filaments and other cytoskeletal proteins from fragmentation caused by stress (Balan et al., 2014). The modulation of these

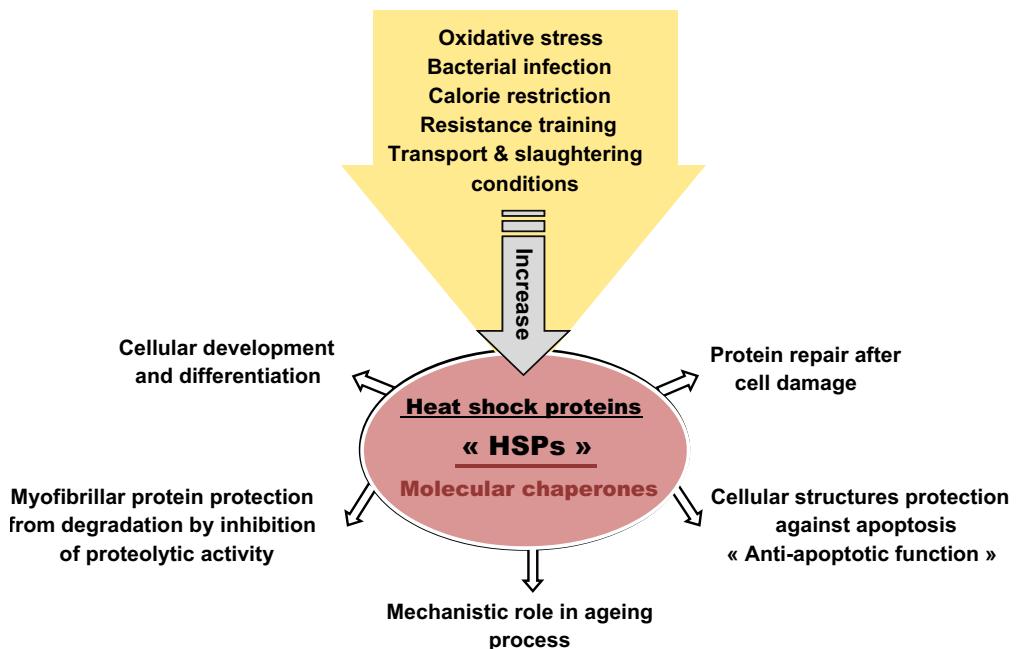


Figure 11.3

Potential roles of heat shock proteins in post-mortem muscle to meat conversion.

proteins during the post-mortem period suggests a change in the stabilization of the myofibril proteins (Jia et al., 2007; Bjarnadóttir et al., 2010). Considering that meat tenderness is dependent upon the degradation of myofibrillar proteins post-mortem (see Section 3.4), small HSPs have therefore been implicated in the development of tenderness (Lomiwes et al., 2014).

Previous studies by Pulford et al. (2008) and Kim et al. (2008) reported that the expression of HSP20 and α B-crystallin peaked at the early post-mortem of beef *Longissimus* muscle. On the other hand, it has been postulated that alterations in abundance of HSP20 may lead to decreased stability of the actin filament, which may be associated with increased tenderness (Zapata et al., 2009). Protein levels of HSP70 were also found to be decreased in *Longissimus* muscle from Norwegian Red bulls 48 h post-mortem, as well as two spots corresponding to HSP27 (Bjarnadóttir et al., 2010). These findings confirm that these chaperones are important in tenderness development, with HSP70 as negative markers, in contrast to HSP20 (Guillemin et al., 2012). We have reported similar findings in a previous study showing significant higher levels of HSP27 and HSP20 in the more tender *Longissimus thoracis* in comparison to *Semitendinosus* muscle (Guillemin et al., 2011). Recently, an inverse relationship between tenderness and proteins from the small HSPs (HSP20, 27, and α B-crystallin) according to muscle type and breed has also been highlighted (Picard et al., 2014). These proteins were found to be negatively related to tenderness in the *Semitendinosus* muscle and positively in

the *Longissimus* muscle. It seems evident that the involvement of HSPs in beef tenderness is achieved through both their constitutive and inducible expression.

11.3.2 Energy Metabolism

Several studies reporting post-mortem changes in beef (Jia et al., 2006a,b, 2007; Laville et al., 2009; Bjarnadóttir et al., 2010) indicated a clear shift in energy metabolism post-mortem compared to the muscle of the living animal. Differentially abundant proteins between extreme groups of tenderness for *Longissimus thoracis* muscle were glycolytic enzymes such as enolase, particularly enolase 3 (ENO3), malate dehydrogenase (MDH1), triosephosphate isomerase (TPI1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH A), pyruvate kinase (PKM), and phosphoglucomutase (PGM1) (Table 11.2). Relatively few proteins of oxidative metabolism were revealed: aldehyde dehydrogenase (ALDH2), creatine kinase M (CKM), and succinate dehydrogenase (SDH) (Table 11.2).

Enolase 1 (ENO1) and 3 (ENO3) are two isoforms catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis. ENO1 is ubiquitous in cell cytoplasm, whereas ENO3 is mainly present in skeletal muscle. Their correlation to meat tenderness is isoform specific as ENO3 has been correlated with tenderness (Table 11.2). For example, Jia et al. (2006b) showed an increase of ENO3 early post-mortem. After longer aging period (longer than 21 days), an increase of ENO3 fragments has been observed (Laville et al., 2009), indicating the slowdown of the glycolytic process. Moreover, this protein has been reported to exhibit positive correlation with lightness (Gagaoua et al., 2015c) and surface redness of beef (Joseph et al., 2012).

An increase of PGM1 after 5 and 21 days of aging has also been reported (Laville et al., 2009). PGM1 catalyzes the production of 3-phosphoglycerate and ATP from 1,3-bisphosphoglycerate and is present as two isoforms: one with a strongly decreased concentration during the early post-mortem hours while the other strongly increased only after longer aging (Jia et al., 2006a,b).

The enzyme PKM, involved in the catalysis of the transfer reaction of a phosphate group from phosphoenolpyruvate to ATP using a molecule of pyruvate, is found in the form of four isoenzymes among which two, PKM1 and PKM2, predominate in skeletal muscle. A relationship between gene polymorphism of PKM2 and glycogen content in the pig muscle has been reported with increased expression of PKM2 related to a more favorable evolution of glycolytic metabolism early post-mortem (Fontanesi et al., 2003). Likewise, an increased amount of PKM2 correlates with decreased tenderness in Thai chicken meat (Mekchay et al., 2010). One isoform of PKM2 which increases only after longer aging was identified in beef (Table 11.2).

TPI, a glycolytic enzyme which catalyzes the conversion of dihydroxyacetone phosphate to GAPDH, was reported to be related with WBSF (D'Alessandro et al., 2012b; Marino et al.,

2014; Grabež et al., 2015). TPI was also proposed as a potential biomarker for intramuscular fat (Kim et al., 2008) and discoloration during post-mortem storage in beef (Wu et al., 2015).

The oxidative enzymes revealed to be related to tenderness are associated to mitochondria and ATP regeneration (Ouali et al., 2013). In striated muscles, the protein CKM is associated with the transfer of a phosphate group from phosphocreatine to ADP to generate ATP. An increase of fragments of this protein during the aging of meat has already been described (Jia et al., 2007; Laville et al., 2009; Bjarnadóttir et al., 2010).

For the *Longissimus thoracis* muscle of young bulls from the main French beef breeds, a study by our group showed that several proteins representative of glycolytic metabolism were negatively related with tenderness (Chaze et al., 2013). On the contrary, several proteins of oxidative metabolism were positively related with tenderness. This is in accordance with the earlier-cited study of Morzel et al. (2008) showing that abundance of SDH was positively correlated with initial and overall tenderness in *Longissimus thoracis* muscle. We have recently validated these relationships in other young bulls of French breeds (Picard et al., 2014). However, we demonstrated that in young Aberdeen Angus bulls, which are characterized by higher oxidative and less glycolytic metabolism comparatively to French beef breeds, these relationships were reversed. This is in accordance with the results of D'Alessandro et al. (2012a) showing a positive relation between proteins of glycolytic metabolism and tenderness in Chianina cattle (with oxidative muscles). Laville et al. (2009) in Charolais young bulls also demonstrated that tender meat had higher abundance of mitochondrial membrane fragments in the early post-mortem period, suggesting increased degradation of the mitochondrial membrane in slow oxidative muscles. Mitochondria play a central role in the apoptotic process and a large set of proapoptotic proteins are translocated from these organelles to the cytoplasm and/or to the nucleus according to their biological function (Ouali et al., 2013; Sierra and Olivan, 2013).

11.3.3 Oxidative Stress

During muscle to meat conversion, muscle is subjected to various reactive oxygen species (ROS) which are able to target both the lipid and protein fractions (Brand, 2010). Among the protective scavenger agents revealed by proteomic beef tenderness studies, we found peroxiredoxins (PRDX1, 2, 3, and 6), superoxide dismutase (SOD), DJ-1 (encoded by *PARK7*), and aldehyde dehydrogenase (Jia et al., 2009; Guillemin et al., 2012; Polati et al., 2012; Grabež et al., 2015). Among them, SOD1 was positively correlated with WBSF in *Semitendinosus* muscle (Guillemin et al., 2012). SOD is an antioxidant which allows a fast dismutation of O_2^- to O_2 and H_2O_2 . In a recent study by Grabež and coworkers, superoxide dismutase (Cu-Zn) was more abundant in tough meat (Grabež et al., 2015). The authors suggest that stronger oxidative stress may cause ROS accumulation in tough meat and reduce cell protection by Cu/Zn-SOD.

DJ-1, also known as *PARK7*, is another protein revealed by proteomic studies to be related to meat tenderness (Jia et al., 2007, 2009; Polati et al., 2012; Picard et al., 2014; Gagaoua et al., 2015b). This highly conserved protein has the ability to oxidize and translocate to mitochondria in response to oxidative stress, thus serving a role as sensor and protector against toxicity from oxidative stress and limiting mitochondrial fragmentation (Thomas et al., 2011). It was further reported to play an antiapoptotic role by the involvement of its antioxidant activities. Furthermore, it has also been reported to increase in abundance in the bovine *Longissimus* muscle during the early tenderizing period (Polati et al., 2012), after 10 h (Jia et al., 2007) and after 5 and 21 days post-mortem (Laville et al., 2009).

Among the PRDX family members reported to play an important role in the determinism of beef tenderness and to maintain cell homeostasis, PRDX6 was identified as a potential marker of beef tenderness Jia et al. (2009). PRDX6 is the only 1-cysteine peroxiredoxin that uses glutathione (GSH) rather than thioredoxin as the electron donor and is a bifunctional enzyme with both GSH peroxidase and acidic Ca^{2+} -independent phospholipase A2 (PLA2) activities (Fisher, 2011). This protein was found to be related with meat tenderness as well as other quality traits, such as pH decline and meat color (Gagaoua et al., 2015c). Other PRDXs were also identified by proteomic studies (Table 11.2). PRDX1 is involved in regulating the cell redox state and plays an important role in the elimination of peroxide produced during metabolism. Polati et al. (2012) reported a decrease in the abundance of this protein after the early tenderizing period and a strong increase from early to late in the tenderization period. Further, thioredoxin and thioredoxin-dependent peroxide reductase (PRDX3), a mitochondrial precursor was more abundant in tender meat (Grabež et al., 2015). Thus, thioredoxin/PRDX3 was suggested as a very efficient system concerning the regulation of ROS production in mitochondria (Jia et al., 2007).

Finally, in addition to their role in glycolysis (Jia et al., 2006b), identified mitochondrial ALDH are reported to protect cells from the oxidative stress through the oxidation of cytotoxic aldehyde derivates (Ohta et al., 2004). Overall, the proteins identified to be involved in oxidative stress were proposed as negative biomarkers of tenderness mainly in glycolytic muscle types (Guillemin et al., 2012). However, it is presently difficult to get conclusions about their real role in post-mortem muscle and thus, more investigations are needed in order to clarify the exact relationship with ultimate meat tenderness.

11.3.4 Structural Proteins

During post-mortem tenderization there are major changes in the myofibrillar structure and thanks to proteomics, their study and identification were possible (Lametsch et al., 2003). Among the structural proteins identified by proteomic investigation that were shown to be related to beef tenderness, we found α -actin (Lametsch et al., 2003; Hwang et al., 2005; Morzel et al., 2008; Zapata et al., 2009; Bjarnadottir et al., 2012; Polati et al., 2012). This

protein was more abundant in the tender group in the *Longissimus* muscle of young bull from three French breeds (Chaze et al., 2013). The authors also identified the structural protein CapZ β with a higher abundance in the tender group. A study by Laville et al. (2009) identified an actin fragment of 31 kDa to be upregulated in the tender meat of Charolais young bulls compared to tougher Charolais meat. Ouali et al. (2013) suggested that α -actin, a major constituent of contractile apparatus in muscle tissue, constitutes a good marker of post-mortem apoptosis. It was found to be modulated during maturation of bovine muscle (Bjarnadóttir et al., 2010). Full-length α -actin was also observed with decreased abundance in the tender group by iTRAQ and 2-DE (Kim et al., 2008; Laville et al., 2009; Bjarnadóttir et al., 2010). α -actin has also been reported to be present in less abundance in bovine muscle samples taken 36 h post-mortem and to correlate with WBSF at 72 h post-mortem (Zapata et al., 2009).

Chaze et al. (2013) demonstrated a lower abundance of several spots of TnnTfast in the tender groups of French beef breeds. Moreover, it has also been shown that in bovine *Longissimus* (Jia et al., 2006b, 2007) and in pig muscles (Lametsch et al., 2002) intact TnnT decreases during the post-mortem period, while its fragments increase.

Myosin heavy chain (MyHC) fragmentation of bovine *Longissimus* muscle was found to correlate with tenderness at 7 days post-mortem (Sawdy et al., 2004). It was shown that heavy (MyH1) and light (MyL1, MyL2, and MyL6B) myosin II chains have altered abundance and notably most fragmented chains accumulate during tenderization. Two myosin light chain MLC1 and 2 were reported to be closely related to tenderness of meat by several authors (Zapata et al., 2009; Jia et al., 2009). Bjarnadóttir et al. (2012) reported that MLC1 was more abundant in muscle biopsies from tender meat, while MLC2 was present in less abundance in tender meat. The correlation with tenderness of the two MyHC-I (MyH7) and MyHC-IIx (MyH1) was specific of muscle contraction type. Correlation with tenderness of the fast-twitch MyHC was positive in fast-twitch muscles, and negative in slow-twitch muscles (Picard et al., 2014).

Similarly, Anderson et al. (2012) in beef *Adductor* (tough muscle) and *Longissimus* (moderately tender muscle) revealed that proteins such as actin, MyH1 fragment, myomesin-2, and α -actinin-3 undergo muscle-specific changes during aging.

11.3.5 Proteolysis

The enzymatic machinery that underlies muscle to meat conversion involves synergistic activities of numerous endogenous proteolytic enzymes. There are several proteolytic systems present in muscle, which could participate in post-mortem proteolysis, including the calpain, cathepsin, proteasome systems, and more recently caspases (Sentandreu et al., 2002; Ouali et al., 2006, 2013). These proteolytic systems has been extensively reviewed (Sentandreu et al., 2002; Huff Lonergan et al., 2010) and thus are not discussed in this chapter but only the input of proteomics is presented.

In a proteomic-based approach, putative partners of calpain enzymes were revealed (Brulé et al., 2010). Calpains are members of a large family of 14 related calcium-dependent cysteine proteases. The isoforms μ -calpain and m -calpain are, respectively, two typical and ubiquitous isoforms consisting of a distinct large catalytic subunit, CAPN1 or CAPN2, encoded by *capn1* and *capn2* genes, respectively (Goll et al., 2003). Both enzymes were found to interact with several proteins belonging to numerous biological pathways, namely Ca^{2+} homeostasis, structure, glucose metabolism, heat stress, mitochondria, and apoptosis. The association between Ca^{2+} -regulating proteins and Ca^{2+} -dependent calpains suggest a complex nature of the mechanisms governing post-mortem proteolysis and thus meat tenderness. For example, ryanodine receptor (Brulé et al., 2010) and oxidized HSP70-1A/B (Gagaoua et al., 2015c) were shown to be potential substrates for calpains. Furthermore, the use of proteomic tools revealed that μ -calpain undergoes oxidation by forming an intermolecular disulfide bond and that the oxidation results in the loss of proteolytic activity. This finding highlighted the adverse influence of protein oxidation on meat tenderness. Moreover, changes in the proteome of purified myofibrils caused by a 4-day in vitro incubation with CAPN1 under simulated post-mortem conditions was reported (Lametsch et al., 2004). Recently, with analyses of bovine muscle proteome using protein–protein interactions and correlation networks, additional protein partners (potential interacting proteins) rather than calpastatin (a specific inhibitor of calpains) were revealed to be associated with these enzymes (Guillemin et al., 2011; Gagaoua et al., 2015b).

Furthermore, both proteases were used in an immunological tool as biomarkers to predict beef tenderness (Guillemin et al., 2011, 2012; Picard et al., 2014). Guillemin et al. (2011) showed that the proteolysis contribution to tenderness is different between *Semitendinosus* and *Longissimus* muscles. In *Semitendinosus*, they found a positive correlation with tenderness for CAPN1, in contrast to the negative correlation for CAPN2. As CAPN1 is known to play a major role in tenderness, they supposed that in *Semitendinosus* the role of CAPN2 is less important. The authors stressed forward that this inverse relation compared to *Longissimus* is in coherence with the inverse correlation between contractile and metabolic proteins found with tenderness in the two muscles, already observed by Picard et al. (2014).

11.3.6 Transport, Signaling, and Apoptosis

In addition to the earlier-discussed proteins, numerous other biological pathways such as transport, signaling, and apoptosis are reported. Several of the proteins presented earlier are also related to apoptosis. The hypothesis of the involvement of apoptotic programmed cell death in muscle cells was first proposed by Ouali et al. (2006) as a possible explanation of the high variability in meat tenderness. Later on, numerous studies showed close connections between the apoptotic pathway and meat tenderization (Kemp and Parr, 2012; Ouali et al., 2013; Sierra and Olivan, 2013; Gagaoua et al., 2012, 2015a).

Among the identified proteins we found the annexins (Table 11.2), which are members of a large structurally related and Ca^{2+} -sensitive protein family. Expressed in all eukaryotic cells,

they participate in a variety of cellular processes including apoptosis and intracellular signaling (Monastyrskaya et al., 2009). Bjarnadottir et al. (2012) observed lower annexin A6 levels in tender beef meat. In post-mortem muscle, annexin A6 may act as a brake to apoptosis through inhibition of dynamin-related protein 1 and subsequent intensification of cytochrome C release occurring soon after animal death (Ouali et al., 2013). Bjarnadottir et al. (2012) suggested that the lower abundance of annexin A6 in the tender samples may lead to an accelerated release of Ca^{2+} and thus affect the activity of calpains. Furthermore, a change in the concentration of annexin A1 (also known as lipocortin-1) probably related to apoptosis development, has also been reported to change in post-mortem muscle (Zhao et al., 2010).

Galectin 1, a β -galactose-binding lectin involved in apoptotic process, was proposed to be linked with beef tenderness (Zapata et al., 2009; Bjarnadottir et al., 2012). Both studies suggested that a lower expression of galectin 1 is associated with greater tenderness. However, these somewhat preliminary results warrant further investigations since proteomic studies have yet to confirm these findings.

Numerous post-mortem events are controlled by muscle Ca^{2+} homeostasis (Ouali et al., 2013) and it is well documented that proteins involved in Ca^{2+} regulation are important for meat quality (Picard et al., 2010). Parvalbumin was found more abundant in tender muscles in Charolais and Limousin cattle (Bouley et al., 2004b). Parvalbumin has a strong affinity for Ca^{2+} ions and has been reported to participate in their transfer between cytoplasm and sarcoplasmic reticulum (Berchtold et al., 2000) especially in fast fibers. Inositol 1,4,5-triphosphate receptor type 1 (IP3R1), is another protein involved in Ca^{2+} channel activity that was reported by Kim et al. (2008) to be higher in animal groups with superior tenderness and proposed recently, as related to Hsp70-Grp75 (Fig. 11.4), to play a great role in muscle to meat conversion (Gagaoua et al., 2015b).

11.4 Protein Biomarkers and Their Role in Determining Beef Tenderness

Bioinformatics and statistical analyses of the identified proteins have elucidated the biological mechanisms behind beef tenderness development (Fig. 11.4). Knowledge of the biological mechanisms allowed the construction of a correlation network between several biomarkers of tenderness (Gagaoua et al., 2015b). This revealed some robust correlations as they were observed in three continental breeds and two muscles studied. These correlations allowed a better understanding of the underlying mechanisms. For example, the protein DJ-1 was found to be positively correlated to small HSP (HSP20, HSP27) and to glycolytic enzymes such as Eno3, LDH-B, and MDH1. These correlations originated through the oxidative stress as DJ-1 was reported to play an antiapoptotic role by the involvement of its antioxidant activities (Fan et al., 2008). These authors reported that deficiency in DJ-1 induced an increase of apoptosis through a decrease of *Bax*, a proapoptotic protein regulator, expression and an inhibition of caspase activation, thus affecting muscle structures and meat tenderness development. As such, we proposed that the post-mortem increase of ROS production would increase antioxidant and sHSP proteins expression (Fig. 11.4).

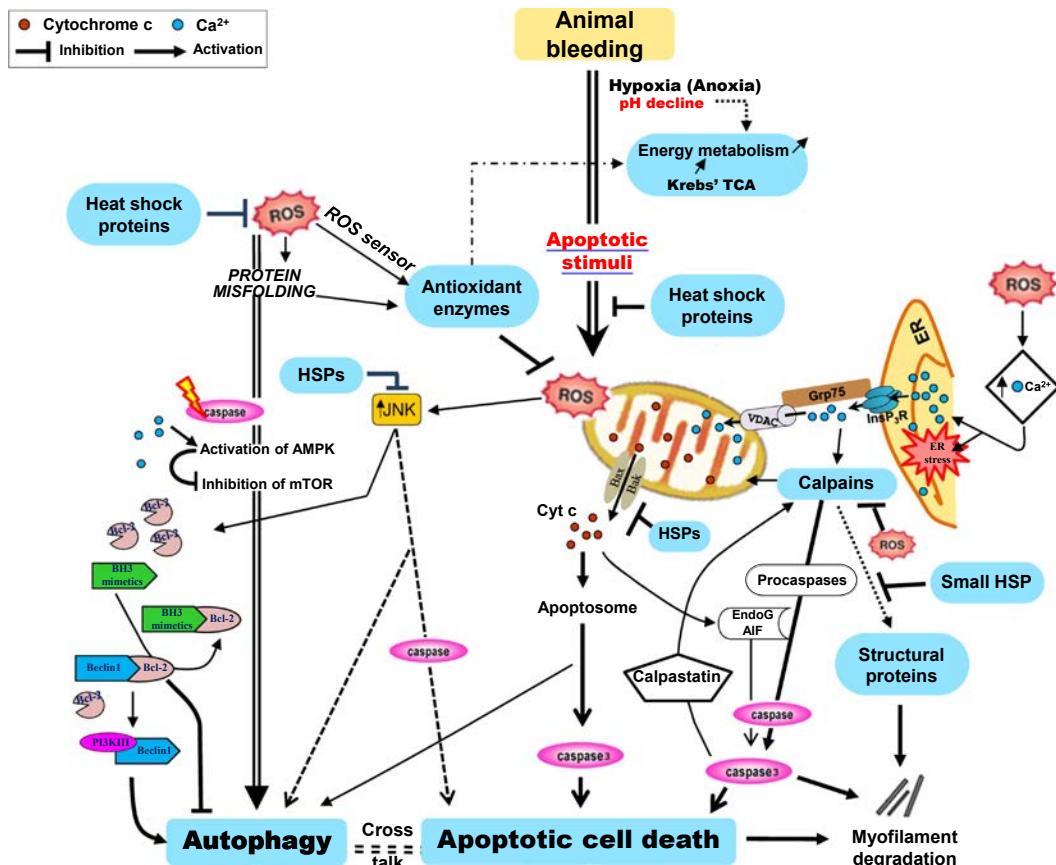


Figure 11.4

Postulated biological mechanisms of post-mortem muscle to meat conversion involving numerous biological pathways proposed thanks to proteomics.

Another robust correlation was found between μ -calpain and PRDX6 (Gagaoua et al., 2015b). It was hypothesized that the correlation could be explained by the PLA2 group of PRDX6 being able to hydrolyze phosphatidylcholine. They proposed that at the first stage of apoptosis post-mortem, the inversion of membrane polarity would induce modifications in the fluidity of the membrane increasing the permeability to ions such as Ca²⁺ and increasing the μ -calpain activity. The activity of μ -calpain was also found to be correlated with several members of HSP70 family in LT muscle but not in ST muscle. HSP70 expression is induced by hypoxic and ischemic conditions and by ROS production (Wang et al., 2012). They are involved in apoptotic pathways through the sequestration of Bcl-2 proapoptotic factor. Under oxidative stress conditions, HSP70 are also reported to collaborate with HSP40, a biomarker of beef tenderness and play an antiapoptotic action (Bernard et al., 2007). According to Gotoh et al. (2004), the antiapoptotic actions may begin with the formation of a complex with active

caspases and slow down the process of cellular death which will constitute an obstacle to good meat aging and consequently to meat tenderness. Further, HSPs may also sequester cytochrome C after its release from the mitochondria (Bruey et al., 2000). Finally, the ROS accumulation can trigger both autophagy and apoptosis, but their precise roles are not known, and their establishment is strongly variable. Further, oxidative stress and mitochondrial metabolism seem to play major roles in initiating a cascade of reactions affecting the determinism of tenderness (Fig. 11.4).

11.5 Conclusions and Future Prospects

Proteomic studies applied to beef tenderness over the last 15 years has led to substantial increase in knowledge in this field confirming the important role of energy metabolism and proteolysis in muscle to meat conversion. Furthermore, proteomics studies have implicated biological functions such as apoptosis, oxidative stress, and autophagy in tenderness. The cumulative findings give researchers a better understanding of the post-mortem modifications and the impact on meat tenderness development. Muscle type and breed effects have been elucidated identifying biological pathways that are muscle-type specific and others independent of the muscle type (Picard et al., 2014; Gagaoua et al., 2015c). Proteins linked to specific biological functions have been proposed as biomarkers which could see application in the future for routine prediction of tenderness. The development of phenotyping tools, making use of these biomarkers, may in time be used by livestock farmers, breeders, and industrials of the beef sector either on carcasses or on live animals. The recent advancements in proteomic technologies and availability of bioinformatic tools (Kaspric et al., 2015) will enable further progress in biomarker discovery and quantification. Moreover, the proteomic strategy applied to beef tenderness starts to be applied to other meat traits, such as color (Joseph et al., 2012), pH drop (Damon et al., 2013; Gagaoua et al., 2015c; Di Luca et al., 2016), water holding capacity (Di Luca et al., 2016; Gagaoua et al., 2016), juiciness and flavor (Cassar-Malek and Picard, 2016), and adiposity (Damon et al., 2006; Kim et al., 2008; Mao et al., 2016).

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Protein Modifications in Cooked Pork Products

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12.1 Introduction

Muscle proteins (~20%) represent the primary constituents of meat and are classified on the basis of their solubility. The myofibrillar proteins are salt soluble (~45%–55%), the sarcoplasmic fraction is water soluble and comprises the main metabolic enzymes (~30%–35%), and the connective tissue (or stroma) proteins are soluble in dilute acidic solutions (~10%–15%). Their physiological role is summarized in [Table 12.1](#).

In raw meat muscle proteins are organized into supramolecular structures via self-assembling and self-organization machinery that acts to build a soft scaffold to protect muscle cells and to permit the physiological mechanisms that convert chemical energy from nutrients into mechanical energy. Such architectonic supramolecular structures are necessary for the contractile properties of the monomeric unit, known as the sarcomere ([Fig. 12.1](#)). The conversion of muscle to meat is due firstly to the enzymatic action of cytoplasmic enzymes, m- and μ -calpains, and then, with pH diminution, to the action of lysosomal enzymes, the cathepsins. These enzymes act to break the supramolecular structure of sarcomere weakening the Z-discs and destroying the anchoring of the actomyosin complex and myofibrillar proteins ([Fig. 12.1](#)). Biochemical and structural changes increase the tenderness of meat in turn affecting quality attributes of meat palatability such as flavor and juiciness traits ([Maltin et al., 2003](#); [Calkins and Hodgen, 2007](#)). Consumer behavior has had implications in terms of both food safety and the development of better sensory attributes ([Mottram, 1998](#)). The first attempts at food processing by cooking meat is thought to have originated with primitive humans that savored roast meat accidentally produced by forest fires. Cooked meat was found to be more palatable and easier to chew and digest. The consumption of cooked meat thus began with the ability to generate and control fire thus contributing to human evolution. Successive animal

Table 12.1: Physiological role of the main meat proteins.

| Proteins | | Characteristics | Function |
|-------------------|--|---|---|
| Myofibrillar | Myosin | Two large polypeptide chains, “heavy chains” (200 kDa), and three small chains “light chains” | Contractile action |
| | Myosin light chain I | Light chain isolated with alkali treatment (20.7–25 kDa) | |
| | Myosin light chain II | Light chain isolated with 5,5'-dithiobis(2-nitrobenzoic acid) (19–20 kDa) | |
| | Myosin light chain III | Light chain isolated with alkali treatment (16–16.5 kDa) | |
| | Actin | G-actin: globular actin polymerizes to form F-actin: fibrous actin | |
| | Tropomyosin | Regulatory protein (66–68 kDa) composed of two polypeptides: α -chain (34 kDa), β -chain (36 kDa) | |
| | Troponins C, I, T | Protein involved for the regulation of contraction-relaxation processes TnC binds Ca^{++} , TnI inhibits actomyosin ATPase; TnT binds tropomyosin | |
| | α - and β -actinin | α -actinin (95 kDa) and β -actinin (34–37 kDa) bind actin | |
| | M-protein | Binds to myosin (165 kDa) | |
| | C-protein | Binds to myosin (140 kDa) | |
| Sarcoplasmic | Titin | Massive protein. Molecular weight of around 1 million Dalton | Scaffold proteins |
| | Desmin | Intermediate filaments. These proteins represent longitudinal intrafibrillar and transverse interfibrillar bridges | |
| | Nebulin | | |
| | Vimentin | | |
| | Synemin | | |
| | Glyceraldehyde phosphate dehydrogenase | Enzymes of the glycolytic pathway | |
| Connective tissue | Aldolase | | Energetic and other globular cytoplasmic proteins |
| | Creatine kinase | | |
| | Myoglobin | Myoglobin (17 kDa) stores oxygen in muscle cells; it is a pigmented protein | |
| | Collagen | Type I (epimysium) Type II and III (perimysium) Type III, IV, and V (endomysium) | |
| | Reticulin | Collagen type III | |
| | Elastin | Fibrillin (glycoprotein) | |

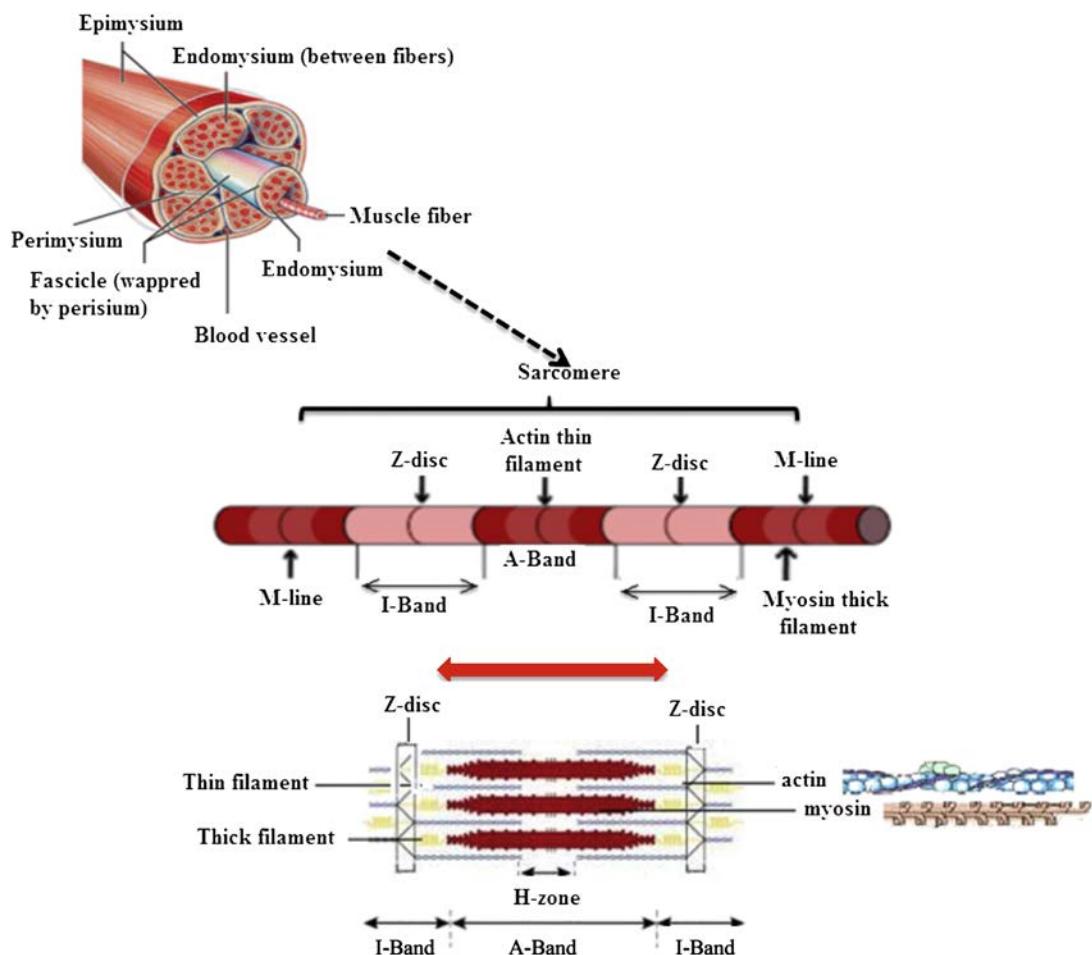


Figure 12.1

From supramolecular architecture of myofibril to the proteins of contractile monomeric unit, sarcomere.

domestication and breeding allowed humans not only to consume the meat but to store the meat by producing both dry cured meats and cooked meat (Civitello, 2007).

Heat treatment induces unavoidable modifications to the structural arrangement of the main meat constituents (Barbera and Tassone, 2006; Tornberg, 2005), especially proteins as a direct consequence of degradation, denaturation, oxidation, and polymerization events (Barbieri and Rivaldi, 2008; Gatellier et al., 2009). When heat is applied to meat, two general changes occur: the muscle fibers become tougher and the connective tissue becomes tenderer.

Specifically, actin, myosin, and other muscle fiber proteins undergo significant changes. The polypeptide chains composed of amino acids (the basic components of proteins) unfold, in a process termed denaturation, and may undergo structural rearrangements such as coagulation.

The result of this process is shrinkage, moisture and fat loss, and toughening of the muscle fiber. The tenderizing effect of moist heat on connective tissue results from the conversion of collagen to gelatin. The extent to which these changes occur in a piece of meat depends on both the time and temperature of cooking.

The denaturation of the muscle proteins influences the structural characteristics (Tornberg, 2005) and the water distribution of the meat (Bertram et al., 2006). Such structural changes lead to substantial loss of water (cooking loss) in the range of 15%–35%. However, the amount of cooking loss is highly dependent on the cooking method, the cooking time/temperature, and the end-point temperature (Aaslyng et al., 2003). Thermographic studies of meat have shown that protein denaturation generally takes place in three steps: (1) myosin (rod and light chain) denaturation occurs at ~40–60°C; (2) denaturation of sarcoplasmic protein and collagen occurs at ~60–70°C; and finally (3) denaturation of actin occurs around 80°C (Deng et al., 2002; Stabursvik et al., 1984; Stabursvik and Martens, 1980; Wright et al., 1977; Wright and Wilding, 1984).

Bertram et al. (2006) found that denaturation of specific muscle proteins resembled the change in water characteristics during cooking. Thus, the greater changes in water characteristics took place between 40°C and 50°C, which suggested that the denaturation of myosin heads was important for subsequent cooking loss. Moreover, the denaturation of myosin rods and light chains at ~53–58°C also affected the water distribution, whilst the denaturation of actin at ~80–82°C was correlated directly to expulsion of water from the meat (cooking loss). Straadt et al. (2007) demonstrated pronounced shrinkage of fibers upon cooking giving rise to large gaps between the cooked muscle fibers, as well as at the level of the individual myofibrils.

During shrinkage the triple-stranded helix is destroyed to a great extent and the decreased dimensions of the fibers and retractile strength is directly related to the number of cross-links formed (e.g., HLNL, hydroxylysine norleucine; DHLNL, dihydroxylysine norleucine; HHMD, histidinohydroxymerodesmosine; PYR, pyridinoline). Concomitantly, water expulsion from the myofibrillar matrix takes place and this was postulated to be one of the mechanisms responsible for cooking loss given by Bertram et al. (2006) and Micklander et al. (2002). Shaarani et al. (2006) identified three distinct water populations in raw meat: free water located in the sarcoplasmic area (Honikel, 1988) that could be easily mobilized, e.g., through minor physical forces formed upon shrinking of myofibrils at the time of rigor mortis; immobilized water that was bound either by steric effects of attraction between the filaments or hydrogen bound with muscle proteins or other macromolecules; and water that was closely associated with macromolecules.

In cooking meat or its products the reorganization of protein structures and water-holding properties have, unquestionably, a greater importance for their commercial value and consumer acceptance. In this chapter we explore the disruption of the supramolecular structure of muscle proteins and the rebuilding of a different supramolecular architecture by self-assembling and self-organization in a hierarchical fashion as a consequence of the heat treatment.

12.1.1 The History of Common Pork Products

Two different models of cooked meat products are generally manufactured, from a whole anatomical pork cut (e.g., leg or shoulder) and comminuted pork pieces from different anatomical cuts. Cooked hams from leg or shoulder preserve the structural organization of anatomical cut, whilst mortadella and würstel lose the muscular filamentous network due to extensive grinding resulting in particles of size less than 0.9 mm.

The origin of cooked ham dates back to the mid-15th century, as quoted noted in *Libro de coquinaria* written by Martino of Como (c.1465), where it was obtained from the hind limb of a pig, from which the fat was removed, cut, boned, massaged, processed, and, finally, steamed. “Praga” ham is a related specialty from the Trieste area of Central Europe, which formerly belonged to the Austro-Hungarian Empire. For this style of ham, the cooking stage is carried out in special hot-air ovens rather than by exposure to wet steam. Following this, the smoking stage is entirely natural and is based exclusively on beech wood.

Cooked hams are produced by heating in an oven to a core temperature of 70°C, following brine injection and tumbling. Generally, a cooking time of 1 h/kg of meat product is required, which means an overall time of 10–12 h.

One of the most important quality attributes of cooked ham is the juiciness; therefore, the cooked ham production starts with the choice of raw ham piece with higher pH values and ionic strength, which are associated with better water-holding capacity (Puolanne et al., 2001; Puolanne and Halonen, 2010). The water-holding capacity is linked to the pH of the ham; pH values within the range 5.8–6.2 may assure good water retention.

Comminuted meat products include mortadella di Bologna, a typical Italian cooked sausage, and würstel, a sausage manufactured worldwide. The origin of the word “mortadella” is somewhat controversial, but one of the most reliable hypotheses is that it derives from the late Latin “mortarium,” which described the mortar and pestle, in which the friars in Bologna (Italy) prepared the mixture of pounded meat mixed with fat and spices. Nowadays, comminution is accomplished by grinders that reduce the granulometry of the meat to less than 0.9 mm and the cooking is performed in stages (drying, precooking, firing, and second firing) with temperatures as high as 80°C for 19–20 h overall (Barbieri et al., 2013). The würstel is linked to the butcher Johann Georg Lahner, who in 1807, invented the frankfurter sausage that gradually spread to the entire Austro-Hungarian Empire (Lahner, 1969). Würstels are cooked at variable times and temperatures, typically for about 2 h or until the whole product reaches a temperature of 70°C. Following this, the sausages are traditionally smoked with beech wood, to give them a characteristic flavor. Therefore, the heat-induced effects on the structural and conformational arrangement of the muscle proteins within these products are expected to be different.

12.1.2 Effect of Heat Treatment on Meat Proteins in Cooked Pork Products

An undesirable effect of heat on the cooked meat processing is the shortening of fibers. The cooking process increases the intramuscular connective tissue contribution to toughness, in the range 20–50°C and only above 60°C is there a prominent myofibrillar contribution (Bendall and Restall, 1983). The transverse shrinkage of the fiber starts at 35–40°C and then increases almost linearly as a function of temperature and at 65–80°C shrinkage of meat is visible through a volume reduction in the muscle fibers, increasing their toughness (Lepetit et al., 2000). Therefore, the shrinkage of the connective tissue per se starts at 60°C and around 65°C it contracts more intensely. However, the amount of shrinkage varies substantially from about 7% area up to 19% area. The discrepancy in results could, among other things, be due to large biological variation within a muscle and among different muscles. The shrinkage phenomenon is strongly reduced by applying pressure to the entire meat cut in a mould or to finely minced meat pieces. Through application of pressure a linear structure similar to that of native collagen (triple-stranded helix) is maintained, thus avoiding the shortening phenomenon that is due to denaturation and the consequent restructuring of the random coils in the collagen network. In highly comminuted meat products, collagen fibers are already broken such that the shrinkage is not perceived.

In addition, heat treatments and processing result in decreased solubility of the sarcoplasmic proteins in cooked ham and comminuted products, which are attributed to the increased hydrophobicity that occurs as a consequence of heat denaturation and coagulation (Promeyrat et al., 2010). This is caused by destruction of the hydrogen bonding and/or electrostatic interactions at high temperatures wherein the orientation of the dipole of water is disturbed, destabilizing the protein–water solvation system that exists in the “native” state. The conformational transitions and the exposure of hydrophobic amino acids, which, in normal conditions, are hidden within the inner core of the globular proteins (Chelh et al., 2007; Santé-Lhoutellier et al., 2008), promote the aggregation of proteins due to hydrophobic interactions. Davey and Gilbert (1974) found that heat aggregation of sarcoplasmic proteins could occur up to 90°C, and they suggested that during cooking, sarcoplasmic proteins might contribute to the consistency of the meat by forming a gel that links together several structural elements.

12.2 Proteomic Studies of Cooked Meat Products

Most of the proteomic investigations and related studies have examined the change in protein pattern during the transformation of fresh pork meat either to cooked or dry cured ham (Di Luccia et al., 2005; 2015; Barbieri and Rivaldi, 2008; Pioselli et al., 2011; Paredi et al., 2012).

Cooked hams and emulsion sausages represent two different models of cooked meat products. As noted previously, the former are generally manufactured from a whole anatomical

cut (leg) that preserves the structural arrangement of the muscle and the latter are produced with comminuted pork meat pieces from different anatomical cuts. Therefore, the heat-induced effects on the structural and conformational arrangement of the muscle proteins are expected to be different. A more recent study confirmed this expectation and showed different patterns of protein modifications as a result of heat treatments and processing (Di Luccia et al., 2015). One of the main observations in this work was the almost complete insolubility of sarcoplasmic proteins in cooked ham and comminuted products, which was attributed to the increased hydrophobicity, as a consequence of heat denaturation and coagulation (Promeyrat et al., 2010).

The two-dimensional electrophoresis (2-DE) map of the muscle proteins from raw pork and cooked samples is shown in Fig. 12.2. As expected, myofibrillar proteins migrated within the acidic side of the 2-DE map, whereas sarcoplasmic proteins tended to be spread toward the alkaline side, above pH 7.0. Despite the tumbling and heat treatments that cooked hams undergo, the main myofibrillar and sarcoplasmic proteins are characterized by a higher total number of detectable protein spots (211 and 242, respectively) compared to raw pork (156). In the case of emulsion sausages protein extract is characterized by a very limited number of detectable spots (70 and 68, respectively) and surprisingly, myofibrillar proteins such as TPMs, myosin light chains, and other minor proteins involved in muscle contraction were almost completely missing. Table 12.2 shows the total number of spots (ns) for the various cooked products, the percentage of variation compared to raw pork, and the total volume of the spots. It is noteworthy as the values of ns for Parma and “Praga” cooked hams represent 135% and 156%, respectively, of the raw pork ns ; the values of ns of mortadella and würstel were 45% and 44%, respectively, compared to the raw pork. The total volumes increased as the number of spots increased. These findings were defined by the variation of spot density as the ratio ($D_{ns}/|DV|$), where D_{ns} is the variation in spots and $|DV|$ is the absolute value of the variation in the volume, derived from the image analysis. The variation in spot density determines the degree to which the protein changes in the cooked products with respect to raw pork. The variation of spot density was negative for emulsified sausages and positive for cooked hams. Spot matching, as assessed by image analysis, showed the similarities and differences between the cooked products and raw pork. Parma and “Praga” cooked hams matched raw pork for 58% and 53% of the spots respectively, while mortadella and würstel matched for 22% and 21% respectively. The sarcoplasmic proteins of emulsion sausages also occurred at lower spot numbers and intensities than in cooked hams (Fig. 12.2). These qualitative and quantitative differences in the 2-DE protein patterns depended on a series of factors, including (1) decrement of the stain intensity of sarcoplasmic spots that made it easier to single out individual proteoform within a contiguous train of spots; (2) the appearance of newly formed spots around pI 7.0 in cooked hams, which probably arose due to the heat-induced breakdown of large protein chains (e.g., myosin); and (3) the detection of additional spots in the 50–100 kDa MW range, probably due to partial protein aggregation.

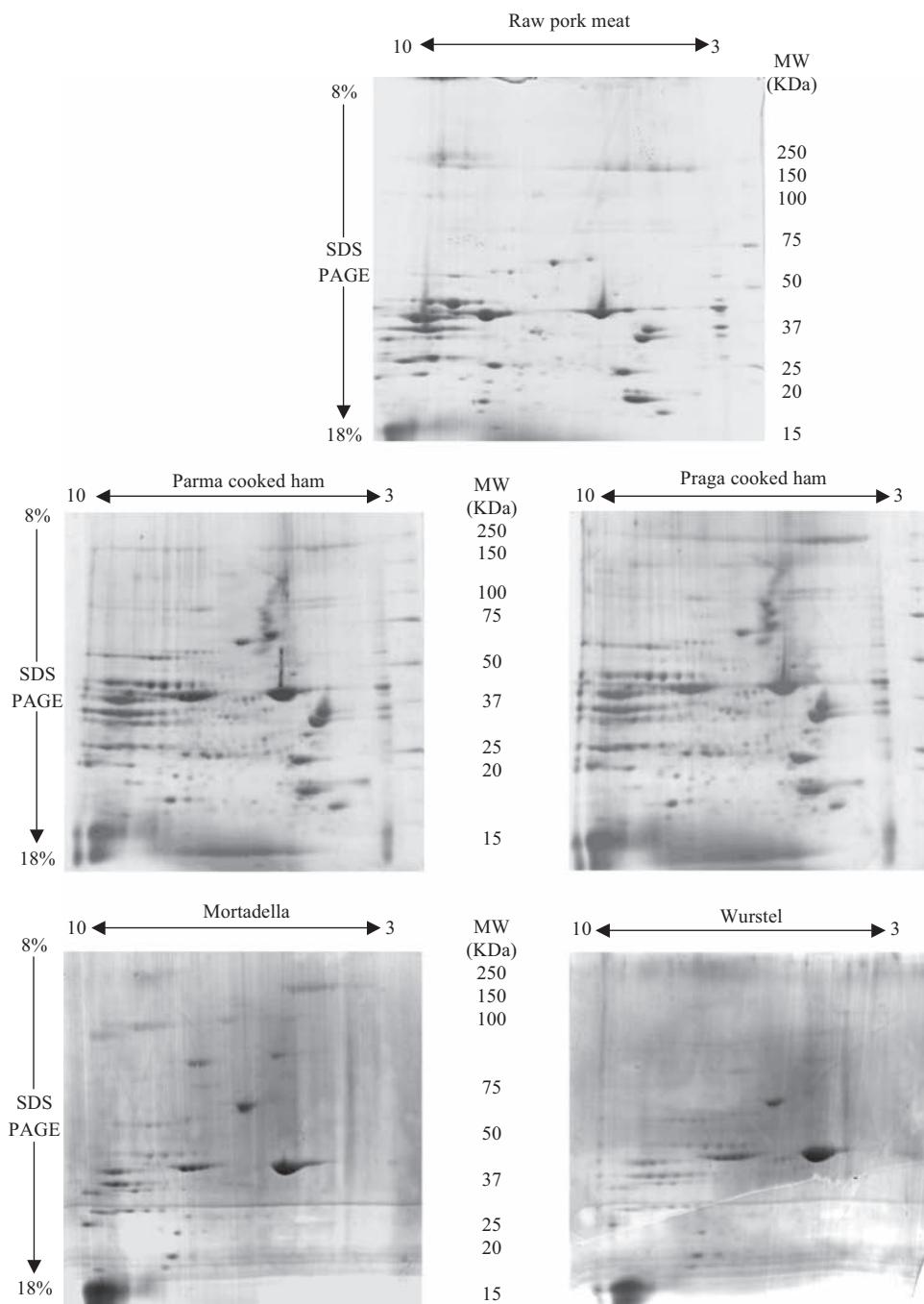


Figure 12.2

2-DE analysis of meat proteins extracted with denaturing and reducing buffer (DRB) from raw meat, cooked hams and emulsion sausages (I extraction). From Di Luccia, A., la Gatta, B., Nicastro, A., Petrella, G., Lamacchia, C., Picariello, G., 2015. Protein modifications in cooked pork products investigated by a proteomic approach. *Food Chem.* 172, 447–455.

Table 12.2: Spots identified from the 2-DE gel electrophoresis of total proteins obtained from first extraction by Image Master analysis.

| Samples | Number of Total Spot | | (V _t) | n _s / ^a V _{trp} ·10 ⁻³ |
|------------------|----------------------|-----|-------------------|--|
| | (n _s) | | | |
| Raw pork meat | 156 | 100 | 87,383.47 | 0.00 |
| Parma cooked ham | 211 | 135 | 116,648.36 | 1.85 |
| Praga cooked ham | 242 | 156 | 116,752.10 | 2.90 |
| Mortadella | 70 | 45 | 35,920.16 | -1.67 |
| Würstel | 68 | 44 | 34,399.42 | -1.66 |

^aV_{trp} = total volume of raw fresh pork sample.

From Di Luccia, A., la Gatta, B., Nicastro, A., Petrella, G., Lamacchia, C., Picariello, G., 2015. Protein modifications in cooked pork products investigated by a proteomic approach. *Food Chem.* 172, 447–455.

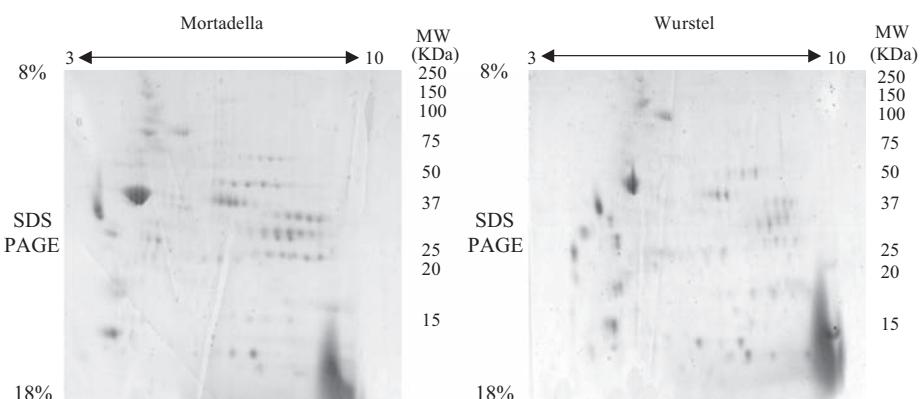


Figure 12.3

2-DE analysis of proteins obtained from sonication-aided denaturing and reducing buffer (DRB) of mortadella and würstel (II extraction). From Di Luccia, A., la Gatta, B., Nicastro, A., Petrella, G., Lamacchia, C., Picariello, G., 2015. Protein modifications in cooked pork products investigated by a proteomic approach. *Food Chem.* 172, 447–455.

The missed detection of several expected major myofibrillar and sarcoplasmic proteins in the 2-DE of emulsion sausages (Fig. 12.2) suggested that they probably constituted an insoluble protein fraction (IPF). Indeed, the 2-DE analysis, aided by ultrasonication, of cooked pork products that were extracted by a denaturing and reducing buffer (DRB), revealed several additional proteins for mortadella and würstel (Di Luccia et al., 2015) and, to a lesser extent, for cooked hams. In particular, the analysis of the DRB fraction of proteins extracted from emulsion sausages showed the presence of intense spots of both sarcoplasmic and myofibrillar proteins, primarily actin. Sequential extraction by DRB (extraction I) and ultrasonication-aided DRB (extraction II) showed that ultrasonication increased the ratio of myofibrillar-to-sarcoplasmic *ns* values (M/S) from 0.23 to 0.93 and from 0.17 to 0.88 for mortadella and würstel, respectively. However, the total spot volumes were stunningly different between the two products, the values for mortadella being much higher (Fig. 12.3).

The percentage contribution of actin to the total volumes also increased more than twice with ultrasonication (extraction II), indicating that it has an important role in the formation of soluble and insoluble protein aggregates in emulsion sausages. The different ratios and compositions of the two protein fractions reported by [Di Luccia et al. \(2015\)](#) suggested that insoluble protein fraction aggregates were formed by nonreducing covalent cross-linking and differed both qualitatively and quantitatively between the two emulsion sausages. These findings indicated that the meat preparation and heat treatment of the production processes induced the formation of peculiar gel networks due to protein aggregation, and this was mainly through the mechanisms of coagulation, denaturation, oxidation, and interchain cross-linking of polymers.

Other proteomic studies performed to define the quality and optimize the processing parameters through the identification and monitoring of molecular markers. [Barbieri and Rivaldi \(2008\)](#) and [Pioselli et al. \(2011\)](#) analyzed the exudate during the manufacturing of cooked ham. The exudate is formed due to the injection of brine, extensive tumbling, and increased temperature. Basically, three steps are crucial to this process ([Barbieri and Rivaldi, 2008](#)): (1) formation of an exudate rich in myofibrillar proteins; (2) the formation of a network at a temperature near 62°C; and (3) the loss of exudate in cooking subsequent to the formation of a myofibrillar matrix. These authors identified desmin in the exudate as a marker of the heat-induced modifications. Desmin steadily increased in any sample of the exudate of the cooked ham sample, but it decreased or even disappeared once the meat structure reached a steady state, and the cooking process could be stopped. On the other hand, the lack of desmin in the exudate indicates that the meat is not sufficiently cooked or that the meat structure is too strong for a good network to be formed. In the cooking phases of production, raw ham and raw pork undergo changes. Muscle meat proteins denature, which causes deep structural changes, such as the destruction of cell membranes ([Rowe, 1989](#)), the transversal and longitudinal shrinkage of meat fibers, the aggregation and gel formation of sarcoplasmic proteins, and the gelation and solubilization of connective tissue ([Tornberg, 2005](#)). To avoid shrinkage and fiber separation, and to obtain homogenous products, the application of pressure on the pork leg is necessary during cooking in order to ensure that the muscle fibers cohere by gel formation, due to the denaturation of proteins and the solubilization of connective tissue. In a system in which the fibers are forced to remain in a firm structure, an increase in temperature first causes myosin heavy chain (MHC) denaturation, followed by an increase in emulsification, sarcoplasmic coagulation, and successive incorporation within the myofibrillar network. Finally, the fibers adhere to each other due to a collagen gelation that acts like glue. The result is a compact and homogenous product in which the fibers are no longer distinguishable. In this case, protein networks are generated mainly through disulfide links and hydrophobic interactions, as demonstrated by a very small insoluble residue obtained from total protein extraction in the DRB. This also explains the progressive decrease of myofibrillar proteins in the exudate from ham during the cooking process.

Emulsion sausages (e.g., mortadella and würstel) lack the fiber structure that serves to increase the solubilizing effect on the muscle proteins. Moreover, the process of comminution results in incorporation of air into a system that is composed primarily by fat, proteins, and water. In such a system, the conditions for generating both metal-catalyzed and myoglobin/H₂O₂ oxidative phenomena, that underlie the formation of reducible and nonreducible link formations, exist (Bhoite-Solomon et al., 1992).

The dissolution of the myofibrillar protein into the water phase during comminution and blending represents the most important factor for judging the quality of the protein network and the structure of the emulsion sausage. Solubilization and heat denaturation of the myofibrillar and soluble sarcoplasmic proteins leads to a protein solution with emulsifying properties that acts to conglomerate the exposed hydrophobic residues with fats and is also prone to oxidation. These events underlie the formation of aggregates and, likely, the polymeric protein networks. With extraction using a denaturing and reducing buffer, we obtained larger amounts of protein residue from the emulsion sausages than from the cooked hams. Moreover, the complete solubilization of the aggregate by breaking the disulfide bonds (Singh et al., 1990) and, probably, other covalent bonds was achieved by combining the use of sodium dodecyl sulfate with sonication. This explains the different 2-DE patterns of proteins obtained from the total protein extract and from the IPF supporting the existence of two types of aggregation in the gel matrix residue, one characterized by the presence of actin alone and the other with interactions between actin and the remaining myofibrillar proteins and some of the associated sarcoplasmic proteins.

12.3 Spot Identification by MALDI-TOF MS

Protein identification has also been accomplished in previous 2-DE analysis (pI range 3–10) for muscle proteins from different mammals (Bendixen, 2005). We note the occurrence in cooked pork products of a neo-formed series of spots in the 65–110 kDa range; this is particularly evident in the 2-DE maps of cooked hams. They were identified as α -actinin, MHC, and BSA. The MHC spots migrated at similar pIs, but with lower MW than the parent protein, which indicated an extensive cooking-induced breakdown of the chain during the cooking process. Interestingly, almost all of the tryptic peptides of the MHC fragments mapped to the central and C-terminal protein region, indicating that the 2-DE spot arose from the fibrous (C-terminal) region of myosin. On the other hand, the N-terminal heads (up to the actin-binding domains) of MHC are for the most part engaged in the formation of the coalesced heat-induced gel networks, and this could be why they are resistant to extraction (Sharp and Offer, 1992; Tornberg, 2005). The majority of the other identified spots corresponded to intact protein chains. This was also established by comparing the theoretical and experimental 2-DE coordinates (MW/pI), which demonstrated that heat-induced proteolysis is a rather uncommon event and affects MHC almost exclusively. Many of the methionine residues were partially oxidized, as demonstrated by MALDI-TOF MS mapping (Fig. 12.4) of the tryptic peptides of actin (Di Luccia et al., 2015).

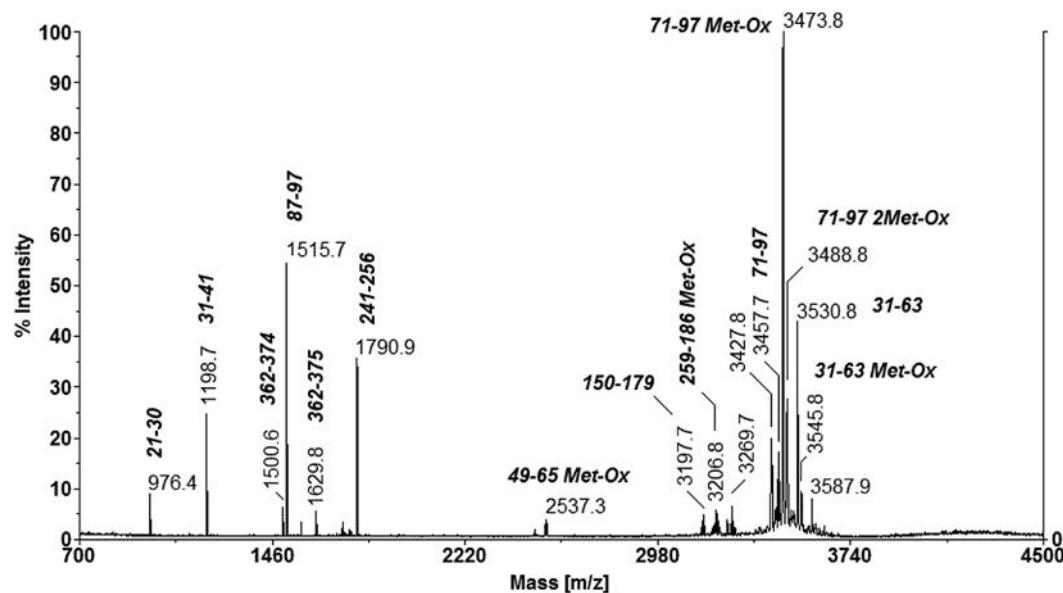


Figure 12.4

MALDI-TOF spectrum of Actin. Met-Ox indicates peptides containing oxidized methionine.

12.4 Supramolecular Rebuilding of Meat Proteins in Cooked Pork Products

During cooking, muscle proteins undergo conformation changes and alterations to their aggregation state dependent on the cooking method. The transition from a native conformation to an unfolded one in the two models (whole anatomical cuts and comminuted emulsified sausages) involves two different self-assembling mechanisms resulting in a new architectural network. Molecular self-assembly is a powerful approach to build up novel supramolecular architectures. Supramolecular self-assembly is mediated by weak noncovalent bonds, notably hydrogen bonds, ionic bonds (electrostatic interactions), hydrophobic interactions, $\pi-\pi$ stacking, van der Waals interactions, and water-mediated hydrogen bonds. Proteins interact and self-organize to form well-defined structures that are associated to their intrinsic ability to respond to environmental conditions.

The supramolecular assemblies determine the greater network with an architecture that includes meat proteins. This example also illustrates that different quaternary structures can be generated from the same biopolymer through small changes linked to the environmental conditions. Understanding the changes to the induced structure in meat proteins is important for the production of meat products. On heating, a filamentous network is formed where each strand is composed of myosin filaments; however, recently [Di Luccia et al. \(2015\)](#) found that actin plays an important role in this supramolecular filamentous network.

Protein aggregation from the denatured proteins involves the formation of higher molecular weight complexes as supramolecular structures (Schmidt, 1981) that are stabilized by cross-links formed at specific sites on the protein chains or by nonspecific bonding occurring along the protein chains. Protein–protein hydrophobic interactions are usually the main cause of subsequent aggregation (Cheftel et al., 1985) providing that the protein concentration, thermodynamic conditions, and other conditions favor the formation of a new tertiary architecture (Schmidt, 1981).

Cross-linking of protein aggregates, following denaturation, usually involves oxidative chemical reactions of proteins resulting from the covalent interaction of their functional groups. In fact, oxidative protein cross-linking can involve different amino acids: basic and aromatic amino acids and cysteine are particularly prone to reacting with free radicals during the cooking process (Petruk et al., 2012; Santé-Lhoutellier et al., 2008). Basic amino acids are oxidized to generate carbonyl products (Stadtman and Levine, 2003; Uchida, 2003). The thiol groups of cysteine can be oxidized with the formation of disulfide bridges (Lund et al., 2011). Tyrosine in myofibrillar proteins can generate dityrosine cross-links (Gerrard, 2002; Lund et al., 2011) and the combination of thiol radicals can lead to thiol-tyrosine bonds (Lund et al., 2008; Martinie et al., 2012; Petruk et al., 2012). Finally, the interaction of reactive amino acids, such as lysine and cysteine, with (bifunctional) aldehyde products arising from lipid oxidation can also induce protein networking through the formation of Schiff bases (Gardner, 1979; Refsgaard et al., 2000). In cooked meat products such as comminuted cooked sausages and cooked ham (Estévez et al., 2005, 2007; Estévez and Cava, 2004, 2006; Sun et al., 2010), the extent of protein carbonylation was also investigated. Carbonylation is an irreversible and nonenzymatic modification of proteins that involves the formation of carbonyl moieties induced by oxidative stress and other mechanisms (Berlett and Stadtman, 1997). These modifications may be directly derived from oxidative damage to proteins and/or by inducing conformational changes leading to denaturation (Xiong, 2000). The presence of these various links results in the formation of larger aggregates or/and insoluble polymeric protein networks. In the light of these results, it is possible to hypothesize that the cooking of a whole anatomical cut or of comminuted pork pieces generates different aggregates and/or insoluble polymeric protein networks.

Cooked ham, from whole anatomical cuts, maintains the supramolecular architecture as raw meat but the protein denaturation, caused by heat treatment, rearranges meat proteins during cooked meat processing (Di Luccia et al., 2015). In emulsion sausages, the comminution causes mechanical disruption of the native supramolecular organization, reducing fiber dimensions before protein denaturation through heat treatment can reorganize the large aggregates as supramolecular structures (Fig. 12.5). As a matter of fact, in both cases, of whole and comminuted cuts, the disrupted structure of stroma and myofibrillar proteins existing as random coils, which are soluble in water, is called gelatin. However, the response to heat treatment is dependent on the chemical and physical properties of the meat protein fraction.

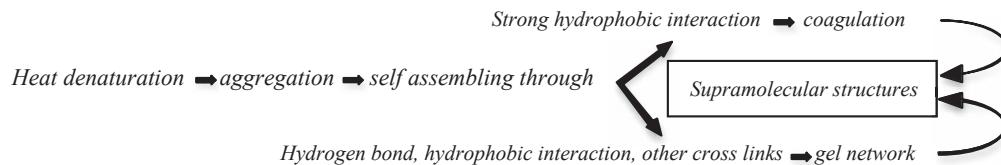


Figure 12.5

Schematic representation of the mechanisms involved in supramolecular structures formation by heat treatment.

12.5 Conclusions

Proteomic approaches have proved that emulsion sausages contain larger amounts of insoluble residue, such as gelled collagen, than cooked hams. The presence of an insoluble protein fraction with a high myofibrillar-to-sarcoplasmic protein ratio can be recovered from the insoluble residue of emulsion sausages. Actin is the main protein in emulsion sausages, and it forms aggregates with tropomyosins and myosin light chains that occur almost exclusively in the insoluble protein fraction. Proteins in cooked pork products are subjected to an oxidative environment, the effects of which are evidenced by the MS identification of a high proportion of oxidized methionine in the muscle proteins. The differences observed between the protein composition of cooked ham and emulsion sausages were ascribed to the degree of solubilization of the myofibrillar proteins, their distribution in the network gel of cooked products, the temperature cycle utilized, the presence of fats, and the status of the pork cut (whole cut or comminuted). The gel properties of cooked pork products determined the nature of the myofibrillar network and their inclusion within the coagulated sarcoplasmic protein matrices, giving rise to a homogenous gel matter bonded by covalent and noncovalent links. Denatured MHCs, and probably solubilized actin, contributed to the homogeneity of these products by emulsifying the fat matter within the myofibrillar and sarcoplasmic gel. Finally, proteomic studies have led to a deeper understanding of the rebuilding of denatured meat proteins into novel supramolecular structures determined by self-assembling and self-organization machinery.

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Poultry and Rabbit Meat Proteomics

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13.1 Introduction

Proteomics, like transcriptomics and metabolomics, is considered one of the so-called postgenomics tools. It is one of the fastest growing fields in biological sciences and its importance in the context of animal health and animal science has been steadily increasing as recently reviewed (Eckersall et al., 2012; de Almeida et al., 2014; Almeida et al., 2015). Proteomics may be defined as the science that studies the proteome i.e., the proteins present in a given cell, tissue, organ, organism, population or biological fluid, or any resulting food such as meat or dairy products. Ultimately proteomics aims to characterize the changes in protein expression in that cell, tissue, organ, organism, population, or biological fluid as a consequence of a provided stimulus or condition. Generally speaking, proteomics in meat science has been predominantly applied in two approaches: two-dimensional gel electrophoresis (2DE) coupled to protein identification using mass spectrometry (MS) techniques such as MALDI TOF/TOF (Matrix Assisted Laser Desorption Ionization–Time-of-Flight) and the so-called shotgun proteomics, purely based on the identification and quantitation of proteins using MS often coupled to high performance liquid chromatography (HPLC). For further information, kindly refer to the review by Almeida et al. (2015). While there is a rapid increase in the number of proteomic studies in the diverse fields of animal sciences, these studies have been hindered by the quality and accuracy of public databases. These are highly dependent on the degree of representability of a domestic animal (Soares et al., 2012). As illustrated in Table 13.1, the composition of public databases varies considerably from species to species. In fact, in the last decade the cattle (*Bos taurus* and *Bos indicus*), pig (*Sus scrofa*), and rabbit (*Oryctolagus cuniculus*) genomes have been thoroughly studied and/or sequenced (Ferreira et al., 2013; de Almeida and Bendixen, 2012; Miller et al., 2014) translating to higher numbers of proteins annotated in the public databases. Yet for other domestic species, such as small ruminants including sheep (*Ovis aries*) and goats (*Capra hircus*) or ducks (*Anas platyrhynchos* and *Cairina moschata*), the number of proteins automatically populating the databases (for example in NCBI) might be similar, but the number of manually curated proteins with experimental evidence in public databases such as SwissProt is extremely limited. Despite this drawback the importance of proteomics in the field of meat sciences has recently been addressed (Paredi et al., 2012, 2013) including investigations of some less-characterized species.

Table 13.1: Number of entries for the most important farmed animal species, man, rat, and mice in the redundant NCBI, nonredundant RefSeq, and curated SwissProt databases.

| Species | Scientific Name | NCBI ^a | RefSeq ^a | SwissProt ^a |
|----------------|------------------------------|-------------------|---------------------|------------------------|
| Cattle | <i>Bos taurus</i> | 126,991 | 64,771 | 5,998 |
| Pig | <i>Sus scrofa</i> | 69,414 | 47,478 | 1,416 |
| Chicken | <i>Gallus gallus</i> | 68,401 | 46,448 | 2,274 |
| Rabbit | <i>Oryctolagus cuniculus</i> | 47,083 | 37,656 | 890 |
| Sheep | <i>Ovis aries</i> | 106,407 | 95,766 | 461 |
| Goat | <i>Capra hircus</i> | 35,794 | 30,250 | 117 |
| Mallard | <i>Anas platyrhynchos</i> | 50,337 | 30,778 | 53 |
| Muscovy duck | <i>Cairina moschata</i> | 191 | 13 | 18 |
| Turkey | <i>Meleagris gallopavo</i> | 27,386 | 26,463 | 57 |
| Quail | <i>Coturnix japonica</i> | 39,907 | 39,111 | 87 |
| Geese | <i>Anser anser</i> | 319 | 13 | 15 |
| Red deer | <i>Cervus elaphus</i> | 976 | 39 | 32 |
| American bison | <i>Bison bison</i> | 36,184 | 35,554 | 15 |
| Reindeer | <i>Rangifer tarandus</i> | 450 | 13 | 8 |
| Man | <i>Homo sapiens</i> | 1,015,503 | 101,808 | 20,232 |
| Mice | <i>Mus musculus</i> | 312,831 | 78,325 | 16,794 |
| Rat | <i>Rattus norvegicus</i> | 144,042 | 60,109 | 7,959 |

^aNCBI protein database (<http://www.ncbi.nlm.nih.gov/protein/>).

To date the majority of meat proteomics papers have been published on topics related to beef and pork. Despite the fact that the domestic chicken (*Gallus gallus*) genome has been sequenced, little information has been published on poultry meat proteomics. In a similar manner even though the rabbit is an extensively studied species, little information seems to be published on rabbit meat proteomics with a preponderance instead for publications in the physiology field, particularly cardiac and skeletal muscle proteomics (Miller et al., 2014). In this chapter, we aim to provide an overview on the major studies conducted using proteomics at the level of meat science in the rabbit and poultry species. As explained in Fig. 13.1, we will focus on the major achievements of such studies encompassing four major areas: characterization of both the chicken and rabbit muscle proteomes; breed differentiation; effect of stress on muscle proteomics; and the use of proteomics in the characterization of *foie gras* manufacturing process. This chapter will finally end with a general analysis of the major achievements in poultry and rabbit meat proteomics, suggesting major trends for future research in this field.

13.2 Poultry Muscle and Meat Proteomics: A Tool to Study Muscle Growth and Allow Breed Differentiation

Unlike other agriculturally important species, such as cattle and pig, studies conducted using proteomics to unravel the mechanisms of skeletal muscular growth in chickens are limited.

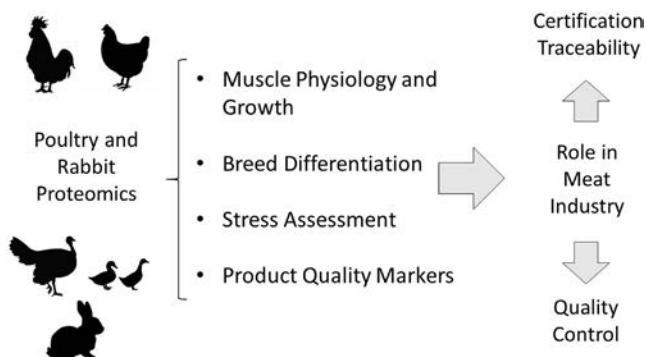


Figure 13.1

Major areas of application to date of poultry and rabbit meat proteomics and their implications and importance in the framework of meat science.

[Doherty et al. \(2004\)](#) conducted the first of such studies. Comparison of the pectoralis muscle of male chicks of a laying strain at different stages of growth (1, 5, 9, 13, and 27 days) using 2DE and protein identification using MALDI TOF/TOF was undertaken. A total of 53 proteins were identified that showed differential expression with apolipoprotein A1, ovotransferrin, and serum albumin showing a trend for decreased expression during the growth phase, whilst other proteins including α - and β -enolase, α - and β -tubulin, adenylate kinase, creatine kinase, triosephosphate isomerase, and phosphoglycerate mutase showed increased expression with muscular growth ([Doherty et al., 2004](#)). The pattern of protein expression was correlated with the growth process in the chicken muscle, in particular with protein turnover in these tissues. The chicken was proposed as a good model for muscle developmental studies. A similar study published by [Teltathum and Mekchay \(2009\)](#) examined the pectoralis muscle by 2DE, but differed in the use of Thai indigenous chickens and more mature ages in the growth process (0, 3, 6, and 18 weeks). Several of the proteins previously determined by [Doherty et al. \(2004\)](#), for example phosphoglycerate mutase, also increased in expression with age and muscle growth, but several others including triosephosphate isomerase and apolipoprotein were found to have a decreased expression as a consequence of muscle growth in contrast to the previous study.

These studies clearly demonstrated both the importance of the use of proteomics and the chicken model to understand the biological and structural aspects of muscle growth and differentiation. Interestingly, and given the differences in protein expression profiles between the two studies, the applications of proteomics in the context of breed differentiation could also be inferred. This is an important aspect in meat science as ultimately it allows the development of tools for product authentication and the ability to detect fraudulent addition of unlabeled ingredients. [Zanetti et al. \(2011\)](#) compared the pectoralis muscle of three Italian chicken breeds: *Pepoi*, *Padovana*, and *Ermellinata di Rovigo* using 2DE and MALDI TOF/

TOF for protein identification. Several proteins were found to have differential expression including structural proteins such as myosin light chain 1 and annexin A5, several metabolism proteins including cofilin-2, lactoylglutathione lyase, and bromodomain-containing protein 4, as well as stress responsive protein heat shock protein β -1. Furthermore, this study demonstrated that the muscle proteome is tightly linked to breed and hence to animal selection processes. Principal component analyses and a neighbor-joining tree clearly demonstrated strong breed differences, finally confirming the importance of proteomics as a tool to characterize poultry meat products of outstanding importance in a context of product value-adding, traceability, and differentiation. [Phongpa-Ngan et al. \(2011\)](#), published an interesting piece of work on the use of proteomics to differentiate groups of chicken with different productive characteristics. Within the same genotype, the pectoralis proteome of animals with different growth rates and different water holding capacity were compared. A total of 22 protein spots were found to show differential expression and proteins such as creatine kinase, pyruvate kinase, triosephosphate isomerase, ubiquitin, heat shock proteins, as well as several structural and contractile proteins were identified. Several of these proteins were proposed as markers of water-holding capacity and also of growth rate, demonstrating once again the potential of proteomics in meat authentication, specifically in the selection of quality and productive traits markers.

13.3 Poultry Muscle and Meat Proteomics: A Tool to Study Restraint- and Transport-Derived Stress

Animal welfare and particularly the reduction of stress in farm animals is one of the most relevant contemporary issues in animal science. This subject, particularly the importance of proteomics in stress studies, has been thoroughly addressed on a recent multispecies review by [Marco-Ramell et al. \(2016\)](#). In poultry, a special concern has been dedicated to slaughter-associated stress, particularly aspects related to the conditions the animals are subjected to during transport to the slaughterhouse. This feature is particularly important as animals are transported together in crates, with no defined social hierarchy and frequently in open trucks exposed to hot or cold temperatures. Such transport conditions have the conditions to potentiate losses due to animal and carcass bruising, not to mention deaths during transport.

Proteomics was first used to address this issue by [Hazard et al. \(2011\)](#) in a study that used a multiomics-based approach combining transcriptomics and metabolomics, in addition to proteomics. Control chickens were compared to chickens that were restrained and transported for 2 h. This integrated analysis conducted at the level of the tight muscle led to the determination of numerous proteins and transcripts with altered expression, most of which belonged to two molecular pathways primarily related with cytoskeleton structure or carbohydrate metabolism. The authors finally related prolonged transport stress to a repression of glycogenolysis and glycolysis and a reinforcement of myofibrils in thigh muscle of chickens. The use of complementary “omics” techniques allowed careful examination of the most

important biochemical aspects associated to chicken transport revealing the important changes in energy metabolism and the structural organization of muscle. The proteomics results were corroborated by transcriptomics. In this study the authors opted to work with the tight muscle instead of the more commonly used pectoralis muscle and for that reason comparison with other studies may be more difficult. Later in 2013, Zanetti et al. studied the same subject in chickens of different ages/weights and transport periods (90 and 220 min). Protein expression profiles were determined using 2DE revealing 12 differentially expressed proteins: eight were related to the age effect, two to time in transit, and two to the interaction between age and time in transit. Both factors affected the muscle proteome with biological processes linked to the cellular housekeeping functions, mainly to metabolism, cell division and control of apoptosis being the most affected pathways. Principal component analysis clustering was used to assess differences between birds. The recommendations of this study included the need to redesign alternative strategies and policies in order to reduce the impact of stress related to time in transit in poultry. Probably the most important aspect of both studies is the fact that the muscle (and hence poultry meat) proteome is severely affected by transport. The data obtained by these two laboratories could be used to design for instance kits of stress detection in muscle and meat, being therefore a valuable tool in the context of meat certification. These studies have contributed to the definition of thresholds for maximum transport time and handling procedures poultry should be subjected to. The cumulative findings are useful for the poultry meat producing sector, the consumer, and regulating authorities.

13.4 Proteomics and Poultry Meat: The Special Case of Foie Gras

Foie gras is a highly valuable meat-derived product produced from the fatty liver of ducks (*Anas platyrhynchos*, *Cairina moschata* or their crosses, known as mule ducks) and to a lesser extent geese (*Anser anser*). The animals are force-fed with high-energy rations in a procedure called *gavage* that induces a fatty liver condition or steatosis. This is a very old and traditional poultry product produced mainly in France and being sold for high prices in gourmet restaurants or grocery stores. Paradoxically and given the low volume of *foie gras* production in the whole context of poultry production, it is noteworthy to mention that this is one of the poultry subsectors where proteomics has been used more often, mainly through the effort of French researchers.

Bax et al. (2012) used 2DE to compare different time points in fatty liver induction demonstrating that steatosis leads to proteome alterations, with enzymes, translation factors, and structural and antioxidant proteins being the most affected. More recently, using a similar approach, Zheng et al. (2014) compared the liver proteomes of two strains of *Anas platyrhynchos* differing in the ability to deposit fat, at different ages. Authors demonstrated that fat ducks had an overexpression of glycolysis proteins, as well as protein catabolism. This is a process somehow similar to *foie gras* production.

Proteomics has been extensively used in the study of *foie gras* production. Theron and co-workers (Theron et al., 2011) used proteomics to establish *post-mortem* markers of fat loss during the cooking of mule duck (*Anas platyrhynchos* × *Cairina moschata*) *foie gras*. The soluble protein fraction at low ionic strength was analyzed by 2DE while the nonsoluble fraction was analyzed by shotgun proteomics. The combined analysis of both datasets suggested that at the time of slaughter, livers with low fat loss during cooking were in an anabolic state with regard to energy metabolism and protein synthesis, whereas on the contrary, livers with high fat loss during cooking developed cell protection mechanisms. Heterogeneity in the state of fatty livers found in commercial production was the main causes for such results. The same research laboratory (see Theron et al., 2013) conducted a follow-up study using the same techniques to study protein expression in fatty livers during chilling. The 2DE analysis revealed 36 (21 identified) and 34 (26 identified) protein spots of interests in the low-fat-loss and high-fat-loss groups, respectively. The expression of proteins was lower after chilling, which revealed a suppressive effect of chilling on the biological processes prior to slaughter. The shotgun strategy allowed the identification of 554 and 562 proteins in the low-fat-loss and high-fat-loss groups respectively. The majority were cytoskeleton and associated proteins. Similar to their previous study, the authors associated the heterogeneity of livers as the main cause for the changes in technological yield observed in *foie gras* processing plants. Finally, in 2014, the same research group conducted a 2DE-based study that established protein markers that were linked to several quality traits in the finished product: liver weight, melting rate, protein contents of crude, or dry matter (François et al., 2014). *Foie gras* can therefore be considered and exceptionally interest study case and what has been described in this section is of relevance to the whole industry, highlighting the importance in establishing quality markers specifically associated to the different steps in the production process. This rationale could be readily applied to a broad range of different poultry meat products.

13.5 Rabbit Muscle and Meat Proteomics

The rabbit is one of the most studied laboratory and production species. Proteomics has been extensively used in this species in a variety of subjects, particularly those related to skeletal and cardiac muscle physiologies (Miller et al., 2014). However, in the context of rabbit meat proteomics for food production, very few papers have been published on this subject. The majority were dedicated to muscle proteome characterization and one paper concerned the muscle proteome as affected by breed and nutritional management.

In 2005, Maughan et al. used one-dimensional polyacrylamide gel electrophoresis and protein identification using peptide mass fingerprinting representing one of the first attempts to characterize the skeletal muscle rabbit proteome. The focus of this study was to determine the concentrations of glycolytic enzymes and other cytosolic proteins in muscle and correlate these with protein–protein interactions on the basis of stoichiometry. Later in 2009, Almeida et al.

used an approach based on 2DE gels and peptide mass fingerprinting for protein identification. In that early study, the authors identified and mapped 45 different proteins that were classified according to different major roles: cell structure contractile apparatus; metabolic and cell defense proteins. The study aimed essentially to serve as a model for future studies on the gastrocnemius muscle proteome using 2DE. More recently, a Chinese team has thoroughly characterized the rabbit skeletal muscle sarcoplasmic reticulum (Liu et al., 2013) and the mitochondrial (Liu et al., 2015) proteomes using shotgun proteomics. The results are strikingly different when compared to the earlier approach using 2DE (de Almeida, 2013) leading to the identification of over 450 different proteins in both cases taking rabbit skeletal muscle proteome characterization to a new level. Using a totally different approach, Staunton and Ohlendieck (2012) used one-dimensional gradient gels and on-membrane digestion to study the rabbit skeletal muscle sarcoplasmic reticulum proteome. The study showed the presence of 31 different protein species in the sarcoplasmic reticulum, including key Ca^{2+} -handling proteins such as the ryanodine receptor, Ca^{2+} -ATPase, calsequestrin and sarcalumenin providing an additional step in the characterization of a difficult to analyze fraction of the rabbit skeletal muscle. These works serve as a basis for future proteomics studies on rabbit muscle and consequently rabbit meat research.

Almeida et al. (2010) conducted a study comparing the reaction of New Zealand White rabbits (the major domestic and meat producing breed) and Iberian Wild rabbits to weight loss (20% weight decrease) using 2DE and peptide mass fingerprinting. Their results showed that L-lactate dehydrogenase, adenylate kinase, α - and β -enolase, fructose bisphosphate aldolase A, and glyceraldehyde 3-phosphate dehydrogenase, which are enzymes involved in energy metabolism, were differentially expressed in restricted diet experimental animal groups. These were suggested as biomarkers of weight loss and putative objects of manipulation as a selection tool toward increasing tolerance to weight loss. A similar role was proposed for the important structural proteins tropomyosin β -chain and troponin I. Akin to the studies described for poultry breed differentiation, this work demonstrates the role, importance, and potential for proteomics in the context of rabbit meat differentiation and characterization of rabbit meat product, despite the scarcity in published studies to date.

13.6 Conclusions and Future Perspectives

Proteomics and its allied disciplines have been extensively used in meat research, particularly on species such as cattle and pig that have a high individual value. Despite the potential demonstrated in the published literature and moreover the importance of poultry meat in human nutrition worldwide, proteomics has seen limited application in poultry and rabbit meat research. There are a few exceptions with breed differentiation in chickens and rabbits being a key example. To date, these studies have been more directed toward understanding physiology than to meat science. Additionally, it must be stated that proteome mapping and

characterization studies are still required in part owing to the lag in the availability of complete, annotated genomes for these species. Once the fundamental studies have been undertaken, the stage will be set for broader use of proteomic technology in poultry meat research. A noteworthy exception is the rather extensive application of proteomic studies in the *foie gras* production process leading to the identification of several putative protein markers of quality. The success story of proteomics in this specific field is perhaps a paradox given the relatively low importance of the *foie gras* industry in the whole poultry meat context. However, it is not surprising given the importance of certification and quality control in this highly valued product. It seems therefore that with a few relevant exceptions poultry and rabbit meat proteomics is still in its infancy. This is probably also a consequence of the fact that poultry research is often conducted by multinational companies that tend not to publish their research results and outcomes, unlike pork and beef research that is essentially done through academic laboratories. As access to the technology becomes cheaper and moreover, as the protein databases become more accurate and complete, a bright new future seems to be on the horizon for all aspects of poultry and rabbit meat proteomics.

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Using Peptidomics to Determine the Authenticity of Processed Meat

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14.1 Introduction

Peptidomics is a field of molecular science that enables versatile analysis of all peptides in a biological sample. Peptidomics has evolved from proteomics as the result of the development of modern advanced analytical and computation technologies in recent years, mainly mass spectrometry-based techniques and bioinformatics tools (Dallas et al., 2015). Standard proteomic approaches identify hundreds of proteins in complex biological samples, but peptidomics provides deeper insight into the protein sample composition due to fast analysis of low molecular mass protein fragments at the peptide sequence level. Similar to the concept of proteome simply defined as a set of all proteins present in a cell, tissue, or even the entire organism, the concept of peptidome has been defined as the peptide contents of a cell, tissue, or organism (Clynen et al., 2008). The primary goal of peptidomics was to study endogenous peptides (i.e., peptides derived from the action of endogenous proteases or the mature functional peptides) that are present *in vivo* within a given sample; this includes also all transient products of *in vivo* protein degradation. Recently, peptidomics has referred frequently to a strategy based on simultaneous detection and identification of all peptides derived from digested protein preparations by the use of exopeptidases or endopeptidases (mostly trypsin). This strategy has opened the field of peptidomic analysis for food authenticity and discovery of peptide markers derived from larger endogenous proteins. It enables the analysis of thousands of small polypeptides present in complex biological samples subjected to enzymatic digestion, and thus the discovery of relevant peptide markers in the areas of clinical medicine or food science, for instance peptide biomarkers of a given disease or peptide markers for food authentication. The analysis is based on the identification of unique species-, tissue- and/or protein-specific peptide fragments, which differ in mass and amino acid composition allowing differentiation between two or more analyzed materials. This chapter describes the application of peptidomics to determine the authenticity of meat and processed meat products.

14.2 Authenticity Issues

In a globalized world, food is often manufactured from ingredients shipped over long distances, imported from different countries and continents. For that reason, to improve food safety and control quality, traceability systems to monitor food management at all stages of the food supply chain, from production, via food processing and its distribution, have become mandatory in the EU and other countries. Honest and reliable food labeling is the foundation of any traceability system, but the authorities must ensure rigorous verification of food safety as well as the composition of food products to prevent and detect fraudulent practices. Since cases of food fraud are widely publicized by the media, food adulteration increases public concern at the international level. In modern society, consumer choice is affected not only by economic factors, but also by ethical, religious, or dietary concerns (Nakayinsige et al., 2012; Sentandreu and Sentandreu, 2014).

Nonauthentic or adulterated food is defined as food in which nature or quality is not consistent with the information declared on the label. In the case of processed meat products, the most frequent fraudulent practices include the substitution of meat components with less valuable ingredients, such as cheaper meat species, offal, fat, connective tissue, or other proteins of nonmeat origin (Montowska and Pospiech, 2011a). The growing popularity of traditional and regional meat products may also increase the risk of their mislabeling, since they are perceived as natural, unique, high-quality products and consumers are willing to pay a higher price for their exceptional sensory traits (Montowska and Pospiech, 2012a).

For the earlier reasons, an effective system of control in the meat industry and trade is extremely important, especially for processed meat products for which visual assessment is highly unreliable. Unlike raw meat, processed meat loses its morphological attributes, and its structure undergoes disintegration as a result of processes such as mincing, curing, and cooking so that visual discrimination of the individual ingredients is barely possible. Recent food labeling legislation has imposed requirements for the declaration of every meat species and its quantity in the product. The content of other nonmeat ingredients, e.g., mechanically recovered meat, heart, liver, lung, fat, and connective tissue, must also be indicated on the label. Enforcement of these mandatory regulations requires robust and reliable analytical methods, especially ones applicable to complex and processed meat products. The studies described in following sections of this chapter have shown that peptidomics has great potential in the field of verification of food authenticity issues in compliance with legislation.

14.3 Protein and Peptide Discrimination

Muscle proteins are most commonly divided into three groups: myofibrillar, sarcoplasmic, and connective tissue proteins. With a share of 40%–50% of the total amount of protein, myofibrillar proteins are the largest group of muscle proteins in muscle fibers. These proteins are divided into contractile apparatus (actin, myosin, tropomyosin) and cytoskeletal

proteins (titin, nebulin). The sarcoplasmic proteins are the second group of muscle proteins, comprising 30%–35% of the total protein. These are primarily enzymatic and regulatory proteins and chromoproteins. The largest chromoproteins found in meat are the muscle pigment myoglobin and the blood pigment hemoglobin. Since they are most abundant in muscle fibers, the earlier-mentioned proteins are the main target for meat authentication purposes. Connective tissue proteins, of which the most abundant are collagen and elastin, are difficult to extract. Some of them are insoluble even in solutions of strong bases and acids. Among the proteins found in connective tissue, authenticity discrimination is based mostly on collagen.

Protein expression and composition varies depending on the species of origin, but also on many other factors, including the duration of meat aging, the existence of different protein isoforms, and posttranslational modifications during or after their biosynthesis. For authentication purposes, the most important feature is protein species and tissue specificity. Therefore, when analyzing the protein composition, a distinction can be made between the muscle, milk, and connective tissue proteins derived from the same species if they are present in the meat product. Using standard proteomic approaches, i.e., ultra performance liquid chromatography and two-dimensional electrophoresis (2-DE) coupled with liquid chromatography-mass spectrometry (LC-MS), detection of nondeclared pork in raw beef burgers and horse meat in mixtures with beef has been proposed based on myoglobin as a marker (Giaretta et al., 2013; Di Giuseppe et al., 2015). Interspecies differences in skeletal muscle myosin light chain isoforms between cattle, pig, chicken, turkey, duck, and goose have been observed on the basis of specific properties of the proteins associated with their structure and mobility in 2-DE gels (Montowska and Pospiech, 2011b). The species-specific differences were retained after processing in mixtures of minced meat and various processed meat products, not only for MLC isoforms but also for some regulatory proteins, metabolic enzymes, and blood plasma proteins (Montowska and Pospiech, 2012b, 2013).

Proteins in processed meat products, treated with high temperature or pressure, then subjected to curing, smoking, cooking, and drying, undergo denaturation and considerable degradation. Proteins can lose their solubility and aggregate during thermal treatment, though the primary structure of protein is often not affected. In some cases, proteins such as myoglobin and ovalbumin undergo enhanced enzymatic digestion by thermal denaturation, as shown by MALDI-MS (Park and Russell, 2000). However, the formation of insoluble protein aggregates has been shown to occur because of the conformational changes after processing, and is thus less susceptible in certain regions to tryptic digestion; this seems to be the reason for the slightly lower sequence coverage observed in meat digests (Montowska et al., 2014a).

Since some proteins can survive the processing and the amino acid sequence of peptide fragments (primary structure of protein) is not severely affected by thermal treatment, peptidomic techniques have advantages when applied to authenticate highly processed meat

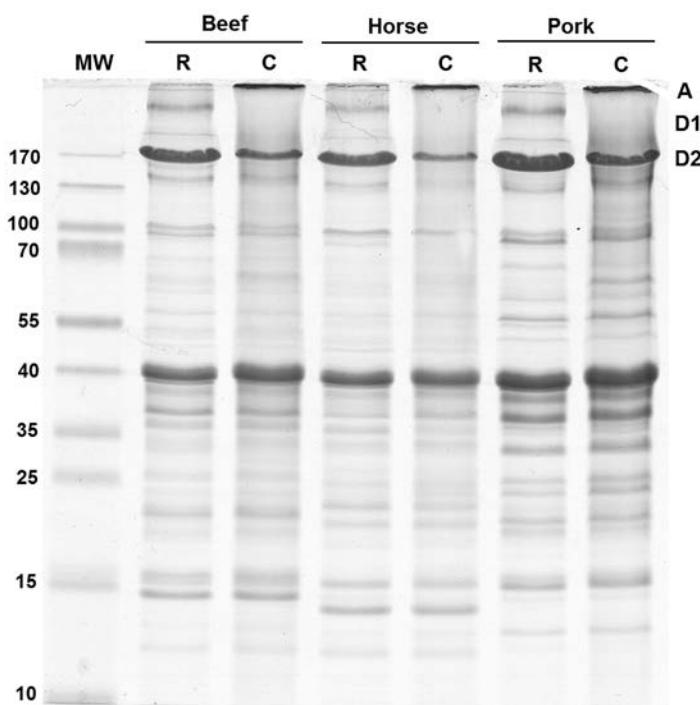


Figure 14.1: The impact of cooking on meat proteins.

SDS-PAGE profiles of meat proteins extracted from raw and cooked *longissimus* muscle. Meat slices of about 25 mm in thickness were wrapped in aluminum foil and heated in a Rational Combi convection oven under harsh conditions at 190°C until reaching a core temperature of 99°C. MW, Thermo Scientific Prestained Protein Ladder (kDa); R, raw meat; C, cooked meat. Protein bands show the extent of protein aggregation in relation to raw meat (A), the degradation of very large nebulin and titin proteins or their degradation products (D1), and the degradation of myosin (D2).

products (Buckley et al., 2009, 2013; Sentandreu and Sentandreu, 2014). Fig. 14.1 shows that high temperature, which is the most destructive of the all technological factors applied in industrial processing, leads to the formation of protein aggregates and degradation of high molecular weight proteins in parallel (Hofmann, 1977). However, the application of the standard peptidomic sample preparation workflow using proteolytic digestion procedure results in efficient enzymatic hydrolysis of protein aggregates and excellent peptide recovery, which enables detection and identification of specific peptide markers (Montowska et al., 2015; Montowska and Pospiech, 2016). The uniqueness of the particular peptide is usually confirmed by BLAST search (basic local alignment search tool) for peptide specificity against all known peptide sequences stored in protein databases such as UniProtKB/Swiss-Prot hosted by the UniProt consortium or the Protein database (incorporating GenBank and RefSeq) hosted by the National Center for Biotechnology Information.

14.4 Analytical Approaches

Advanced progress in the analysis of protein at the sequence/peptide level using mass spectrometry technologies became possible thanks to the application of two critical soft ionization techniques, i.e., electrospray ionization and matrix-assisted laser desorption/ionization (MALDI), in conjunction with instrumentation capable of the fragmentation of parent ions to provide added-value information about the amino acid sequence of the examined ion. Another extremely important factor was the simultaneous advance in liquid chromatography (LC) and capillary electrophoresis for in-line separation. These new technological advances in mass spectrometry methodology enable the identification of peptides with femtomole (10^{-15} mol) to attomole (10^{-18} mol) sensitivity in complex biological samples (Mallick and Kuster, 2010). Recently, taking advantage of considerable improvements in mass spectrometry instrumentation, mass accuracy, and sensitivity, species-specific proteins and peptides have been identified using standard LC-MS techniques in soybean (Leitner et al., 2006), fish (Carrera et al., 2011), and meat species (Buckley et al., 2009; Sentandreu et al., 2010; Balizs et al., 2011; Dosi et al., 2012; von Bargen et al., 2013).

At present, however, standard peptidomic analytical approaches require multistep and time-consuming procedures involving protein extraction, digestion, fractionation, detection, and quantification. Therefore, ambient mass spectrometry techniques have been developed in parallel over the past decade. Ambient MS, introduced in 2004 (Takáts et al., 2004), has advantages over more traditional analytical approaches because of its ability to rapidly detect compounds in open air with minimal sample preparation. The general analytical workflow for peptidomics in meat science is shown in Fig. 14.2. Both the traditional workflow with peptide fractionation and the direct ambient method are presented in the diagram. Since its introduction, there has been a rapid development of a range of ambient MS techniques, which seek to exploit ways of gaining mass spectrometry information directly from a sample surface (Harris et al., 2011). One of the recently introduced approaches is liquid extraction surface analysis mass spectrometry (LESA-MS), a new chip-based nanoelectrospray technique, which extracts analytes directly from the surface by continuous flow liquid microjunction without a requirement for peptide prefractionation (Kertesz and Van Berkel, 2010; Montowska et al., 2014b). The LESA source is a TriVersa NanoMate (Advion, Ithaca, NY), which also operates in direct infusion mode. To date, despite the limited application to protein analysis, there is evidence that LESA-MS can be applied to measure proteins in dried blood spots (Edwards et al., 2011), rat brain tissue (Quanico et al., 2013), human liver tissue (Sarsby et al., 2014), and processed meat products (Montowska et al., 2015).

14.5 Authentication of Processed Meat

The potential for the application of peptidomics in meat authentication remains unfulfilled, likely due to limited knowledge about the identity of heat stable species-specific peptide

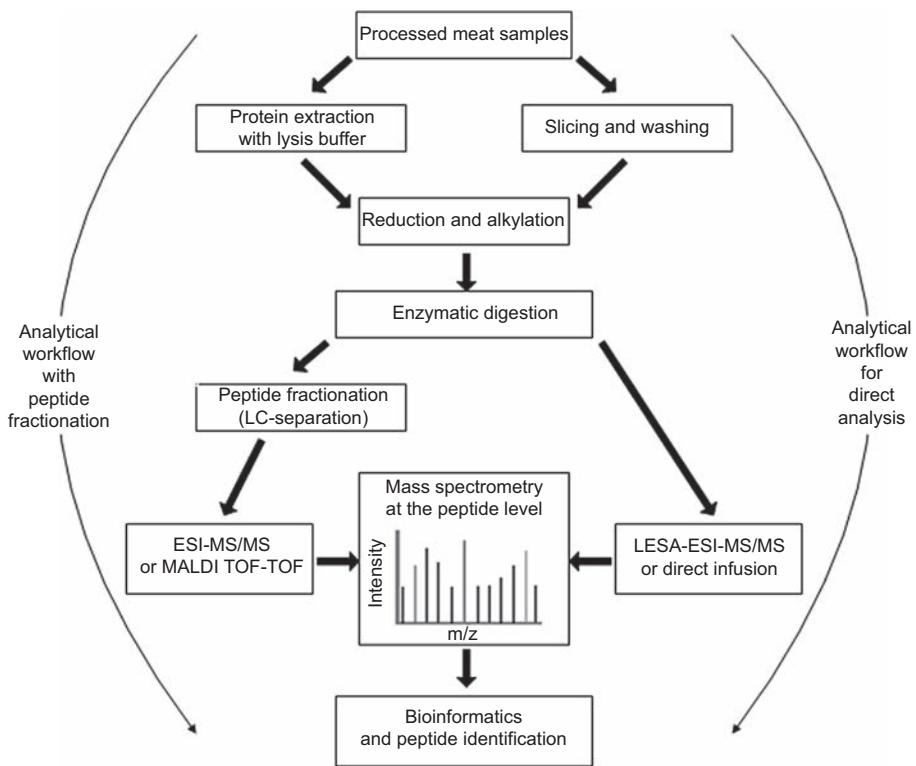


Figure 14.2
General analytical workflow for peptidomics in meat science.

markers, which could lead to the detection of undeclared ingredients in heavily processed meat products. The first step is to apply a nontargeted strategy that would enable the detection and identification of unknown peptide markers unique to the species and/or compound of interest. For this purpose, a shotgun proteomic approach is the most effective way to analyze the complex peptide matrix derived from digested sample preparations. Recent examples found in the literature emphasize its potential for meat authentication (Table 14.1). [von Bargen et al. \(2013\)](#) applied a shotgun peptidomic approach using LC-high-resolution mass spectrometry to select peptide markers for raw pork and horse meat species. The protein source of the 12 detected peptide markers were troponin T, myosin heavy chain (MHC) isoforms, and myoglobin. The species specificity of these markers was evaluated by the analysis of various meat products bought on the market. Having identified specific targets by the nontargeted approach, the authors developed a targeted multiple reaction monitoring (MRM) method that enabled the detection of low amounts (0.55%) of pork or horse meat in beef. Subsequently, the method was optimized for the detection of pork or horse meat in highly processed meat products, such as meatballs, sausages, salamis, and canned meat ([von Bargen et al., 2014](#)). In this case, detection of 0.24% pork or horse meat in a cooked beef

Table 14.1: Application of peptidomics for the meat speciation in meat products.

| Sample | Species | Protein | Exemplary Peptide Markers | References |
|--------------------------------------|-------------------------------------|--|--|--------------------------|
| Raw meat | Cattle, pig, horse, lamb | Myoglobin | HPSDFGADAQAAMSK (cattle) HPGDFGADAQGAMTK (horse) | Watson et al. (2015) |
| Raw and cooked meat | Chicken | MLC3 | DQGTFEDFVGLR ALGQNPTNAEINK | Sentandreu et al. (2010) |
| Raw and cooked meat | Cattle, pig, horse, chicken, turkey | Myosin, myoglobin | ALEDQLSELK (cattle myosin-1) VAEQELLDATER (chicken myosin) | Montowska et al. (2014b) |
| Cooked meat | Pig | Lactate dehydrogenase, serum albumin | LVVITAGAR (lactate dehydrogenase) EVTEFAK (serum albumin) | Sarah et al. (2016) |
| Raw meat and processed products | Pig, horse | Troponin T, myosin, myoglobin | EEFEIGNLQSK (horse myosin-2) TLAFLFAER (pig myosin-4) | Von Bargen et al. (2013) |
| Raw meat and processed meat products | Horse | Myoglobin, myosin | VEADIAGHGQEVLR (horse myoglobin) TDAGATLTVK (horse myosin-2) | Claydon et al. (2015) |
| Processed meat products | Pig, horse | Troponin T/uncharacterized protein, myosin | YDIINLR (pig troponin T/horse uncharacterized protein) TLAFLFAER (pig myosin-4) | Von Bargen et al. (2014) |
| Processed meat products | Cattle, pig, horse, chicken, turkey | Myosin, myoglobin, GAPDH, beta-enolase | SALAHAVQSSR (pig myosin-1/4) WGDAGATYVWESTGVFTTMEK (pig GAPDH) | Montowska et al. (2015) |
| Processed meat products | Cattle, horse, pig | Fast and slow type MHC isoforms | LLSNLFANYAGADTPVEK (pig myosin-7) MLSNLFANYLGAAPIEK (horse myosin-7) | Montowska et al. (2015) |

matrix (boiled meatballs) was achieved using MRM experiments. Pork-specific peptides derived from sarcoplasmic proteins have also been detected in heated meat using LC-QTOF-MS analysis (Sarah et al., 2016). One peptide originating from lactate dehydrogenase and three peptides from serum albumin were found to be the most suitable for quantitative analysis via MRM acquisition in boiled and autoclaved porcine meat.

The nontargeted strategy has also been designed using ambient LESA-MS methodology to differentiate between raw (Montowska et al., 2014b) and cooked (Montowska et al., 2014a) whole meat tryptic digests of beef, pork, horse, chicken, and turkey meat using multivariate data analysis. In this case, the advantage of LESA-MS analysis is the availability of a stable nanoelectrospray interface, which enables the rapid detection and identification of hundreds of peptides simultaneously using an automatic data-dependent mode of MS analysis. This is a desirable approach for rapid high-throughput screening of processed food. Since the intention was to develop a rapid and high-throughput method, the authors avoided widely used chemical denaturants, such as urea, thiourea, dithiothreitol, or guanidine hydrochloride, which have a strong potential to interfere with the electrospray signal despite enhancing the efficiency of digestion. Although the sequence coverage of proteins in processed meat products was observed to be lower when compared to raw and cooked meats (Montowska et al., 2014a), it was found that this methodology was robust, specific, and competitive with LC-MS methods, especially when monitoring particular heat stable peptides. Three factors were noted to enhance the efficiency of the analysis: (1) washing all samples; (2) purification/centrifugation to remove undigested material and reduce the mixture complexity and the subsequent effect on ion suppression; and (3) achieving stable nanoelectrospray during MS data acquisition. To distinguish meat species in cooked beef, two-component mixtures of beef spiked with 1% pork, horse, chicken, and turkey meat were analyzed using PCA-X and OPLS-DA multivariate data analysis. Excellent differentiation was achieved with good values of predictive variation. The established subsequent targeted LESA-MS/MS methodology could detect unique chicken peptides down to 5% (w/w) in cooked beef.

The developed LESA-MS/MS could also provide efficient recovery of peptides from industrially processed samples (Montowska et al., 2015). As a result, a set of 25 both species- and protein-specific heat stable peptide markers has been identified in different foodstuffs purchased at supermarkets, e.g., cooked ham, corned beef, various types of sausages, and frankfurters. The identified peptides belong to the most abundant myofibrillar (myosin heavy and light chains) and sarcoplasmic (myoglobin, GAPDH, beta-enolase) proteins. This peptidomic approach is fast, since the entire MS/MS analysis (excluding sample preparation) takes approximately 10 min. This is very important when the quality of quickly deteriorating meats and meat products is monitored. Alternative but more laborious MS-based proteomic methods using OFF-GEL isoelectric focusing followed by SDS-PAGE and subsequent identification of selected peptides by LC-MS/MS have been tested (Sentandreu et al., 2010; Surowiec et al., 2011). Based on two peptides obtained from tryptic digests of myosin light chain isoform 3, quantitative

detection of 0.5% of chicken meat mixed with pork was successful when using an absolute quantification method with stable isotope peptides (AQUA method) (Sentandreu et al., 2010).

A semitargeted workflow for meat speciation to select peptides specific to horse meat in highly processed food was reported by Claydon et al. (2015). Proteins extracted from baby food, corned beef, as well as raw and processed horse meat, and its mixtures with beef, pork, and chicken meat, were separated using SDS-PAGE gels, followed by in-gel digestion and protein identification by the use of LC-MS/MS. Subsequently, the full amino acid sequences of the identified proteins were aligned across nine relevant species (horse, cattle, pig, sheep, goat, chicken, turkey, rabbit, and donkey) and a database of selected species-specific peptide markers was created and used for semitargeted species discrimination. The limits of detection (LODs) were assessed for processed horse meat in mixtures with pork, beef, and corned beef revealing detection as low as 0.5% processed horse meat. This methodology supported by the database comprising species-specific peptide markers will serve as the foundation for the detection of low-level undeclared meat contamination in processed meat products.

A targeted strategy strictly based on myoglobin corresponding peptides across four species of meat, namely beef, pork, horse, and lamb, has been proposed by Watson et al. (2015). The concept of “corresponding proteins corresponding peptides” was founded on previously established knowledge of species specificity of some corresponding proteins, in this case myoglobins. The term “corresponding peptides” refers to the peptides that arise from aligned cleavage sites of the aligned full amino acid sequences of these proteins. Taking myoglobin for example, tryptic digestion of beef, pork, and horse myoglobins may generate three corresponding peptides HPSDFGADAQAAAMSK, HPGDFGADAQGAMSK, and HPGDFGADAQGAMTK, respectively. The sequences of these peptides are similar with a difference of up to three amino acids (indicated by underlined letters) among them. The authors developed an MRM method based on ratios of transition peak areas for corresponding peptides, assuming that the impact of environmental conditions would be the same on corresponding proteins, and thus corresponding peptides would behave very alike during food processing, sample preparation including digestion, desalting, and mass spectrometric analysis. The study introduced the concept by the identification of pairs of corresponding peptides that would allow the detection of one meat in binary mixtures down to 1% (w/w).

Quantitative analysis was performed on the intensities of MRM transitions for horse in beef, beef in lamb, and pork in raw lamb mixtures.

Actin, being a highly conserved protein, has primary structure very similar across vertebrates. On the other hand, myosin is a protein that exists in many variants/isoforms differing, on average, by several amino acids, which is reflected in fiber composition and the velocity of contraction of striated and smooth muscles. In adult mammals, muscles may consist of pure and hybrid fibers. The pure fibers of slow type I red and fast type white IIA contain a single MHC isoform (1, 2A, 2X, and 2B). The hybrid fibers may be expressed as several MHC isoforms

(1/2A, 2A/1, 2AX, 2XA, 2XB, and 2BX), where each isoform is encoded by a separate gene (Pette and Staron, 2000). Such myosin diversity may be applicable to detect some unlawful practices in meat products made from different meat components. Recently, discrimination between fast and slow type MHC isoforms has been performed in various processed meat products using the LESA-MS/MS technique (Montowska et al., 2015). Six fast type II and five slow/cardiac type I MHC marker peptides unique to cattle, pig, and horse have been identified in foodstuffs, such as cooked ham or fried horse sausage. Interestingly, slow MHC isoforms were detected more frequently over the fast MHC isoforms, which suggests that the meat products under investigation were likely manufactured from smaller red or intermediate muscles. The results indicate that peptide markers of specific tissues and proteins may help in the discrimination between good-quality or less-valuable ingredients, or even trace substitution of ingredients. Therefore, peptidomics may serve as a viable tool for both meat speciation and product quality, especially when the criterion of a low LOD is achievable at levels below 1%.

14.6 Authentication of Proteins of Nonmeat Origin

Cheaper proteins of nonmeat origin might be fraudulently added to meat products to substitute more expensive meats of various species of animals. Mechanically recovered meat, milk, blood plasma, and connective tissue as well as vegetable soy proteins are most commonly used for adulteration purposes. Table 14.2 presents recent examples of the application of peptidomics to authenticate proteins of nonmeat origin in processed meat products. According to the European regulations, the term “meat” means the skeletal muscles of mammalian and bird species recognized as fit for human consumption, together with the diaphragm and rumen, with the exception of the heart, tongue, the muscles of the head other than the masseters, the muscles of the wrist, the tarsus, and the tail, along with naturally included or adherent tissue. In the meaning of obligatory regulations, mechanically recovered meat must not be called meat; however it is allowable to add mechanically recovered meat to processed foodstuffs provided that it is indicated on the label. Mechanically recovered meat is produced by high-pressure treatment of bones with meat remains after the removal of the muscles attached to the bones, followed by processing through a sieve of a suitable mesh size, which leads to the loss or modification of muscle fiber structure. Mechanically recovered meat differs in protein composition and has a high content of calcium and cholesterol compared with hand-deboned meat. Day and Brown (2001) found a higher concentration of hemoglobin in chicken mechanically recovered meat using capillary gel electrophoresis (CGE). Surowiec et al. (2011), when comparing hand-deboned chicken and mechanically recovered meat, identified potential chicken mechanically recovered meat peptide markers derived from hemoglobin subunits.

In response to the outbreak of bovine spongiform encephalopathy in the 1990s, the European Union introduced restrictions on feeding livestock with highly processed animal proteins, such as fishmeal and meat and bone meal (MBM). Currently, feeding farmed animals with animal proteins derived from the same species is prohibited in Europe (EC regulation No.

Table 14.2: Application of peptidomics for the authentication proteins of nonmeat origin in processed meat products.

| Sample | Authenticated Aspect | Species | Protein | Exemplary Peptide Marker | References |
|-------------------------|-----------------------------|-----------------------------------|--|--|---|
| Raw meat | Mechanically recovered meat | Chicken | Hemoglobin | MFTTYPPTK | Surowiec et al. (2011) |
| Gelatin | Gelatin | Cattle, pig | Collagen | GPPGSAGSPGK (cattle) GPPGSAGAPGK (pig) | Zhang et al. (2009) |
| Bone | Collagen | Cattle, pig, sheep, goat, chicken | Collagen | GPSGEOGTAGPOGTOGPQ- GLLGAOGFLGLOGSR (sheep) | Buckley et al. (2009, 2010) |
| Bone | Meat and bone meal | Cattle, pig | Osteocalcin | RFYGPV (cattle) RFYGIA (pig) | Balizs et al. (2011) |
| Processed meat products | Soy protein isolate | Soy | Glycinin, β -conglycinin | HFLAQSFNTNEDIAEK (glycinin G4) | Leitner et al. (2006) |
| Soybean varieties | Soybean seeds | Soy | Glycinin, β -conglycinin, Kunitz trypsin inhibitor | DTVDGWFNIER (Kunitz trypsin inhibitor) | Houston et al. (2011) |
| Processed meat products | Milk and cheese | Cattle | Casein alpha-S1 | HQGLPQEVLNENLLR | Montowska et al. (2015) |
| Processed meat products | Blood-binding agent | Cattle, pig | Fibrinopeptides A and B | EDGSDPPSGDFLTEGGV (cattle fibrinopeptide A) AEVQDKGEFLAEGGGV (pig fibrinopeptide A) | Grundy et al. (2007, 2008) |

999/2001, and the amending regulations No. 1292/2005 and 956/2008). MBM is treated in accordance with the regulations at a temperature of 133°C for 20 min, and a pressure of 300 kPa. The high degree of processing has not been an obstacle to the analysis of meat and bone meal in feed and food matrices by means of mass spectrometry by detecting hydrolyzed gelatin, a derivative of collagen (Fernandez Ocaña et al., 2004; Zhang et al., 2009), as well as gelatinized bone collagen from cattle, pig, sheep, goat, or chicken (Buckley et al., 2009). Difficulties were encountered only in differentiating between sheep and goat due to the loss of some key peptides with harsh treatment at 145°C for 20 min at 3 bar pressure (Buckley et al., 2009). However, a peptide of 33 amino acids from sheep and goat collagen was sequenced using MALDI-MS/MS, with the results showing a difference of two amino acids between these species (Buckley et al., 2010). Another MS-based method using osteocalcin, a small protein constituent of calcified bone, as a source of species-specific peptides for porcine and bovine MBM has been reported recently (Balizs et al., 2011). The concentration of osteocalcin in bone is estimated to be 10%–20% of noncollagen proteins; however, since the protein was found to be susceptible to thermal processing, resulting in a decreased intensity of specific peptide markers, the method employed high-resolution mass spectrometry (LC-Q/TOF) to enhance the sensitivity.

Nonmeat proteins, for instance soy, milk, egg white, blood plasma, and connective tissue proteins, also fulfill many important functions in meat processing. They are used to emulsify fat, stabilize the whole system of the stuffing emulsion, and reduce drip losses because of their high water- and fat-holding capacity, which is particularly important for cured and finely comminuted products. The addition of nonmeat proteins is essential for the manufacture of meat products with a high fat content, where there is a shortage of meat proteins. In the processing of coarsely minced meat products, nonmeat proteins improve the binding of meat pieces, give the product texture, as well as reduce mass losses. Thus, proteins of nonmeat origin are frequently added to processed meat products both to improve functional properties and for economic reasons.

With respect to protein functional properties, soybean proteins are of particular importance, having excellent water-binding and fat-emulsifying properties. Having the ability to form gels, they can also serve as fat replacers for low-fat products. Since soy is listed as a highly allergenic food, there is an obligation to declare even trace amounts of soy in the product, because for allergic consumers this is the only way to avoid an allergic response. So far, immunoassays, mainly based on ELISA, have been commonly used for the detection of soybean proteins in processed meat products. For the time being, alternative proteomic methods have been reported for the detection of soy proteins in commercial heat-processed meat products by the use of reverse-phase HPLC (García et al., 2006; Castro et al., 2007) and multidimensional LC-MS (Leitner et al., 2006). Advanced peptidomic approaches for relative and absolute quantification have been applied to examine and compare the expression of 10 soy allergens in 20 varieties of mature soybean (Houston et al., 2011).

Whey proteins and caseinates from among milk proteins are most often used in meat processing. Sodium caseinate matches the emulsifying properties of lean meat protein; however whey concentrates have more beneficial functional properties due to their high water-binding capacity as they form strong heat-stable gels after denaturation. At present, producers wanting to make their products more attractive for consumers have introduced an increasingly wider range of meat products with the addition of cheese on the market, for instance smoked and dried sausages and frankfurters where meat has been replaced with 10%–20% cheese. As a result of recent advances in peptidomics, the detection of unique milk and meat peptides simultaneously in a single analysis in processed products is achievable. The LESA-MS/MS technique allows for the simultaneous identification of proteins and peptides of meat as well as milk origin, such as casein, in whole meat product digests (Montowska et al., 2015). Two peptides unique to casein alpha-S1 (HQQLPQEVLNENLLR and EPMIGVNQELAYFYPELFR) were identified in two processed meat products, namely kabanos sausage manufactured with the addition of 7.5% cheese and frankfurters with the addition of milk proteins.

A combination of whey proteins with extracts of blood plasma is sometimes used to create mixtures of functional proteins. These mixtures have good emulsifying and gelling properties, allowing them to be used for the production of a wide range of meat products. However, the inclusion of undeclared blood derivatives raises a severe issue for consumers that have ethical or religious dietary restrictions regarding the consumption of pork or products containing even small traces of blood. Since fibrinogen is the main protein in blood-based preparations, it has been found to be a good source of specific marker peptides (fibrinopeptides) that are indicators of the presence of blood in the product (Grundy et al., 2007, 2008). These peptides were released from fibrinogen during gelling in the presence of thrombin, the blood-clothing enzyme. Fibrinopeptides A and B were found to be reliable markers in fresh meats and various commercial products made from chicken, beef, pork, or lamb, spiked in-house with 5% commercial bovine (Grundy et al., 2007) and porcine (Grundy et al., 2008) blood-binding agent for validation purposes. The developed methods were based on a conventional LC-MS peptidomic approach. More recently, a collaborative study was conducted by five food testing laboratories across Europe and the USA to determine the transferability of the previously established LC-MS method to different instruments in various laboratories. Since the species of origin of blood-binding preparations based on bovine and porcine fibrinopeptides A and B was correctly assigned for all tested samples, the method displays sufficient specificity to address authenticity issues in processed foods (Grundy et al., 2013).

14.7 Conclusions

The authentication of processed meat products involves a range of key considerations, such as the meat content (speciation), confirmation of the product specification (declared quantities), and the fraudulent addition of undeclared ingredients, such as mechanically recovered meat,

blood plasma, milk, and vegetable proteins. Peptidomic approaches based on mass spectrometry techniques have advantages regarding the differentiation between species- and tissue-specific proteins. It is relatively straightforward to discriminate between proteins derived from the meat of a given species and proteins of nonmeat origin at a qualitative level due to the differences in the amino acid sequences of particular proteins. However, quantitative analysis faces additional challenges, requiring careful calibration and costly synthesis of labeled peptides. Several studies performed on highly processed material and discussed in this chapter have demonstrated the greater stability of peptide markers in comparison with DNA biomolecules, affirming the potential of peptidomic analysis in the authentication of highly processed foodstuffs.

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Proteomic Characterization of Nonenzymatic Modifications Induced in Bovine Milk Following Thermal Treatments

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15.1 Introduction

Consumption of bovine milk and related dairy products has found a widespread diffusion in human nutrition as a result of the important components present therein. With the aim of preserving microbiological safety and prolonging shelf-life, milk is not consumed as a raw material but is submitted to thermal treatment prior to sale and consumption. These processes may induce physicochemical changes in several milk components, depending on the duration/extent of the heating procedure. Pasteurization and ultrahigh-temperature (UHT) treatments poorly influence the composition and nutritional/sensing quality of the final products, but ensure a shelf-life of few days and months, respectively. Sterilization, in vacuo concentration/sterilization and ultimately milk caramel formation generate novel chemical products produced as result of the complex network of nonenzymatic processes occurring during heating of amino acids in the presence of reducing sugar mixtures, known as the Maillard reaction (van Boekel, 1998). This reaction generates a multitude of products depending on: (1) the different processes occurring in parallel; (2) the various (~2500) proteins present in bovine milk; (3) the reactive amino acids present in each protein; (4) the main sugars present in the raw material, i.e., lactose (more abundant), D-glucose, and D-galactose, which are in part transformed during processing into lactulose and are epimerized to epilactose and D-tagatose, respectively. The impact of temperature is also an important issue for infant formulas, where bovine milk-derived constituents (caseins or whey proteins, lactose, D-glucose, D-galactose, and triglycerides) are combined with different additives to meet the dietary requirements of children.

A simplified scheme of the Maillard reaction is summarized in the Hodge diagram (Hodge, 1953) (Fig. 15.1). The initial step (step A) involves the condensation of a carbonyl group from a reducing sugar, e.g., lactose, with the protein N-terminus or the ε-amino group of Lys

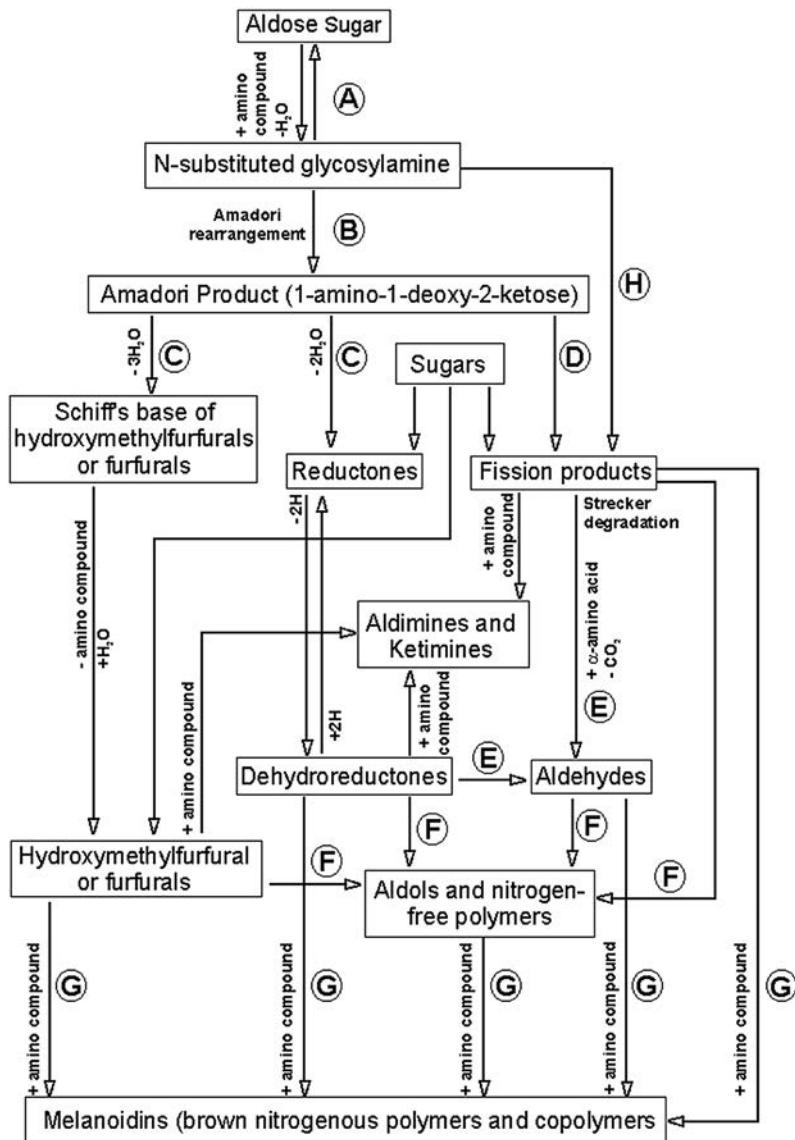


Figure 15.1: The Hodge Diagram.

The initial reaction between a reducing sugar and a protein amino group forms the unstable Schiff base (step A). The latter derivative rearranges to form the corresponding AP (step B). Degradation of AP (step C). Formation of reactive carbonyl and α -dicarbonyl compounds (step D). Formation of Strecker aldehydes of amino acids and aminoketones (step E). Aldol condensation of furfurals, reductones, and aldehydes produced in step C, D, and E without intervention of NH_2 -containing derivatives (step F). Reaction of furfurals, reductones, and aldehydes produced in step C, D, and E with NH_2 -containing derivatives to form melanoidins (step G). Free radical-mediated formation of carbonyl fission products from the reducing sugar (Namiki pathway) (step H).

residues, yielding a Schiff base (aldimine) product. This unstable compound may rearrange to form a 1-amino-1-deoxy-2-ketose (ketoamine) derivative known as the Amadori product (AP) (Fig. 15.1, step B); in the case of lactose (Lact), this compound is N^ε-(1-deoxy-D-lactulos-1-yl)-Lys, also known as lactulosyl-lysine (LL). It has been demonstrated that some milk products, such as skim milk powder, present about 50% of the lysine as LL (Henle et al., 1991). This Amadori derivative is an early stage glycation adduct that may be further degraded through different molecular pathways into advanced glycation end-products (AGEs) (Henle, 2005; Arena et al., 2014), ultimately leading to the production of protein unbound pyranones, furfurals, reductones, and fragmentation derivatives (Fig. 15.1, steps C and D). AP degradation product N^ε-carboxymethyllysine (CML) was identified as the major AGE formed in heated milk models and commercial products (Ahmed et al., 2005; Hegele et al., 2008; Assar et al., 2009; Lima et al., 2010; Nguyen et al., 2016); this compound can also derive from other glycation and lipid peroxidation processes. A nonoxidative pathway based on intramolecular enolization, followed by elimination of galactose or water, can eventually precede these reactions yielding the early glycation protein-monosaccharide intermediates N^ε-(5,6-dihydroxy-2,3-dioxohexyl)-Lys and N^ε-(2,3-dihydroxy-5,6-dioxohexyl)-Lys. The α-dicarbonyl moiety of these monosaccharide adducts can in turn modify other amino acids, leading to stable protein cross-linked AGEs such as glucosepane and crossline. Alternatively, these adducts may lose a carbon unit by retro-Claisen ester condensation to generate the corresponding pentose derivatives, which in turn can modify protein residues to yield other AGEs. A prototype example of five carbon-containing AGE product is pentosidine (PENT), which has been detected in processed milk products (Henle et al., 1997). In parallel, sugar adduct fragmentation occurs by retro-aldolization (Fig. 15.1, step D), generating stable α-dicarbonyl compounds, such as glyoxal (G), methylglyoxal (MG), 3-deoxypentosone (3-DPen), glucosone (GONE), 1-deoxyglucosone (1-DG), and 3-deoxyglucosone (3-DG). These reactive intermediates can also form directly from unbound reducing sugars subjected to thermal degradation and/or autoxidative reactions (Fig. 15.1). For example, lactose thermal degradation in milk generates GONE, galactosone (GAONE), 3-DG, 3-deoxygalactosone (3-DGal), 3-DPen, 3,4-dideoxypentosone, GO, and MGO. Similarly, D-glucose degradation produces significant amounts of GONE, 3-DG, 3-DPen, tetrosone, triosone, GO, and MGO; interconversion of 3-DG and 3-DGal was observed. These α-dicarbonyl compounds are able to react with proteins (Lys and/or Arg side chains) yielding various AGEs, including Strecker aldehydes of the amino acids, aminoketones, and other products (Fig. 15.1, step E); some compounds subsequently condense to form pyrazine derivatives, contributing to the aroma of heated foods. Ascertained examples of linear and cross-linked AGEs deriving from the earlier-mentioned α-dicarbonyl compounds include (1) G-, MG-, 3-DG-, and 3-DGal-derived hemiaminals (G-He, MG-He, 3-DG-He, and 3-DGal-He); (2) G-, MG-, 3-DG-, and 3-DGal-derived-dihydroxyimidazolines (G-DH, MG-DH, 3-DG-DH, and 3-DGal-DH); (3) G-, MG-, 3-DG-, and 3-DGal-derived hydroimidazolones (G-H, MG-H, 3-DG-H, and 3-DGal-H); (4) G-, MG-, and 3-DG-derived Lys dimers (GOLD, MOLD, and DOLD); (5) G-, MG-, and

3-DG-derived imidazolium cross-link products (GODIC, MODIC, and DOGDIC); (6) MG-derived N^ε-carboxyethyllysine (CEL), di-hydropyrimidine and tetra-hydropyrimidine (DHP and THP); argypyrimidine (RPYR); (7) 3-DG-derived pyrraline (PYR) and G-derived CML (Henle, 2005; Mittelmaier and Pischetsrieder, 2011; Arena et al., 2014). CEL, PYR, MG-H, and CML have been directly detected in heated milk product hydrolysates (Ahmed et al., 2005; Hegele et al., 2008; Assar et al., 2009; Lima et al., 2010; Nguyen et al., 2016). Final stages of the Maillard reaction are associated with steps F and G of Fig. 15.1, where polymeric molecules contributing to color/fluorescence of the final products are generated. Step F reports the aldol condensation of the aldehydes, reductones, and furfurals, without the intervention of NH₂-containing molecules. Conversely, step G refers to the reactions between the same intermediates and NH₂-containing compounds, leading to the final products melanoidins. These pyridine, pyrrole, and furan rings-containing polymers, having a brown-black color and a mass above 100 kDa, may incorporate redox-active molecules thereby acting as antioxidants.

Nature and relative concentration of each AGE depend on the identity of the reducing sugar and the complex network of concomitant reactions (Fig. 15.1). AGEs may thus derive from protein nonenzymatic glycation by reducing sugars in pre-AP and post-AP reactions and from processes where AP is not the precursor. Accordingly, they may be formed in both the early and late stages of the Maillard reaction. On this basis, the term advanced glycation end-products is not correct; however, the concept of early, intermediate, and advanced glycation products is simple and has found a wide diffusion.

Thermal treatment in the presence of oxygen can also promote milk protein oxidation, and Met, Cys, and Trp are amino acids highly susceptible to direct oxidative modifications (Bachi et al., 2013). Although these reactions do not directly depend on the Maillard reaction, it was shown that lactose somehow facilitates protein oxidation in the course of milk thermal treatment (Meltretter et al., 2007). This may be ascribed either to (1) sugar degradation-derived α-dicarbonyl compounds that can directly induce the oxidation of the Lys side chain yielding amino adipic semialdehyde via the Strecker-type degradation (Meltretter et al., 2007) or (2) reactive oxygen species (ROS) that are generated in the course of the Maillard reaction (Mossine et al., 1999). In this context, the most abundant oxidized derivative present in heated milks is methionine sulfoxide (MetO) (Baxter et al., 2007; Meltretter et al., 2008, 2014).

Depending on the dairy product, variable levels of oxidized Cys and Trp derivatives have also been detected, i.e., cystine, sulfenic acid, sulfonic acid, hydroxytryptophan, kynurenine, and N-formylkynurenine (NFK) (Hoffman and van Mil, 1999; Puscasu and Birlouez-Aragon, 2002; Cho et al., 2003; Meltretter et al., 2007, 2014). Cysteine is responsible for the formation of heat-induced protein aggregates in milk products. Worth mentioning are also carbonylated derivatives eventually deriving from the addiction of lipid peroxidation products (Fenaille et al., 2005; Meyer et al., 2012). Additional sugar-independent protein modifications occurring during milk heat processing are (1) deamidation of Asn side chain (Meltretter et al., 2014);

(2) oxidative deamination of N-terminal amino acids (Meltretter et al., 2014); (3) cyclization of N-terminal Glu to pyrrolidone (Meltretter et al., 2007, 2014); and (4) β -elimination of Cys and Ser residues to yield dehydroalanine. The latter amino acid is subjected to the nucleophilic addition of Lys or His to yield cross-linked lysinoalanine (LAL) and histidinoalanine (HA), respectively (Pischetsrieder and Henle, 2012).

The afore-mentioned amino acid adducts have been widely used as markers of the nutritional value of dairy products (Erbersdobler and Somoza, 2007; Hellwig and Henle, 2014; Wada and Lonnerdal, 2014). Their accumulation in heat-treated raw material generally modifies the availability of essential amino acids and may affect function, digestibility, and allergenic potential of individual proteins (Muscat et al., 2009; Heilmann et al., 2014).

15.2 Single Protein-Centered Characterization of the Modifications Induced by Thermal Treatments

The occurrence of these amino acid derivatives in various heated milk samples was initially demonstrated by procedures based on gas or liquid chromatography (GC or LC) coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS), which were applied to the analysis of whole protein extracts further subjected to extensive enzymatic or acid hydrolysis (Henle et al., 1991, 1997; Ahmed et al., 2005; Erbersdobler and Somoza, 2007; Baxter et al., 2007; Hegele et al., 2008; Assar et al., 2009; Delatour et al., 2009; Troise et al., 2015). Using isotope-enriched internal standards, selected ion monitoring MS and/or multiple reaction monitoring (MRM) procedures allowed an accurate evaluation of trace quantities of these derivatives. Comparative studies on various dairy products ascertained that heat treatment progressively induces milk protein modifications, whose extent is dependent on the time and/or temperature of exposure. The same conclusions were reached through dot blot, ELISA, and western blotting experiments that allowed detection of LL, CML, NFK, and carbonylated adducts in heat-modified milk proteins (Fogliano et al., 1997; Pallini et al., 2001; Scaloni et al., 2002; Fenaille et al., 2005; Ehrenshaft et al., 2009; Meyer et al., 2011, 2012). The sheer diversity of the derivatives to be assayed by dedicated reagents was the major challenge in simultaneously studying milk protein damage on the whole.

Additional disadvantages in using these analytical methods include the lack of information on the nature of the modified proteins, their extent of modification, together with the assignment of the corresponding adducted amino acids. Obtaining these data is crucial for linking a specific protein modification to a certain technological/nutritional milk product characteristic. To this end, various MS methods were used to identify and characterize heat-modified milk proteins purified from real food matrices, taking advantage of the improved instrumental sensitivity, resolution, mass accuracy, and ability to produce high-quality MSⁿ spectra (Siciliano et al., 2013; Arena et al., 2014). In parallel, in vitro studies using specific chemicals (sugars or α -dicarbonyl compounds) able to induce glycation/glycoxidation and oxidation

were also performed on purified milk proteins to predict their reactivity under real dairy transformation conditions. Thus, MS detection of intact protein components showing a (multiple) mass increase of +324 or +162 Da (for reaction with lactose or D-glucose/D-galactose, respectively), with respect to the unmodified counterpart, demonstrated that AP derivatives are the most abundant species occurring in milk samples subjected to mild-moderate thermal treatments (Siciliano et al., 2013). In this context, studies on β -lactoglobulin (LG) (Leonil et al., 1997; Morgan et al., 1998; Fogliano et al., 1998; Siciliano et al., 2000; Hau and Bovetto, 2001; Fenaille et al., 2004; Czerwenka et al., 2006; Monaci and van Hengel, 2007; Carulli et al., 2011), α -lactalbumin (LA) (Siciliano et al., 2000; Czerwenka et al., 2006; Hau and Bovetto, 2001; Carulli et al., 2011), and α S1-casein (CN) and β -CN (Scaloni et al., 2002; Johnson et al., 2011) are prototype examples in which the detection of the intact protein adducts provided important information on the prevalent reactions occurring in heated milk models and dairy products, which were later used for the elucidation of the thermal history of milk samples (Losito et al., 2007; Johnson et al., 2011; Sassi et al., 2015). In fact, comparative quantitative studies on raw, pasteurized, UHT, sterilized, powdered, condensed and liquid milks, whey concentrates, and powdered infant formulas ascertained that heat treatment promotes nonenzymatic glycation of milk proteins, whose extent is dependent on time/temperature at which the raw material is exposed. A progressive increase in the number and content of AP and oxidized products was observed in the spectrum profile, together with the concomitant production of additional uncharacterized derivatives. It was also demonstrated that use of dry state processing conditions generally increased the extent of protein modification (Morgan et al., 1998; Fenaille et al., 2004).

Qualitative studies on the nature of the modifications and the number of assigned, modified residues employed comparative nanoLC-ESI or MALDI-TOF MS peptide mapping experiments on isolated milk proteins (Leonil et al., 1997; Morgan et al., 1998; Fogliano et al., 1998; Siciliano et al., 2000; Scaloni et al., 2002; Meltretter et al., 2007, 2008; Lima et al., 2009; Carulli et al., 2011). These investigations demonstrated a time-dependent and temperature-dependent formation of LL, hexose-associated AP derivatives, and CML at specific Lys sites, as evidenced by the detection of peptide adducts at +324, +162, and +58 Da, respectively. More extensive modification of residues exposed on the protein surface or having a chemical-assisted higher reactivity was observed for poorly glycated intact proteins (Fogliano et al., 1998); this condition was followed by nonspecific modification of lysines when moderately/highly glycated intact proteins were considered. These studies also demonstrated a significant formation of Met/Trp/Cys oxidation products, lysine aldehyde, and pyrrolidone in samples heated for variable times or at moderate temperatures, as ascertained by the detection of peaks at +16, -1, and -18 Da, respectively (Meltretter et al., 2007, 2008; Carulli et al., 2011). When milk models were treated with D-glucose or MG at high temperatures for prolonged times, the occurrence of MG-H and MG-DH adducts was also observed at specific Arg residues, as evidenced by detection of peaks showing a mass increase of +54 and +72 Da,

respectively (Lima et al., 2009). Uncertainty due to the concomitant occurrence of multiple modified residues in a single peptide was resolved by performing MS/MS experiments (Morgan et al., 1997; Molle et al., 1998; Lima et al., 2009).

Recently, ultrahigh-performance liquid chromatography (UHPLC)-ESI-MS/MS was applied to the systematic identification and site-specific analysis of thermal-induced modifications of LG in processed milk (Meltretter et al., 2013). To this end, LG was heated with Lact under specific experimental conditions to force the formation of nonenzymatic modifications based on known modifications occurring via oxidation and the Maillard reaction chemistry. The LG digest was subjected to full scan and enhanced resolution scan experiments combined with enhanced product ion scans, which allowed the identification of the main glycation, glycoxidation, oxidation, and deamidation products at Lys, Arg, Met, Cys, Trp, and Asn, as well as at N-terminus. Using these MS data, a sensitive scheduled MRM method suitable for the analysis of milk products was developed. Accordingly, 19 different structures and 26 modified sites of LG were detected in different milk products. As expected, the number of modified species was related to the severity (regarding temperature and time) of the thermal treatment. This approach was then used to evaluate site-specific relative quantification of LG modifications in various dairy products, with the aim of evaluating their thermal and nonthermal origin and to assign candidate markers for milk processing (Meltretter et al., 2014). The results indicated that (1) site-specific analysis of LL may be a more sensitive marker for mild heat treatment than its overall content; (2) CML, N-terminal ketoamide are of thermal origin and may be good markers for intensive heat treatment, whereas CEL reflects thermal and nonthermal processes; (3) the relevance of MG-derived Arg modifications is low compared to that of other modifications; (4) oxidation of Met and Cys is a rather weak indicator of thermal impact; (5) Trp oxidation products are of nonthermal origin and are further degraded during milk processing. Since ROS can be generated by the Maillard reaction, autoxidation of sugars and lipid peroxidation, the influence of milk fat on Met oxidation was also assessed using the MRM approach (Wust and Pischetsrieder, 2016). Quantitative analysis of LG, LA, and α S1-CN oxidation at different Met sites was ascertained in protein samples from raw, UHT, and evaporated milk samples having a different fat content. These experiments demonstrated that oxidation at most protein Met sites was not affected by the presence of milk fat. It was concluded that lipid oxidation products are not the major cause of Met oxidation in milk.

Although MS/MS experiments were widely used to assign adducted amino acids in modified peptides, collision-induced dissociation (CID) spectra of AP-containing species revealed a highly reduced abundance of sequence-informative *b* and *y*-type ions, which hampered sequence-derived molecular identification (Molle et al., 1998; Arena et al., 2010). In the case of lactosylated peptides, the most abundant ions were associated with the cleavage of the glycosidic bond, with retention of the glycosidic O atom by the species carrying the reducing end, plus neutral losses of two to three H_2O molecules to generate the corresponding $\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O}$ furylium ion (−216 Da); neutral losses of H_2O (−18 Da), $2\text{H}_2\text{O}$ (−36 Da), and

$C_6H_{12}O_6 + 2H_2O + HCHO$ (–246 Da) were also observed. Neutral loss (NL) pattern of various H_2O molecules and formation of furylium ions were generally charge-independent for all of the modified peptides investigated. [Fig. 15.2A](#) shows the MS/MS spectrum after CID fragmentation of the $[M+4H]^{4+}$ ion corresponding to the lactosylated peptide (167–181) from α S2-casein. Sequence-informative *b*-type and *y*-type ions are rare in the mass spectrum making confident assignment of the peptide difficult. Despite these challenges, an MRM-based method for quantitative evaluation of lactosylated peptides in LG and LA from stored milk powder samples was developed ([Le et al., 2013](#)). The neutral losses of 162 Da (cleavage of galactose) and 216 Da (the formation of the furylium ion) were specifically selected as MRM transitions; quantification of lactosylated peptides was based on the peak areas of these specific transitions. Good correlation between the MRM and furosine results indicated the feasibility of this method.

To overcome limitations observed during CID-based sequencing of AP-containing peptides, electron transfer dissociation (ETD)-based fragmentation procedures were developed ([Arena et al., 2010](#)). In this case, neither furylium ions nor ions corresponding to NL of H_2O molecules from the glycation site were observed, clearly demonstrating that AP is stable under ETD conditions and the bond cleavage is less dependent on peptide composition and side-chain modification. The abundance and the almost completeness of the *c*-type and *z*-type ion series observed, regardless of the location of the modification site, greatly facilitated peptide sequencing and modification site assignment. Using of supplemental collisional activation (SCA) also increased the number of modified peptides identified ([Arena et al., 2011](#)). This is exemplified in [Fig. 15.2B](#), which shows the MS/MS spectrum under ETD fragmentation with SCA of the $[M+4H]^{4+}$ ion corresponding to the same lactosylated peptide (167–181) from α S2-casein. In contrast to that reported for CID fragmentation, a series of abundant sequence-informative *c*-type and *z*-type ions was detected, which facilitated peptide sequencing and modification assignment to Lys173. These studies demonstrated the importance of using ETD to develop dedicated shotgun procedures for proteomic analysis of milk modifications.

15.3 Proteomic Characterization of the Modifications Induced by Thermal Treatments

To provide an overall picture of the modified proteins present in milk after heating, including assignment of the modified amino acids, classical proteomic approaches based on the integration of two-dimensional electrophoresis (2-DE), enzymatic in-gel protein digestion, and analysis of the resulting peptide mixtures by MALDI-TOF MS or nanoLC-ESI-MS/MS were used. The ability of 2-DE to display and quantify thousands of proteins in a unique experiment allowed the evaluation of small changes in milk protein composition arising from various thermal treatments, but also the ability to purify proteins for further MS characterization. The first proteomic study on commercial milk powder led to a reliable identification of lactose conjugates of LG, LA, and caseins ([Galvani et al., 2000](#)). Similarly, 2-DE, digestion

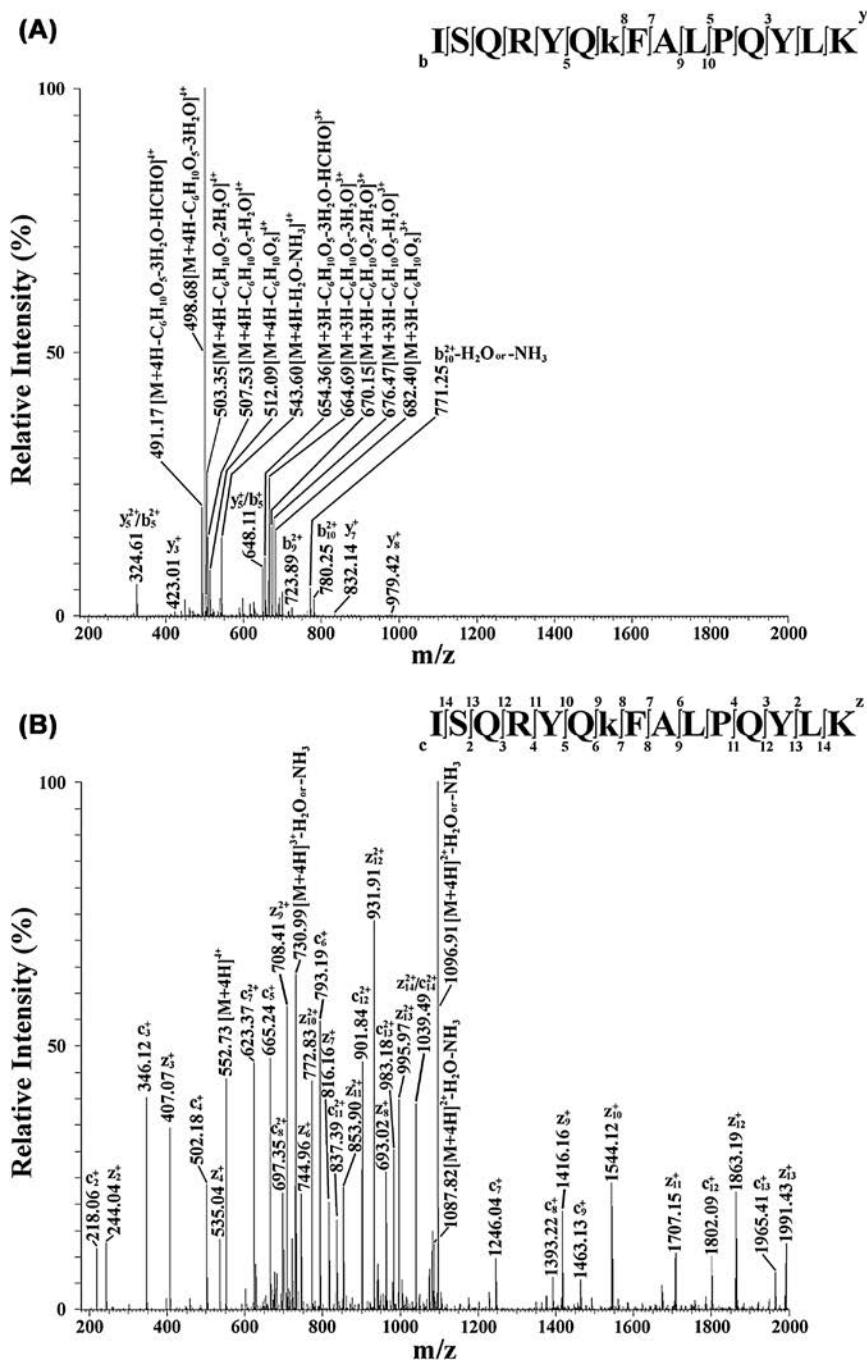


Figure 15.2: Comparison of the spectra obtained for a lactosylated peptide under CID (A) and ETD (B) MS/MS conditions.

Tandem mass spectra of the $[M+4H]^{+4}$ ion at m/z 552.9, which was assigned to the lactosylated tryptic peptide (167–181) from α S2-CN; k represents the Amadori product at Lys173. Identified fragment ions are labeled above and below the peptide sequence. Reproduced from Arena, S., Salzano, A.M., Renzone, G., D'Ambrosio, C., Scaloni, A., 2014. Non-enzymatic glycation and glycoxidation protein products in foods and diseases: an interconnected, complex scenario fully open to innovative proteomic studies. *Mass Spectrom. Rev.* 33, 49–77, with permission from John Wiley and Sons, Copyright 2016.

with trypsin, and nanoLC-ESI-MS/MS were applied to resolve the lactosylation pattern of milk proteins purified from infant formula powders (Marvin et al., 2002). Interestingly, neutral loss MS/MS experiments were carried out to selectively detect lactosylated peptides. Up to 10 lactosylation sites were identified in LG and α S2-CN, 5 modification sites in LA, α S1-CN, and β -CN, and 4 sites in κ -CN. LA was regarded as a potential marker in milk powder sample to detect chemical modification induced by the processing/storage conditions. On the other hand, Holland and coworkers carried out a proteomic analysis of the protein fraction purified from UHT milk samples stored at different temperatures for 2 months (Holland et al., 2011). Two-dimensional electrophoresis maps of these samples were characterized by (1) the appearance of diffuse staining regions above the position of the monomeric caseins caused by nondisulfide cross-linking of α S1-CN, α S2-CN, and β -CN; (2) the appearance of additional acidic forms of proteins (mainly of α S1-CN) caused by deamidation; and (3) the appearance of vertical stacked spots caused by lactosylation of whey proteins. The extent of these proteomic changes increased with augmented storage temperature. MS analysis of in-gel tryptic digests showed that the cross-linked proteins were dominated by α S1-CN, but a heterogeneous population of cross-linked forms with α S2-CN and β -CN was also observed. MS/MS analysis was used to assign deamidation sites in α S1-CN. For stacked spots, it revealed lactosylation of nine lysines in LG and eight lysines in LA, highlighting a strict relationship between the extent of lactosylation and storage temperature. No information was achieved for other milk protein components. A similar link between extent of lactosylation and storage conditions (temperature, relative humidity, and time) was observed in milk protein concentrates by using proteomic techniques. LA was again selected as suitable marker, which was found to be modified by as many as five lactose moieties on eight different Lys residues (Le et al., 2012).

An analytical strategy based on 2-DE, enzymatic in-gel protein digestion, and MALDI-TOF MS was also developed for the detection of milk adulteration and, in particular, for monitoring the addition of milk powder derivatives to commercial milk samples (pasteurized and UHT). Proteomic analyses were initially performed in order to define lactosylated, glycated, oxidized (Met/Trp/Cys), deamidated and aminoacidic semialdehyde-containing or CML-containing peptides specifically found in milk powder, which could be regarded as biomarkers of adulteration. Specific assignment of modified amino acids in LG, LA, α S1-CN, α S2-CN, β -CN, and κ -CN was obtained. Direct MALDI-TOF MS analysis of the tryptic peptide mixtures of whey and casein fractions from intentionally adulterated milk samples allowed these diagnostic peptides to be monitored and detection to 1% of adulteration level was achieved (Calvano et al., 2013).

Disulfide bond formation between milk proteins was specifically evaluated in heated milk by using reducing and nonreducing 2-DE, combined with MS analysis of selected protein digests (Chevalier et al., 2009; Chevalier and Kelly, 2010). Analysis of proteomic profiles in raw milk indicated that about 18%, 25%, and 46% of α S2-CN, LG, and κ -CN molecules, respectively, are involved in S-S-linked homopolymeric and heteropolymeric structures, whereas α S1-CN

and β -CN occur mainly in reduced forms. Following heat treatment, the amount of reduced serum albumin, LG, and κ -CN decreased by 85%, 75%, and 75%, respectively, with the formation of S-S-related polymers. Homopolymers and heteropolymers of κ -casein and α S2-casein were identified by MS in heated samples; polymers involving only α S2-CN or only κ -CN accounted for 43% and 12% of the total polymers present, respectively, indicating the key role of these proteins in the formation of intermolecular disulfide bridging. These results confirmed previous observations on pressurized and thermally treated milk, where copolymers ranging from 440 to 2000 kDa were also observed (Hoffman and van Mil, 1999; Cho et al., 2003; Nabhan et al., 2004). In the case of κ -CN dimers and trimers, the nature of the disulfide-linked peptides and Cys residues involved in disulfide bonds was ascertained (Holland et al., 2008). Analogous studies realized on a milk model were performed to fully characterize κ -CN-containing and LG-containing homomeric and heteromeric dimers and trimers (Livney and Dalgleish, 2004). Interaction and association of milk proteins via disulfide bridges was associated with a protective function in milk micelle stabilization and against formation of fibril aggregates.

15.4 Shotgun Proteomic Characterization of the Modifications Induced by Thermal Treatments

Developments in MS and separation technologies have streamlined the analysis of the entire proteome in the so-called shotgun proteomic approach. In this procedure, a total protein extract is in-solution digested; the resulting peptide mixture is separated via 1D or 2D chromatography and analyzed by tandem MS. However, peptides bearing modifications are typically less abundant and often escape detection. This difficulty can be overcome by adopting specific strategies to trap only the modified peptides and/or to detect them, among the unmodified peptides present in a proteome digest (Bachi et al., 2013). Due to the large variety of chemical adducts generated as result of nonenzymatic glycation/glycoxidation/oxidation reactions associated with milk heating, no dedicated precursor ion scanning or NL experiments can be performed in a single LC-MS/MS acquisition, and all the putative modified species have to be considered for subsequent database searching.

In this context, integrated shotgun proteomic procedures were developed for global characterization of the various glycation and glycoxidation protein targets in raw, pasteurized, UHT, and powdered milk samples (Arena et al., 2010, 2011; Renzone et al., 2015) examining the corresponding adducts and modified amino acids. Soluble milk proteins were enriched for the less abundant components using the ProteoMiner technology (D'Amato et al., 2009) and protein components from purified milk fat globule (MFG) particles were resolved by SDS-PAGE. The ProteoMiner technology acts to equalize the concentration of proteins in a sample. Slices from whole SDS-PAGE lanes were trypsinolyzed (Arena et al., 2010) and extracted protein digests were enriched for modified peptides by affinity chromatography on

m-aminophenylboronic acid-functionalized agarose. This support was previously used to perform proteomic profiling studies on nonenzymatically glycated proteins from human blood; its application in enriching *cis*-diol-containing peptides directly from whole protein digests allowed identifying modified proteins present even at low-abundance levels. We adapted its use for detection of glycated/glycoxidized peptides from milk proteins (Arena et al., 2010, 2011; Renzone et al., 2015). MALDI-TOF mass spectrum profiling of eluted peptide digests from raw, pasteurized, UHT, and powdered milk demonstrated a signal complexity and intensity that correlated with the severity of thermal treatment.

To directly characterize glycated/glycoxidized peptides, simultaneously providing information on the nature of the protein modified, the adduct type, and the modified amino acid(s), peptide mixtures derived from in-gel digestion and eluted from phenylboronate chromatography were directly analyzed by nLC-ESI-LIT-MS/MS with ETD fragmentation using SCA (Arena et al., 2010, 2011; Renzone et al., 2015). This fragmentation technique was selected because (1) CID of lactosylated peptides generated mass spectra having a reduced intensity of sequence-informative ion species and (2) comparative ETD and CID experiments performed on peptides bearing AP with D-glucose/D-galactose (Hex), their dehydrated counterparts, and G-DH, triosone-DH, CML and PYR derivatives revealed improved MS/MS spectra with ETD for the first four adduct types, while no significant differences were observed in the case of the remaining two adducts. To increase the number of modified peptides characterized, each sample was subjected to two nLC-ESI-LIT-MS/MS analyses. Data-dependent product ion scanning of the five most abundant ions with ETD fragmentation was performed with exclusion of identified peptides in the second analysis. For identification of lactosylated and AGEs-containing peptides, nLC-ESI-LIT-MS/MS raw data files were then searched against a non-redundant bovine sequence database selecting carbamidomethylation at Cys as fixed modification, oxidation at Met/Trp/Cys, plus all putative glycation/glycoxidations at Arg/Lys as variable modifications.

When applied to the soluble milk fraction or the ProteoMiner-enriched soluble fraction, this shotgun approach allowed the identification of 271 nonredundant lactosylation sites in 33 proteins (Arena et al., 2010). Most of these protein species escaped detection by Western blotting following 2-DE analysis of skimmed milk or whey samples. Similarly, 157 novel nonredundant modification sites and 35 MFG proteins never reported before as being lactosylated were identified in protein components from purified fat globules (Arena et al., 2011), in addition to the 153 present in 21 proteins detected as adsorbed on this fatty fraction, which were previously characterized in the soluble milk portion (Arena et al., 2010). A total of 428 nonredundant lactosylation sites in 68 proteins mainly involved in nutrient delivery, defense response against pathogens, related inflammatory processes, and cellular proliferation/differentiation were characterized.

The number of modification sites within each protein was variable and their number increased with harsher milk processing conditions. Good reproducibility of the lactosylation data was

noted, since modified sites ascertained in pasteurized milk were also present in UHT and powder milk samples. Similarly, lactosylation sites observed in the UHT milk were also observed in powdered milk. Most of the lactosylation sites were identified in abundant milk proteins, namely LG, LA, α S1-CN, α S2-CN, β -CN, and κ -CN, glycosylation-dependent cell adhesion molecule 1, lactotransferrin, xanthine dehydrogenase, peripilin 2, lactadherin, fatty acid binding protein, and lactoperoxidase. Good qualitative agreement was observed with previous studies on lactosylation sites in the most abundant proteins. The widespread modification measured for proteins from UHT and powder milk samples matched with quantitative amino acid determinations on corresponding whole hydrolysates and MS analysis of intact proteins.

When applied to soluble and MFG milk fractions in searching for AGEs, this shotgun approach identified 308 intermediate and advanced glycoxidation adducts in 31 proteins (Renzone et al., 2015). Aside from confirming lactosylation sites described previously (Arena et al., 2010, 2011), this study also identified 40 novel LL adducts, providing the largest qualitative inventory of nonenzymatically modified species ascertained in commercial samples to date. Similar results were obtained by recent studies in this research field (Milkovska-Stamenova and Hoffmann, 2016a,b). Focusing on intermediate/advanced glycoxidation adducts in milk samples, 289 modified peptides bearing (1) LL- H_2O (+306 Da), LL-2 H_2O (+288 Da), Hex/3-DG-He/3-DGal-He (+162 Da), Hex- H_2O (+144 Da), Hex-2 H_2O (+126 Da), CML/G-He (+58 Da), CEL/MG-He (+72 Da), PYR (+108 Da), 3-DPen-He (+132 Da), and Lact-He (+340 Da) adducts at Lys residues; (2) RPYR (+80 Da), G-H (+40 Da), MG-H (+54 Da), G-DH/G-He (+58 Da), MG-DH/MG-He (+72 Da), 3-DG-DH/3-DGal-DH/3-DG-He/3-DGal-He (+162 Da), 3-deoxylactosone (3-DLact)-DH/3-DLact-He (+324 Da), 3-DLact-H (+306 Da), and triosone-DH/triosone-He (+88 Da) adducts at Arg were recognized. AGE assignment in some cases was not definitive, since the same mass shift value can be associated with different products occurring at the same residue. However, recent studies on hemiaminals reported these compounds as being unstable over prolonged times (Mittelmaier and Pischetsrieder, 2011; Meltretter et al., 2013). Globally, 3, 30, 112, and 281 nonlactosylated modified species were detected in raw, pasteurized, UHT, and powdered infant milk samples, respectively. They were associated with 169 nonredundant intermediate and advanced glycation end-product modified sites in milk proteins that have been already observed as lactosylated. Most frequent intermediate and advanced glycation end-products were the Amadori compounds with D-glucose or D-galactose/3-DG-He/3-DGal-He, LL- H_2O , CML/G-He, G-DH/G-He, 3-DG-DH/3-DGal-DH/3-DG-He/3-DGal-He, and 3-DLact-DH/3-DLact-He derivatives (about 52%, 10%, 8%, 5%, 4%, and 3% of the whole modified, nonlactosylated species, respectively). Three linear peptides bearing intramolecular cross-linked pentosidine, DOGDIC, and DOLD structures were also observed; they were already described in dedicated studies on model proteins heated in the presence of sugars. Concomitant assignment of fragment ions to both bridged residues ensured no ambiguity in the identification of cross-linked peptide adducts. The nature of the observed cross-linked

species and the frequency of the other linear AGEs detected in this study suggested G, 3-DG, and 3-DGal as the main reactive dicarbonyl compounds in heated milks.

Notably, detection of intermediate and advanced glycation end-product containing peptides in the different milk fractions well paralleled with the identification of the corresponding protein species therein (Arena et al., 2010, 2011) and the simultaneous recognition of the corresponding lactosylated peptides in the same sample or in milk samples subjected to softer processing conditions. These findings confirmed that, according to the individual reactivity of lysines present in proteins and to the harshness of thermal treatment exerted, modifiable Lys residues firstly react with the most abundant sugar present in milk, i.e., lactose, to generate the corresponding APs. Then, these early modification products are further modified/oxidized to generate the corresponding intermediate and advanced glycation counterparts. On the other hand, nonreacted Arg and Lys residues in these proteins can be modified by less abundant D-glucose and D-galactose, or by lactose degradation/oxidation products generated following thermal treatment, i.e., G, MG, 3-DLact, 3-DG, 3-DGal, 3-DPen, lactosone, and triosone, ultimately yielding the corresponding AGEs. Progressively augmented detection of intermediate and advanced glycation end-products in milk samples subjected to harshening food processing conditions was in good agreement with quantitative data from GC-MS or LC-MS analysis of amino acid adducts, as reported for commercial milk samples or related model systems (Erbersdobler and Somoza, 2007; Pischetsrieder and Henle, 2012).

A comparison of the data here reported for LG, LA, and various caseins with that recently published in other investigations on specific proteins from raw and commercial milk samples and model proteins subjected to thermal treatment in the presence of sugars demonstrated their good concurrence either in terms of the nature of the AGEs observed in the different products and of the modified Lys/Arg residues present therein. Discrepancies between these studies may be ascribed to the phenylboronate chromatography used in our work, which promoted a general enrichment of diol-containing AGEs-modified peptides in milk fractions.

Visual inspection of the spectroscopic/crystallographic structure of the modified milk proteins revealed that most of the modified residues occur on their macromolecular surface, although few internal amino acids were also affected. Accordingly, the Maillard reaction can influence the 3D structure of these proteins, as already observed in various model systems, with significant consequences on their functionality. Since it has been shown that various proteins are resistant to proteolysis in the gastrointestinal tract (Castell et al., 1997; Lonnerdal, 2003), and may directly exert a number of biological activities within the human intestine or other organs (Lonnerdal, 2003; D'Alessandro et al., 2011), their widespread modification in milk may have important consequences on corresponding nutritional and health-beneficial characteristics. Shotgun studies have demonstrated that various milk proteins involved in the delivery of nutrients, the defense response against pathogens, related inflammatory processes, and the regulation of cellular proliferative/differentiation events (Arena et al., 2010, 2011; Renzone

et al., 2015) are subjected to glycation/glycoxidation after heating. Thus, a severe thermal treatment can influence their activity (Sebekova and Somoza, 2007; Henle, 2007). This can have important consequences when milk is the unique source of bioactive molecules, as is the case for the infant diet.

Since modified Lys/Arg residues are no longer recognized by proteolytic enzymes, the Maillard reaction can also affect the gastrointestinal digestion of milk proteins (Dalsgaard et al., 2007). This issue was recently addressed by comparing the digestibility of the milk proteins present in different commercial samples (Arena et al., 2011; Wada and Lonnerdal, 2014). When major soluble proteins were evaluated, an increased in vitro/in vivo digestion resistance of the intact species was observed in raw and pasteurized samples by SDS-PAGE, compared to the UHT and sterilized ones. However, protein digestibility (as determined by the Kjedahl procedure) showed an opposite trend, as already reported for milk-based infant formulas (Rudloff and Lonnerdal, 1992; Rutherford and Moughan, 2005). This effect was attributed to industrial heating improving the protein digestibility by denaturation, but this enhancement is likely to be offset by heat-derived modifications involved in decreasing proteolysis of milk components. When MFG proteins were evaluated, an increasing resistance to trypsinolysis was observed moving from pasteurized to UHT and powdered milk proteins (Arena et al., 2011). These results confirmed previous studies on model milk proteins treated with various sugars/oligosaccharides (Corzo-Martinez et al., 2010). Not considering possible physiological implications on the eventual consumption of poorly digestible foods, this point has a first important significance for some soluble milk proteins that, after an impaired proteolysis, may present a reduced release of sequence-encrypted bioactive peptides (Meisel, 2005). Reduced biological activity of these components can also be eventually ascribed to undesired peptide glycation/glycoxidation. On the other hand, a reduced digestibility of modified milk proteins may affect the allergic response to specific dairy products; in fact, it has been reported that proteins resistant to digestion may behave as major food allergens (Astwood et al., 1996; Wal, 2001). Extensive glycation/glycoxidation of surface-exposed residues in specific milk proteins should also per se modulate the allergenic impact of these nutrients (Karamanova et al., 2003; Gruber et al., 2004; Heilmann et al., 2014).

15.5 Conclusions and Future Outlook

In conclusion, although important progress has been made in the field of the Maillard reaction regarding dairy products, basic questions from a chemical, biological, and immunological point of view remain unanswered yet. Following investigations on model systems and/or isolated proteins from milk, where the main Maillard reaction products were characterized, focused efforts are now spent in identifying the adducted sites of modification targets and characterizing the corresponding protein adducts in milk products. This information is critical

for milk nutritional, biological, and toxicological properties, since thermally modified milk proteins form a major component of the human diet and as a unique polypeptide source for infants. In this context, controversial data are available on the effect of thermal treatment on the allergenicity and biological properties of proteins (Astwood et al., 1996; Wal, 2001; Karamanova et al., 2003; Lonnerdal, 2003; Gruber et al., 2004; Sebekova and Somoza, 2007; Henle, 2007; Muscat et al., 2009; Corzo-Martinez et al., 2010; D'Alessandro et al., 2011; Shandilya et al., 2013; Hellwig and Henle, 2014; Heilmann et al., 2014; Verhoeckx et al., 2015). This condition probably reflects the different modification and unfolding behavior of the various proteins with respect to thermal treatment. Information on the thermal-induced modifications in milk proteins is also essential to rationalize the technical properties of novel products having milk origin, which are now used for pharmaceutical/cosmetological applications (Ter Haar et al., 2011; Ruffin et al., 2014). Extensive data acquisition on protein modifications will enable the generation of comprehensive milk-focused database which may ultimately allow food manufacturers to optimize the thermal processing of their products in order to obtain manufactured goods with controlled amounts of AGEs. To reach this ultimate goal, future studies applying quantitative MS procedures are warranted to gain information on the amino acids more prone to generate adducts in various milk proteins. In this context, preliminary quantitative proteomic studies have been recently realized (Le et al., 2013; Meltretter et al., 2014; Wust and Pischetsrieder, 2016; Milkovska-Stamenova and Hoffmann, 2016a,b). These investigations confirmed that the site-specific modification degree correlates with the severity of the thermal treatment but also pointed out that it varies considerably among the brands of UHT and powdered infant milk products. Thus, proper adjustments of the industrial processes should allow reduction of the modification levels in both UHT and powdered milk. In addition, they pointed out that lactose-free milks present a glycation degree that varies considerably among different brands, with lactose-free UHT milk and infant formula showing the highest levels. All earlier-mentioned information are important points of interest to connect the extent of the Maillard reaction occurring in different commercial samples with potential nutritional implications. The themes described earlier and those associated with the other modifications present as consequence of milk industrial treatments have to be fully evaluated for a whole estimation of the nutraceutical/toxicological properties of dairy products.

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Proteomics of Egg White

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16.1 Introduction

The avian egg contains all nutrients necessary for the development of the avian embryo (Burley and Vadhera, 1989; Johnson, 2015). However, since the domestication of chicken, which most likely started 10,000–8000 BC in several regions of China and Southeast Asia (Miao et al., 2013; Xiang et al., 2014), the chicken egg has also become an important part of human diet (Miranda et al., 2015; Zaheer, 2015). While most research activities focus on the chicken egg because of its importance for human nutrition and commercial value, the structure, composition, and assembly of eggs is very similar in all avian species (Burley and Vadhera, 1989). The development and composition of the chicken egg has been extensively reviewed (Burley and Vadhera, 1989; Li-Chan and Kim, 2008). The innermost egg compartment, the yolk, is assembled in the ovaries, of which only the left one remains functional in the order Galliformes. Yolk contains the egg's lipids, which constitute approximately 33% of total yolk weight and are bound to protein in low-density and high-density lipoprotein particles (Burley and Vadhera, 1989; Anton, 2013). Approximately 17% of yolk weight is protein, and approximately 47% is water. In addition yolk contains many vitamins and minerals. The yolk is surrounded by a vitelline membrane predominantly consisting of proteins. While the inner vitelline membrane is already formed in the ovaries, the outer vitelline membrane is assembled only after ovulation in the very first section of the oviduct, the infundibulum. In the next section of the oviduct, the magnum, egg white components are synthesized, secreted, and assembled. Egg white is approximately 10% protein and 88% water. The egg white of fresh eggs consists of the highly viscous thick egg whites and the less viscous thin egg whites. The inner thick white, also known as the chalaziferous layer, surrounds the yolk as a thin gel and extends into the ropelike chalazae on the polar ends of yolk. The chalazae cross the inner thin white that separates inner and outer thick white. The outer thick white forms the largest part of egg white and makes contact to the shell membranes at the polar ends of the egg. The outer thin white is a thin layer below the shell membranes in those regions not in contact with outer thick white. Differences in viscosity between the different egg whites tend to disappear with egg age (egg white thinning). Shell formation (Hincke et al., 2012) starts in the isthmus section of the oviduct by secretion and

assembly of the precursors of the proteinaceous eggshell membranes, on the surface of which first mineral deposits aggregate in the terminal section of the isthmus, the red isthmus. Bulk mineralization and cuticle deposition occurs in the shell gland (uterus). The finished shell consists of approximately 96% of calcium carbonate in the form of calcite and an organic matrix of proteins that forms another 2%–4%. The complete migration of the egg through the oviduct takes approximately 24 h, of which 15–30 min are spent in the infundibulum, 2–3 h in the magnum, 60–75 min in the isthmus, and 18–20 h in the uterus. In the fertilized egg, yolk is the predominant source of nutrients for the developing embryo. Egg white is the main source of water, keeps the yolk in place, forms a cushion absorbing shocks, and protects yolk and embryo against microbial invaders by a mixture of antimicrobial components. The shell protects the egg against physical attack, forms a first line of antimicrobial defense, provides calcium to the developing embryo, and regulates gas exchange through its pores.

16.2 Egg White Proteins in the Preproteomic Era

Egg white is an easily available, relatively simple mixture of proteins that has been used frequently to test methods for the separation and characterization of proteins. Early efforts to separate egg white proteins by salt precipitation and related methods, and to characterize the separated proteins by their composition, their ability to crystallize, and/or their response to antisera were summarized by [Hektoen and Cole \(1928\)](#). Since then the introduction of electrophoretic and column chromatographic methods for separation of proteins ([Awade, 1996](#); [Desert et al., 2001](#)), automated amino acid sequence analysis based on Edman chemistry, and nucleic acid sequencing have contributed enormously to our knowledge of egg white protein composition and the characterization of its proteins ([Burley and Vadhera, 1989](#)). Altogether 15 egg white proteins were isolated and characterized by at least partial amino acid sequencing before the advent of proteomics ([Table 16.1](#)). For most of these proteins functions were inferred by *in vitro* activities or domain structure. An important exception is the most abundant egg white protein, ovalbumin, that is supposed to serve as a source of amino acids for the developing embryo, but for which no defined function has been ascribed. In addition to the proteins listed in [Table 16.1](#) the presence of several more proteins was inferred from enzymatic activities in egg white or egg white fractions. These included β -N-acetylglucosaminidase, catalase, α -mannosidase, nucleoside triphosphatase and ribonuclease (summarized in [Burley and Vadhera, 1989](#)), peptidases ([Petrovic and Vitale, 1990](#)), and sulfhydryl oxidase ([Hoover et al., 1999](#)).

16.3 Basic Proteomic Studies of Egg White

Meaningful high-throughput mass spectrometry-based proteomic analysis still depends on the availability of comprehensive and well-curated sequence databases. Thus, the proteomic analysis of egg proteins started only after publication of the chicken genome sequence

Table 16.1: Proteins found in chicken egg white before the advent of proteomic methods.

| Protein ^a | % of Total | Activities ^b |
|--|------------|---|
| Ovalbumin | 54 | Member of the serpin family of protease inhibitors; no inhibitor activity |
| Ovotransferrin | 12 | Iron binding; antibacterial ^b |
| Ovomucoid | 11 | Protease inhibitor |
| Lysozyme | 3.4 | Bacteriolytic enzyme |
| Ovomucin | 1.5 | Gel-forming |
| Ovoinhibitor | 1.5 | Protease inhibitor |
| Ovoglycoprotein | 1.0 | |
| Ovoflavoprotein/Riboflavin-binding protein | 0.8 | |
| Ovomacroglobulin/ovostatin | 0.5 | Protease inhibitor; antibacterial ^b |
| Ovoglobulin G2/TENP ^c | (0.1–0.5) | Belongs to bactericidal/permeability-increasing proteins |
| Cystatin | 0.05 | Protease inhibitor; antibacterial ^b |
| Avidin | 0.05 | Biotin binding; bacteriostatic ^b |
| Clusterin | n.d. | Extracellular chaperone |
| Hep21 | n.d. | Member of the uPAR/CD59/La6 family |
| Ovalbumin-related protein Y | n.d. | Member of the serpin family of protease inhibitors; no inhibitor activity |

^aOnly proteins characterized by at least partial amino acid analysis are included.

^bAdapted from Baron, F., Nau, F., Guérin-Dubiard, C., Bonnassie, S., Gautier, M., Andrews, S.C., Jan, S., 2016. Egg white versus *Salmonella enteridis*! A harsh medium meets a resilient pathogen. *Food Microbiol.* 53, 82–93.

^cIdentical, according to Maehashi et al. (2014)

Adapted from Kovacs-Nolan, J., Phillips, M., Mine, Y., 2005. Advances in the value of eggs and egg components for human health. *J. Agric. Food Chem.* 53, 8421–8431.

(International Chicken Genome Sequencing Consortium, 2004) and the insertion of the derived protein sequences into common databases, such as the now abandoned but previously frequently used International Protein Index (IPI) protein sequence database or its replacement UniProt. However, even this genomic database, used as kind of a gold standard in avian genomics and proteomics research, is still incomplete due to technical problems (Dodgson et al., 2011). Overall approximately 5%–10% of the chicken genome sequence is still missing (Dodgson et al., 2011) and one cannot exclude that this fraction also contains sequences of egg white proteins.

The first chicken egg white proteomic analyses (Raikos et al., 2006; Guérin-Dubiard et al., 2006) were not by today's standards considered to be high-throughput. The more comprehensive study of these two (Guérin-Dubiard et al., 2006) used in-gel digestion of egg white proteins separated by 2-D gel electrophoresis (2-DE), a technique that is not used any more for global high-throughput proteomic analysis. Proteins were identified by a combination of MALDI-TOF peptide mass fingerprinting and LC-MS/MS using a hybrid quadrupole/TOF mass spectrometer. Altogether 16 proteins were identified from 69 excised protein spots. The authors noted that several previously characterized egg white proteins were missing in their list of identified

proteins. This was attributed to protein features not compatible with 2-DE separation, such as extreme molecular mass and/or extreme isoelectric point, or to the possible absence of the respective amino acid sequences in protein sequence databases. Nevertheless, two proteins were identified in egg white for the first time. These were vitelline outer layer membrane protein-1 (VMO-1) and protein Tenp (transiently expressed in neural precursors). Both proteins were also identified in avian eggshell matrix at approximately the same time (Mann, 2004; Mann et al., 2006). Tenp belongs to the family of bactericidal permeability-increasing proteins and has therefore been suggested to be part of the egg's antimicrobial defense (Guérin-Dubiard et al., 2006; Whenham et al., 2014).

The egg white proteome analysis published next (Mann, 2007) used 1-D gradient PAGE for protein separation and in-gel digestion to obtain peptides. Peptide sequencing for protein identification was performed by LC-MS/MS (MS^2) and MS/MS/MS (MS^3) on a 7-Tesla LTQ-FT (linear ion trap Fourier transform) instrument, arguably the most advanced mass spectrometer at that time with superior sensitivity, resolution, and mass accuracy (Olsen and Mann, 2004). The instrument was operated in a data-dependent mode to automatically switch between full scan MS, MS^2 , and MS^3 . The three most intense peaks of each survey scan (MS) were fragmented for sequence analysis and up to three most intense fragment ions of each MS^2 run were subjected to a second round of fragmentation (MS^3) to improve the reliability of identifications (Olsen and Mann, 2004). Peptides and proteins were identified by searching the chicken IPI sequence database. This yielded 78 protein identifications, including 54 identified for the first time in egg white. The list contained almost all proteins identified in egg white previously. Furthermore, the exponentially modified protein abundance index (Ishihama et al., 2005) was calculated as a measure of approximate abundance of proteins, thus discerning major from minor proteins. The results of this calculation were in good agreement with protein concentrations determined previously by conventional methods for major egg white proteins. Among the most abundant interesting proteins detected in chicken egg white for the first time in this proteomic study was a basic 7 kDa polypeptide that was named gallin because of its sequence similarity with previously identified swan egg white cygnin (Simpson and Morgan, 1983) and turkey meleagrin (Odani et al., 1989). This protein was subsequently further characterized as an antimicrobial protein of a larger group of avian defensins called ovodefensins (Gong et al., 2010; Whenham et al., 2015).

A frequent problem in the analysis of proteomes dominated by one or a few major proteins is the limited dynamic range of mass spectrometers. Usually MS spectra produced by modern instruments contain many more peptide peaks than can be analyzed by MS/MS on a reasonable time scale in high-throughput experiments. Therefore, only the most intense peaks are chosen routinely for fragmentation. In egg white, ovalbumin represents approximately 54% of total protein and is almost ubiquitously distributed among fractions derived from chromatographic or electrophoretic separation of egg white proteins. Thus,

ovalbumin-derived peptides tend to be among the most intense peaks in MS spectra of many fractions and are fragmented and identified over and over again, decreasing the likelihood of less intense peaks derived from less abundant proteins to be chosen despite dynamic exclusion times usually set to 30–90 s to prevent resequencing of already fragmented peaks during this time period. A method devised to overcome such problems is the use of combinatorial peptide ligand libraries, CPLL (Boschetti and Righetti, 2009). In this method beads covered with a single species of randomly synthesized hexapeptides is incubated with the protein mixture to be analyzed. Proteins are presumed to bind to one or a few types of peptides by some affinity mechanism. With a similar and suitable concentration of all the possible hexapeptide sequences presented, minor proteins will be enriched until the binding capacity of their respective peptide-covered beads is exhausted, while major proteins will not find enough binding sites and the excess will be removed by washing with a suitable buffer. The mixture eluted from the beads will theoretically contain approximately equal amounts of all proteins present. Application of this method in conjunction with peptide sequencing and protein identification by LC-ESI-MS/MS to chicken egg white yielded 148 unique protein identifications, twice as many as before (D'Ambrosio et al., 2008). However, more than half of these proteins were identified by a single sequence-unique peptide. Usually such identifications are not accepted in high-throughput proteomic studies unless there are compelling reasons.

The advent of faster high-resolution mass spectrometers has circumvented some of the issues encountered owing to protein dynamic range. The LTQ-Orbitrap Velos, which was used in the last egg white proteomic survey to be discussed in this section (Mann and Mann, 2011), is fast enough to isolate and fragment 10 or more peaks simultaneously with the acquisition of one high-resolution mass full scan spectrum (Olsen et al., 2009). Without resorting to single-peptide identifications, 158 proteins were identified, increasing the total number of egg white proteins identified with at least two peptides to 167. Comparing the results of Mann (2007) and Mann and Mann (2011) a depth was reached in the second study of this relatively simple low-complexity proteome that was sufficient to permit detection of a large number of low-abundance intracellular proteins probably not related to egg white function, that may be by-products of secretion or were released by damaged or decaying epithelial cells.

Nevertheless, new potentially interesting trace components may be detected in future research employing specific enrichment procedures, different fractionation methods, or improved mass spectrometers. For instance, Lee et al. (2013), while screening egg white for proteins with cell proliferative bioactivity identified by LC-MS/MS the intracellular protein rho guanine nucleotide exchange factor 11, intracellular serine/threonine protein kinase WNK2, and secreted GP340/DMBT1 (deleted in malignant brain tumors 1) after fractionating egg white by strong anion- and strong cation-exchange chromatography in tandem. Interestingly, they also found an egg white fraction promoting cell proliferation and wound closure, but could not identify the factor(s) responsible.

16.4 Comparative Egg White Proteomics

16.4.1 Egg White Changes Upon Storage

Storage of eggs is known to cause changes in egg white such as, for instance, egg white thinning or the conversion of ovalbumin to S-ovalbumin. Egg white thinning is the conversion of thick egg white to thin egg white usually ascribed to disaggregation of high molecular weight complexes of the gel-forming glycoprotein ovomucin (Burley and Vadhera, 1989; Hiidenhovi, 2007; Omana et al., 2010). Several studies explored the effect of storage time and storage temperature on egg white proteins by MS-based proteomic techniques. Réhault-Godbert et al. (2010) observed fluctuations of anti-Salmonella activity in the egg white of eggs stored at 4, 20, and 37°C for a total storage period of 30 days. These fluctuations, especially a pronounced decrease of activity in egg white of eggs stored at 37°C, coincided with the appearance of protein degradation products observed by SDS-PAGE. The partially degraded proteins were identified by LC-MS/MS after in-gel digestion as ovalbumin and ovotransferrin. Omana et al. (2011) analyzed changes in protein abundance in egg white during storage with the aim to further our understanding of the mechanism of egg white thinning. Egg white proteins of eggs stored over 40 days at 22°C were sampled at 10-day intervals and separated by 2-DE. Spots showing changes in staining intensity at different storage intervals were in-gel digested and the eluted peptides analyzed by mass spectrometry using a Q-TOF MS. This analysis was complicated by the occurrence of most of the identified proteins in different gel spots due to the previously known existence of different isoforms and further due to proteolytic degradation of specific proteins. Major abundance changes, as compared to fresh eggs, were detected in gel spots containing ovalbumin, ovoinhibitor, ovotransferrin, clusterin, and the lipocalin family member prostaglandin D2 synthase. Changes occurred predominantly between days 10 and 20, when egg white thinning was observed. Different spots containing the same protein either increased or decreased in staining intensity, thus indicating a possible redistribution among different forms. In fact the authors reported a shift to lower molecular weight forms especially for ovalbumin and clusterin apparently due to partial proteolytic degradation, and this decrease in abundance of intact molecules was suggested to be among the causes of egg white thinning. Ovomucin, postulated to be a major player in egg white thinning previously, was not analyzed because its high molecular weight renders it unsuitable for 2-DE. Qiu et al. (2012a) analyzed changes in the egg white proteome during storage at different temperatures using essentially the same analytical methods as Omana et al. (2011) with MALDI-TOF MS/MS used for peptide sequencing. Eggs were stored at 4, 20, or 37°C for 15 days before their egg white proteome was compared to that of fresh eggs. As before (Omana et al., 2011), changes were found with protein spots containing ovalbumin, ovoinhibitor, ovotransferrin, clusterin, and prostaglandin D2 synthase. To this list were added lysozyme C, extracellular fatty acid-binding protein, and ovalbumin-related protein Y. Changes were most pronounced upon storage at 37°C. As before, most of these proteins were identified in several spots with increasing or decreasing

staining intensity. The major egg white protein ovalbumin was present in more than half of the 32 identified spots with apparent molecular weights between 18.1 and 170.1 kDa. This indicated the presence of partially degraded forms and complexes of various combinations of complete and partially degraded ovalbumin molecules, increasing in abundance with increasing temperature. The observed degradation of egg white proteins was suggested to be due to the observed general decrease in abundance of intact protease inhibitors ovomucoid and ovoinhibitor during storage. The decreased abundance of the chaperone clusterin and the degradation of major proteins were hypothesized in both reports (Omana et al., 2011; Qiu et al., 2012a) to be a possible cause of the breakdown of structure causing egg white thinning. Clusterin, because of its complete disappearance upon storage at 37°C, was suggested as a possible marker of egg quality (Qiu et al., 2012a).

16.4.2 Egg White Changes During Early Embryo Development

Other studies have explored differences in the egg white proteome of fresh unfertilized and fertilized eggs (Qiu et al., 2013), in egg white during the first 7–9 days of embryo development (Qiu et al., 2012b; Liu et al., 2013; Wang and Wu, 2014) and during the rapid embryonic growth phase (Liu et al., 2015). All of these studies used 2-DE for separation and quantification of proteins and subsequent identification by MS/MS, primarily using MALDI-TOF MS/MS.

During the first days of incubation of fertilized eggs, egg white predominantly loses water and ions that move to the yolk, while protein movements to other compartments of the egg seem to be very limited (Willems et al., 2014). Qiu et al. (2012b) found changes in eight egg white proteins during incubation of fertilized eggs over 7 days at a temperature of 38°C. The proteins involved and the suggested causes for the observed changes were the same as reported in egg white upon storage of unfertilized eggs (Qiu et al., 2012a). In a similar report of this group (Qiu et al., 2013) the focus was on differences between fresh fertilized and unfertilized eggs. Staining intensity differences of more than 10-fold were observed in seven spots, six stronger in egg white of fertilized eggs and one in unfertilized eggs. The six spots all contained ovalbumin-related protein Y, the seventh spot contained ovalbumin. Wang and Wu (2014) analyzed changes in egg white proteins of fertilized eggs occurring during the first 9 days of incubation at 37°C. Peptides eluted after in-gel digestion of 2-DE spots were analyzed on an Agilent 1100 LC/MSD Trap XCT system and a total of 37 proteins were identified from 91 protein spots. Nineteen of these identified proteins were reported to have been identified in egg white for the first time. However, most of the accession codes given for these new proteins belonged to well-established egg white components, like ovomucoid, lysozyme, and ovotransferrin. A closer look at a few selected supposedly novel egg white protein entries, for instance, gil10120552, gil157831883, and gil157831884, revealed that they belonged to lysozyme mutations constructed with recombinant techniques to explore structural consequences of active site mutations by

X-ray diffraction analysis. In two reports (Liu et al., 2013, 2015) 2-DE was combined with CPLL enrichment of low-abundance proteins. As outlined earlier, this method reduces the abundance of major proteins and enriches minor proteins. Although these two procedures do not really seem to be compatible, these equalized proteomes were then applied to 2-DE for quantification, and many changes in spot density were identified when comparing egg white from unfertilized and fertilized eggs incubated at 20 or 38°C for up to 7 (Liu et al., 2013) or 16 days (Liu et al., 2015). Seven and nine proteins, respectively, were identified amongst several dozens of changing spots, including olfactomedin-like protein 3, a secreted protein involved in dorsoventral patterning during early development. This protein was identified in egg white for the first time (Liu et al., 2015). The other proteins with abundance changes were ovotransferrin, ovoinhibitor, clusterin, ovalbumin, ovalbumin-related proteins X and Y, apolipoprotein D, extracellular fatty acid-binding protein, ovoglobulin G2/Tenp, and Hep21. The role of these proteins for embryonic development of the chicken remains unknown at present.

16.4.3 Other Comparative Proteomic Studies

Protein compositional analysis was undertaken in egg whites from six different egg varieties, including white-shell eggs, brown-shell eggs, lutein-enriched eggs, organic eggs, omega-3-enriched eggs, and vitamin-enriched eggs by Wang et al. (2012). Using 2-DE separation and densitometry combined with LC-MS/MS a total of 23 proteins were identified, of which 19 were noted to show significant differences in protein abundance among the six varieties examined. The proteins involved were ovalbumin, ovotransferrin, ovoglycoprotein, clusterin, hemopexin, ovalbumin-related protein Y, a proteinase inhibitor, ovoinhibitor, ovomucoid, prostaglandin D2 synthase/chondrogenesis-associated lipocalin, riboflavin-binding protein, lysozyme, Hep21, and marker protein/Ch21. The latter seems to be very similar, if not identical, to quiescence-specific protein/Ex-FABP. The major conclusion was that these egg varieties differed in egg white protein abundances but not in protein composition.

He et al. (2014) compared the egg white of free-range and barn-raised (supermarket) eggs. Proteins were extracted with trifluoroacetic acid combined with ultrasound treatment. The extracted proteins were then digested and measured using MALDI-TOF-MS and proteins identified by peptide mass fingerprinting. In this nowadays rarely used method one tries to identify proteins by matching measured masses to theoretical masses of in silico digested proteins without support from MS/MS sequencing. Twenty-three and 29 proteins were identified from egg white of free-range and barn-raised eggs, respectively, with an overlap of 16 proteins. Most proteins were previously identified in egg white with the exception of 11 collagen chains that were identified with 24 sequence-unique peptides altogether. However, these peptide identifications were not confirmed by MS/MS. Differences in the proteomes were attributed to the different diets of the chickens.

[Kim and Choi \(2014\)](#) investigated the influence of stress on egg white composition using dietary corticosterone addition as a stress model. After 5 days of treatment SDS-PAGE showed a dramatic decrease of proteins migrating at an approximate M_r of 75 and 50kDa in egg whites of eggs from corticosterone-treated hens. Analysis by 2-DE identified 34 spots that were up- or downregulated when compared to egg white proteomes of untreated hens. From these spots nine proteins were identified by MALDI-TOF/TOF MS/MS after in-gel digestion and peptide extraction. These were ovalbumin, ovalbumin-related proteins X and Y, ovoinhibitor, Tenp, hemopexin, ovotransferrin, extracellular fatty acid-binding protein, and an Ig Y-Fc fragment. While ovoinhibitor and ovalbumin-related X were significantly increased, all others in the list were decreased. The authors concluded that corticosterone modulates the expression of proteins in the magnum and that egg white protein changes may be useful markers of environmental stress.

Most of the studies described in this section (comparative egg white proteomics) relied on 2-DE for protein separation and gel densitometry for protein quantification, combined with MALDI-TOF MS for protein identification. This reliance on 2-DE, despite some disadvantages discussed earlier, stands in contrast to the general trend in proteomic studies to use either 1-D electrophoresis or gel-free sample preparation methods and LC-ESI-MS including label-free quantification for abundance estimation. Despite this, 2-DE is still considered by some to be the best choice for separation and identification of protein isoforms and modifications. However, the only modification explored in egg white studies discussed here was the partial degradation of a few major proteins and the possible formation of complexes between different forms of the same protein or, very rarely, between different proteins, such as ovalbumin and lysozyme ([Qiu et al., 2012a,b](#)).

16.5 Egg White Proteins in Other Egg Compartments

Although yolk is accumulated in the ovaries before egg white components are secreted and assembled in the magnum section of the oviduct, many major egg white proteins, such as ovalbumin or ovotransferrin, were identified in early studies of the yolk ([Marshall and Deutsch, 1951](#)). Such reports were subsequently confirmed by more recent proteomic studies of egg yolk proteins in various yolk fractions ([Mann and Mann, 2008](#); [Nilsson et al., 2008](#); [Farinazzo et al., 2009](#)). The most abundant egg white protein, ovalbumin, was also among the most abundant yolk proteins. Other major egg white proteins, such as ovotransferrin, cystatin, and lysozyme were much less abundant in yolk. Except for lysozyme, all egg white proteins were more abundant in the soluble, plasma fraction of yolk than in the granular fraction. At present it is not known whether egg white proteins have any function in yolk and how they enter yolk during egg assembly in the oviduct.

Less surprisingly, egg white proteins were also identified in the vitelline membrane, which is in direct contact to egg white. Fifty-five of the 137 proteins identified in vitelline membrane preparations by high-throughput mass spectrometry-based proteomic analysis ([Mann, 2008](#)) were also identified in egg white. The most abundant vitelline membrane proteins were the

major egg white proteins ovalbumin and lysozyme C. The latter had already been identified as major vitelline membrane protein previously after purification by gel filtration and identification by enzyme assays (De Boeck and Stockx, 1986). The observed overlap between egg white and vitelline membrane proteomes may be due to the spatial and temporal continuity of outer vitelline membrane and inner thick egg white formation in the infundibulum as described by Richardson (1935) in fowl oviduct and Rahman et al. (2007) in quail oviduct. The latter authors also identified ovalbumin in the outer vitelline membrane of quail.

The major egg white proteins ovalbumin (Hincke, 1995), lysozyme C (Hincke et al., 2000), and ovotransferrin (Gautron et al., 2001) were also identified as major chicken eggshell organic matrix components. Analysis by immunohistochemistry methods localized these proteins within the calcified eggshell matrix showing that these proteins were not surface contaminants that could have been removed by thorough cleaning of shells. The presence of egg white proteins in the calcified layer of the eggshell of chicken and other species was confirmed by several proteomic studies (Mann et al., 2006; Mikšík et al., 2010; Mann and Mann, 2013, 2015; Mann, 2015), showing that most egg white proteins also occur in eggshell. Many egg white proteins were also identified in other eggshell compartments such as the cuticle (Rose-Martel et al., 2012; Mikšík et al., 2014) and eggshell membranes (Ahlborn et al., 2006; Rose-Martel et al., 2015; Cordeiro and Hincke, 2016). In addition, these proteins were also detected in the uterus fluid, the medium where the eggshell is assembled (Sun et al., 2013; Marie et al., 2015). The source of egg white proteins in eggshell could principally be at least twofold. These proteins could for instance be leftovers from egg white assembly in the magnum and migrate with the unfinished egg into the isthmus and shell gland to eventually become incorporated into the shell during shell assembly. Alternatively, isthmus and uterus cells could also produce at least certain egg white proteins. In fact lysozyme and ovotransferrin message expression was detected in isthmus and uterus at very low levels as compared to magnum (Hincke et al., 2000; Gautron et al., 2001). At present it remains unknown whether these messages are translated into secreted protein and, if so, what percentage of the shell egg white proteins is derived from this source. Because of the high abundance of specific egg white proteins in the eggshell matrix it has been proposed that these proteins play a role in the mineralization process. However, in vitro calcium carbonate precipitation assays and crystal structure analysis indicated that such an effect is at best marginal, when compared to the results obtained with eggshell-specific matrix proteins (Hernández-Hernández et al., 2008). Thus the role, if any, of these proteins in eggshell assembly remains unknown at this time.

16.6 Proteomics Applied to Posttranslational Modifications of Egg White Proteins

Many egg white proteins were shown to bear posttranslational modifications previously, but at present there are no published proteomic surveys of egg white protein modifications. However, analysis of the phosphoproteome of the chicken eggshell matrix (Mann et al., 2007) confirmed

the previously determined phosphorylation sites of ovalbumin (Henderson et al., 1981) and cystatin (Laber et al., 1989) isolated from egg white. Another important posttranslational modification, N-glycosylation, was explored using mass spectrometry-based proteomic tools applied to isolated egg white proteins, such as ovomucin (Offengenden et al., 2011), lysozyme (Asperger et al., 2015), and ovomacroglobulin (Geng et al., 2015). Sixteen N-glycosylation sites were identified in α -ovomucin and two in β -ovomucin by analyzing tryptic digests of PNGase F-treated and untreated ovomucin SDS-PAGE bands. The eluted peptides were analyzed using LC-MS/MS and N-glycosylation sites were identified by the mass increase of 0.98 Da resulting from the conversion of asparagine to aspartic acid upon deglycosylation and by the presence of diagnostic glycan ions in spectra of the corresponding glycosylated peptide forms. Using similar methods, Asperger et al. (2015) detected partial glycosylation of lysozyme at two sites not corresponding to the canonical N-X-S/T N-glycosylation site. Instead, N-glycans were attached to the asparagines of \underline{N}_{44} RN and \underline{N}_{39} TQ. Deglycosylation was achieved with PNGase F in 18 O water to unequivocally identify the newly formed aspartic acid residues by a mass increment of 2.99 Da. Geng et al. (2015) analyzed ovomacroglobulin (ovostatin) peptides obtained by digestion with different proteases (to increase sequence coverage) after deglycosylation of the protein with PNGase F. They identified 12 N-glycosylation sites by searching for peptides with deamidated canonical NX(S/T) N-glycosylation consensus sequences. N-glycan heterogeneity was demonstrated by MALDI-TOF MS and MS/MS of released permethylated glycans (Offengenden et al., 2011), by MALDI- and ESI-MS/MS analysis of glycopeptides combined with searching for glycan fragments (Asperger et al., 2015) and by 2-DE separation of protein glycoforms (Geng et al., 2015).

16.7 Proteomic Analysis of Egg White Allergens in Food

The egg white proteins ovomucoid (Gal d1), ovalbumin (Gal d2), ovotransferrin (Gal d3), and lysozyme (Gal d4) are among the most common causes of food allergy (Mine and Yang, 2008; Benedé et al., 2015), with symptoms upon ingestion by hypersensitive individuals ranging from harmless skin reactions to life-threatening anaphylactic shock. Due to the widespread use of egg components in the food industry, egg white proteins can also occur by cross-contamination in food not normally expected to contain these allergens. The most widespread methods to date to detect and quantify such traces of food allergens are enzyme-linked immunosorbent assays (ELISA) that are easy to use, inexpensive, and commercially available for many, but not all, food allergens. However, because of the disadvantages possibly associated to antibody-based assays, such as cross-reactivity, presence of interfering food components, altered reactivity to processed allergens, and lack of multiplexing possibilities, new methods have been explored (Cucu et al., 2013). A promising approach to avoid the drawbacks of ELISA seems to be mass spectrometry-based detection and quantification of allergens (Koeberl et al., 2014). Application of mass spectrometry to the detection of egg white proteins in various food matrices, such as wine, pasta, and bread, demonstrated the

potential of the method (Lee and Kim, 2010; Heick et al., 2011; Tolin et al., 2012; Azarnia et al., 2013; Cryar et al., 2013; Monaci et al., 2013; Pilolli et al., 2014).

When a known set of proteins, such as the egg white allergens, needs to be detected and quantified accurately and reproducibly in samples of different origin and complexity, the proteomic method of choice seems to be selected reaction monitoring (SRM; Picotti and Aebersold, 2012; Koeberl et al., 2014). In this technique selected so-called signature peptides (sequence-unique peptides occurring only in the proteins to be analyzed) are first isolated from the mixture of peptides introduced into the mass spectrometer. Only the molecular ions of these selected peptides are then passed on for fragmentation. Finally, selected specific fragments are measured. Quantification can be achieved by adding a known amount of isotope-labeled signature peptides to samples. Signature peptides have already been identified for ovalbumin and lysozyme and were used to detect these allergens in wine by an SRM approach (Monaci et al., 2014; Pilolli et al., 2014). It remains to be seen whether such methods gain widespread acceptance and diffusion in routine analysis laboratories.

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SECTION 3

Application to Aquaculture

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Proteomics in Aquaculture: Quality and Safety

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17.1 Proteomics Technologies Applied to Aquaculture

Historically two-dimensional gel electrophoresis (2-DE) was the most widely employed proteomics methodology, because it is simple, reliable, and relatively affordable when compared to other methodologies. This technique is based on the protein separation by isoelectric focusing (IEF) followed by molecular mass protein separation. Protein spots from 2D gels are selected for protein identification by mass spectrometry. After selection, the protein gel plugs are enzymatically “in-gel” digested, usually with trypsin, and further analyzed by mass spectrometry (MS). Protein identification is based on either peptide mass fingerprinting (PMF) or by peptide fragmentation and sequencing by tandem mass spectrometry (MS/MS). Despite its wide use, conventional 2-DE has a limited potential to perform high-throughput proteomics and for protein quantitation; moreover, it lacks the sensitivity required to detect low abundance proteins, including many key cell signaling elements and transcription factors. Several strategies have been developed to increase its sensitivity. For example, subcellular proteomics focusing on organelles highly sensitive to environmental stressors such as peroxisomes has been applied to evaluate the effects of classic pollutants (Apraiz et al., 2009). On the other hand, a method based on liquid chromatography (LC) coupled to 2-DE was developed for aquatic biomonitoring (Amelina et al., 2007). This methodology was applied to assess the impact of oil spills in water quality and aquaculture production (Apraiz et al., 2011). Another strategy based on 2-DE with complementary purification techniques led to the comprehensive profiling of glutathione S-transferase isoforms in bivalve mollusks (Martins et al., 2014). Gel-based proteomics has proved also to be effective in noninvasive analyses of aquaculture biofluids by 1D fractionation (Nynca et al., 2014), 2-DE (Braceland et al., 2013), or by shotgun proteomic approaches (Campos et al., 2015). Recently proteomics analysis of the European sea bass mucus (*Dicentrarchus*

labrax) has been presented as a strategy that could be applied in disease diagnosis (Cordero et al., 2015). The protein expression changes of oyster hemolymph have been applied to evaluate the impact of metal pollution on Sydney Rock oysters (*Saccostrea glomerata*) and its consequences (Thompson et al., 2012).

Two-dimensional difference in gel electrophoresis (2D-DIGE) improved the sensitivity and analytical power to 2-DE by incorporating chemical labeling into the conventional methodology. The 2D-DIGE approach was earlier introduced to evaluate the effects of xenobiotics in aquaculture species (Apraiz et al., 2006) and later implemented in aquaculture research to investigate respectively the effects of chemotherapeutics on giant tiger shrimp (*Penaeus monodon*) (Silvestre et al., 2010) or the effects of perfluorooctane sulfonate exposure in the European eel (*Anguilla anguilla*) blood mononuclear cells (Roland et al., 2013).

In the last years, high-throughput proteomics approaches based on online LC coupled with MS have been progressively implemented. Modern and ultrasensitive mass spectrometers enable the high-throughput mass spectral analysis of the peptide products of thousands of protein components in a single sample. Mass spectrometers are also highly effective in protein quantitation and posttranslational modification (PTM) analysis, thus enhancing the completeness of proteome analysis. In LC-MS-based approaches usually the “in-solution” protein extract is enzymatically digested, generally with trypsin. The resultant peptides are separated by C18 reverse-phase LC chromatography and analyzed by MS (bottom-up or shotgun proteomics). Multidimensional LC separations may be performed combining reverse-phase with strong cation exchange or hydrophobic interaction LC. Such methodology has been for instance applied to the comparative proteomics of developing fish (zebrafish and gilthead seabream) (Ziv et al., 2008). Shotgun proteomics is a versatile methodology employed for protein identification and for systematic profiling the dynamics of proteomes. This methodology is now well established in aquaculture research, strategies combining SILAC (stable isotope labeling by/with amino acids in cell culture), ICAT (isotope-coded affinity tag), iTRAQ (isobaric tag for relative and absolute quantitation) labeling, or label-free strategies have been employed mostly in biomarker discovery investigations, leading to the identification of new protein candidates with diagnostic, prognostic, and therapeutic values (Booy et al., 2005; Hou et al., 2016; Nolte et al., 2015; Piovesana et al., 2016). One increasingly popular MS-based protein quantification approach relies on selective quantification of surrogate peptides in a digested protein sample being referred to as multiple or selective reaction monitoring (MRM or SRM). The MS-based targeted proteomic methods have been employed for the accurate measuring of protein targets and in many cases serve as tools to confirm/validate shotgun data. Targeted proteomics was used for instance to validate population-specific plasma proteins in the marine and freshwater three-spined sticklebacks revealed by label-free proteomics (Kultz et al., 2015). Groh developed a targeted proteomics method based on SRM for quantitation of protein expression in zebrafish during gonad differentiation (Groh et al., 2013).

High-sensitive mass spectrometry is also employed for the characterization of PTMs. Simple modifications, such as acetylation, formylation, or phosphorylation, or highly complex PTMs such as glycosylation are enabled with MS. Mucin O-glycosylation on freshwater acclimated Atlantic salmon has been characterized by LC-MS/MS (Jin et al., 2015). Likewise, the complex PTM profiling of mussel adhesive proteins have been investigated (Zhao et al., 2009).

17.2 Proteomics to Evaluate Quality of Aquaculture Species

17.2.1 Proteomics in Seafood Quality

Quality assessment in aquaculture deals with subjects such as (1) geographical origin, traceability, and authentication; (2) production processes; (3) storage and processing; (4) sensory and nutritional qualities; and (5) functionality. There is a distinctive development in this area of research corresponding to the various products from aquaculture. Fish products have received increased attention and more research in quality assessment in comparison to food products from produced crustaceans and mollusks. These differences are also reflected in the number of proteomics studies on the subject.

With regard to product authentication classical methods proved to be insufficient, for instance, to differentiate among Penaeidea shrimp species owing to their phenotypic similarities and to the frequent removal of external carapace during processing. Proteomics approaches based on 2-DE or selected tandem mass spectrometry ion monitoring (SMIM) were used as complementary authentication methodologies for the identification and authentication of commercially relevant shrimp species (Ortea et al., 2009; Pascoal et al., 2012). The characterization of species-specific peptides and proteins demonstrated the adequacy of these approaches for seafood product authentication allowing the differential classification of several commercial, closely related, shrimp species. A comprehensive review of the application of proteomics in the authentication of seafood species is the subject of [Chapter 20](#).

The presence of allergenic proteins has been a critical determinant of the quality of crustacean food products. The major allergen identified in crustaceans is the protein tropomyosin. This protein reacts strongly with human IgG/IgE and triggers a concomitant immune response that generates allergenic symptoms. Proteomic profiling of the allergen tropomyosin with specific monoclonal antibodies was used to analyze the presence and allergenic potential of the protein in shrimp and mollusks (Kamath et al., 2013). The investigation showed for instance that heating of shellfish meat enhanced recognition of multiple tropomyosin variants in the analyzed shellfish species, pointing to a possible alteration of allergenicity potential resulting from this treatment (Kamath et al., 2013). In contrast, a protein profiling study showed that high pressure steaming reduced the levels of tropomyosin in shrimp (*P. monodon*) most likely due to protein degradation therefore reducing the allergenicity of tropomyosin compared with other heat treatments methods (Lasekan and Nayak, 2016).

Gel-based methods were employed to investigate protein quality and digestibility in squid fillet that was treated with high hydrostatic pressure (HHP) and different drying methods (Deng et al., 2015; Zhang et al., 2016). HHP treatments modified the protein and functional properties of squid, and decreased the allergenicity of tropomyosin Tod p1 by protein unfolding and secondary structure modification, thus providing potential for alleviating allergenicity of squid (Jin et al., 2015). Complementary biochemical and proteomics methods were employed to characterize the chymotrypsin from the midgut gland of yellow-leg shrimp, *Penaeus californiensis*, for possible biotechnological applications, food processing, and control of post-mortem muscle softening and melanization (Navarrete-del-Toro et al., 2015).

17.2.2 Proteomics in Fish Quality

Fish quality is mostly considered as the “fish eating quality” presented to the consumer and can be influenced by many factors that have been addressed using proteomics technologies. These factors can be divided in two different categories: (1) fish development and production stages, that is, during the ante-mortem period (influenced by genetics, diseases, nutrition, temperature, stress, and slaughter method); and (2) fish processing techniques, that is, during the post-mortem period (influenced by time, storage temperature, and processing techniques). The latter topic is dealt with in-depth in [Chapter 20](#). Also another approach related to the quality that needs to be considered is the traceability (origin) and authentication of fish products (Berrini et al., 2006; Carrera et al., 2006; Martinez and Jakobsen Friis, 2004; Martinez et al., 2007; Mazzeo et al., 2008; Piñeiro et al., 2001; Volta et al., 2012). The identification of commercial fish species by proteomics is covered in [Chapter 21](#). Proteomics approaches in fish quality have been mostly applied in farmed fish as a monitoring tool to understand the biological mechanisms related to quality changes, in order to present better quality farmed fish and to increase its acceptance by consumers.

During the ante-mortem period fish welfare can definitely play a key role in fish quality treats. Fish welfare is a complex concept that needs a multidisciplinary and holistic approach in its studies. Welfare is also many times strictly related to stress. Nevertheless stress responses will not provide all the necessary information about fish welfare, since in aquaculture this last one is largely associated with tertiary effects of stress response that are generally indicative of prolonged, repeated, or unavoidable stress (Barton, 2002) that is mostly related to maladaptive effects on growth, reproductive function, immune function, and disease resistance (Ashley, 2007). Welfare can in this sense be directly related to most factors that affect fish quality in the ante-mortem period. Several proteomics studies related to welfare have been published in the past few years with emphasis in organs like liver, blood plasma, brain, skeletal muscle, and the osmoregulatory and immune-related organs and tissues. Preslaughter intense muscular activity (Morzel et al., 2006), crowding (Veiseth-Kent et al., 2010), stress

(Alves et al., 2010; Silva et al., 2012), and slaughter techniques (Addis et al., 2012) have been evaluated revealing several fish metabolic pathways being affected by production processes. Diseases involving skeletal malformations are one of the main constraints of commercial aquaculture production with a significant influence on the quality of fish. Proteomics has been employed in order to better understand the mechanisms involved in these skeletal deformities (Silva et al., 2012). Also, in the context in which animal and human health are intertwined aspects of the one-health concept (Marco-Ramell et al., 2016), it is of utmost importance to define markers for stress and welfare. Some metabolic molecular indicators of chronic stress have already been proposed in gilthead bream, *Sparus aurata*, using a comparative proteomics approach (Alves et al., 2010).

Undoubtedly, nutrition plays a main role in fish quality with possible effects on muscle properties that can be in many cases noticeable to consumers. Initially proteomic studies reported the effects at the cellular level on seabream (*S. aurata*) after feeding with feed additives included in the diet to stimulate growth in many fish farms (Rufino-Palomares et al., 2011). A major challenge has been posed to the aquaculture industry over the latest decade with the increasing demand for more sustainable alternatives to the traditional fish diet formulations rich in fishmeal and fish oil (Morais et al., 2012). New fish diets formulations are emerging in the market, richer in new protein sources like the vegetable substitutes with encouraging results for some species such as rainbow trout (Vilhelmsen et al., 2004). Specific enriched diets have also been formulated with the objective of mitigating disease effects in fish as reported by Silva et al. (2012). Proteomics can play an important role in understanding the influence of feed composition on fish metabolism with many studies undertaken in serum, liver, muscle, and other tissues. In the last decade studies have successfully reported the use of this technology in fish nutritional studies (Brunt et al., 2008; de Vareilles et al., 2012; Martin et al., 2001, 2003; Matos et al., 2013; Silva et al., 2012).

The *post-mortem* period is mostly correlated with fish processing techniques and storage. Storage period and temperature have been correlated to several changes in fish muscle proteins abundance and/or oxidation modifications (e.g., carbonylation, thiol oxidation, and aromatic hydroxylation) pointing to proteomics as a useful tool for monitoring the freshness of fish and the quality of storage conditions (Bauchart et al., 2007; Kinoshita et al., 2007; Kjaersgaard et al., 2006; Terova et al., 2011; Verrez-Bagnis et al., 2001). The effect of post-mortem processing techniques was also subjected to proteomics studies that revealed changes associated with muscle proteins composition. In processes such as HHP treatment (Ortea et al., 2010), washing procedures (Martinez et al., 1992), lactic fermentation (Morzel et al., 2000), or canning (Sanmartín et al., 2012), changes in protein abundance and/or degradation were observed. Additionally, the effect of antioxidants, citrate, and cryoprotectants were shown to influence muscle texture in Atlantic cod, *Gadus morhua*, with observations of protein degradation (Badii and Howell, 2002).

Table 17.1: Proteomics in fish quality, information regarding the parameters: tissue, species, and main affected proteins.

| Factors affecting fish quality | Species | Sample | Main proteins identified | References |
|--------------------------------|--|--------------|---|------------------------------------|
| Traceability | European perch, European pikeperch, Nile perch, and sunshine bass | White muscle | Parvalbumin, tropomyosin, actin | Berrini et al. (2006) |
| | Cod, saithe, haddock, mackerel, capelin, Arctic charr, arctic and tropical shrimps | Muscle | Actin, tropomyosin, myosin light chains (MLCs) | Martinez and Jakobsen Friis (2004) |
| | Cod and klipfish | White muscle | Actin, tropomyosin, myosin heavy chains | Martinez et al. (2007) |
| | Hake | White muscle | Parvalbumin, nucleotide diphosphate kinase A | Pineiro et al. (2001) |
| Authentication | Hake (<i>Merluccius</i>) and grenadiers (<i>Macruronus</i>) | White muscle | Parvalbumin | Carrera et al. (2006) |
| | Seabass, seabream, perch, tilapia, cod, hake, halibut, plaice, and sole between others | Muscle | Parvalbumin | Mazzeo et al. (2008) |
| Preslaughter activity | Rainbow trout | White muscle | Triosephosphate isomerase, enolase, pyruvate dehydrogenase, desmin, cap-Z, myosin heavy chain | Morzel et al. (2006) |
| Crowding | Atlantic salmon | Muscle | Actin, myosin, heavy and light chains, tropomyosin, creatine kinase, enolase, phosphoglycerate kinase | Veiseth-Kent et al. (2010) |
| | | Blood plasma | Apolipoprotein A-I, angiotensinogen, complement component C3 | |
| Stress | Gilthead seabream | Liver | Fatty acid-binding protein, heat shock cognate protein, calmodulin, pyruvate dehydrogenase, voltage-dependent anion channel | Alves et al. (2010) |
| | Gilthead seabream | Muscle | Creatine kinase, adenylate kinase, nucleoside | Silva et al. (2012) |

Table 17.1 presents a summary of the factors important in fish quality, the species studied, the fluid/organ/tissue examined, and the identities of the affected proteins for a selection of studies in fish quality.

Overall, many of the changes in fish quality are associated with changes in the muscle protein composition. The challenge will be to develop standardized assays based on proteomics

technologies that can monitor quality parameters and provide the consumer with accurate and reliable information.

17.3 Proteomics to Assess the Potential Risks Associated to Aquaculture

17.3.1 Proteomics in Seafood Safety

Seafood safety is generally associated with the prevention and control of risks associated to the consumption of seafood. The increase of seafood consumption has been correlated with an increase in seafood-related health risks: infections mainly caused by virus, bacteria, and parasites, or risks associated to transferring toxin or pollutants through the food chain. Moreover scientific reports indicate that the risk of occurrence of contaminated seafood is equally distributed in developed or developing countries although the consequences can be mitigated in areas with quick accessibility to health care (Bellou et al., 2013).

Mollusks are filter feeding species and their daily nutrition requires filtration of several liters of water per day. Therefore bioaccumulation of pollutants including toxins, metals, or chemicals and has been extensively reported (Jitar et al., 2015). Initially, proteomics has been extensively utilized for environmental assessment and marine pollution monitoring (Mi et al., 2005). The changes in protein expression from sentinel organisms residing in the monitoring areas provide both spatial and temporal information about the water quality (Amelina et al., 2007; Apraiz et al., 2009). Therefore quantitative proteomic analyses have become the method of choice to evaluate bioaccumulation and safety. Comparative proteomic analyses from tissues of molluscan species exposed to polluted areas have provided a global understanding of the impact of the bioaccumulation at the cellular level. The risk of mercury (Hg) contamination through the food chain has been studied in a laboratory experiment that mimicked the entire food chain from HgCl_2 to a unicellular green algae, *Chlorella vulgaris*, to oysters, and finally mice. The quantitative proteomic analysis could decipher the mechanism of Hg toxicity in the oyster gonad and correlate the toxicity detected in oyster-fed mice indicating that proteomics studies of whole food chain represents a useful tool for seafood safety (Zhang et al., 2013). Recently, the identification of over 2000 proteins in the blue mussel, *Mytilus edulis*, was achieved by applying a combination of shotgun proteomic analysis and proteogenomics. This strategy may be applied to the study of the many marine species for which complete annotated genomes are currently unavailable. Quantitative proteomic analysis revealed the molecular mechanisms driving the adaptation to low salinity stress and how salinity, a possible northern scenario resulting from climate change, modulates the effects of exposure to pollutants (Campos et al., 2016). The first shotgun proteomics analysis of oyster hemolymph after exposure to heavy metal was performed before the oyster genome sequence was released. The quantitative analysis revealed molecular biomarkers of exposure. These studies relied on the construction of an in-house *Bivalvia* database and required manual curation, during the quantification by a spectral counting approach, for

shared and unique peptides between proteins from similar species (Thompson et al., 2011). Characterizing *Mytilus* sp. hemolymph has been pursued for many years starting with low resolution techniques (Galloway et al., 2002), before moving to gel-based proteomics and finally to high-resolution MS. Applying MS-based proteomics techniques around 600 proteins from *M. edulis* have been identified. The method combined analysis by nano-LC-MS/MS with a proteogenomic strategy for protein identification. This systematic analysis reported a network of the elements of innate immunity in the marine mussel *M. edulis* (Campos et al., 2015).

The detection and identification of bacterial pathogens present in seafood is one of the primary goals to improve seafood quality and safety. MALDI-TOF MS analyses allowed researchers to obtain reproducible mass spectral fingerprints of the gram-negative bacteria, *Vibrio parahaemolyticus* which is a causative agent of gastrointestinal illness in humans following consumption of infected seafood. The generated fingerprints could be used to discriminate between the phylogenetically diverse *Vibrio* strains (Hazen et al., 2009). Later MALDI-TOF MS applications have been developed that provide reference libraries for the spectral fingerprints from diverse seafood pathogens and strains. Reference spectral library from 32 reference strains were created and peak masses were assigned as genus-specific and specific-specific biomarkers for rapid bacterial identification. *Vibrio* sp. has been also associated to molluscan mortality, including the most frequent disease associated with oysters. Proteomic analysis based on SDS-PAGE and protein identification by nano-LC-MS/MS were performed to identify the potential virulence factors in the *Vibrio* secretomes (Madec et al., 2014).

Quantitative proteomics has been applied to elucidate the species-specific molecular responses to bacterial infection. A gel-based quantitative analysis of differentially expressed proteins from the lymphoid organ of *P. monodon* after *Vibrio harveyi* infections was reported (Chaikeeratisak et al., 2012). A combined proteomic and metabolomics analysis of the digestive gland of the Mediterranean mussel, *Mytilus galloprovincialis*, infected by gram-positive or gram-negative bacteria provided insights at the protein level (Wu et al., 2013). More recently, shotgun proteomic analysis of *Vibrio metschnikovii* under cold stress conditions has provided a higher coverage of this proteome. Considering that *V. metschnikovii* is a food-borne pathogen found in seafood and long storage under cold is standard procedure for preservation, this methodology could reveal key players involved in this response (Jia et al., 2015). Virus infection can drastically reduce the population of seafood in aquaculture farms. The white syndrome spot virus is one of the most important shrimp pathogens. By applying a gel-based proteomic analysis to shrimp hemocytes exposed to spot syndrome virus, the molecular response was assessed and immune-related proteins were identified (Li et al., 2014). Algal blooms can produce paralytic shellfish poisoning toxins that negatively affect aquaculture industries. Gel-based proteomic methods were explored to identify and distinguish the nontoxic from the toxic phytoplankton

species (Chan et al., 2005, 2006). Protein expression signatures from *M. edulis* exposed to AZA toxins were identified applying SDS-PAGE followed by protein identification by MALDI-MS/MS and ESI-MS/MS (Nzouhuet et al., 2009).

Finally, antibiotic resistance is a major global threat for human and environmental health today. The increasing utilization of antibiotics in aquaculture has emerged as an additional load of antibiotics in the environment (Amos et al., 2014). The aquatic environment is therefore an important reservoir of antibiotic resistance genes (ARGs) and a route for their dissemination and potential transmission to human pathogens. Metagenomics has been applied for establishing a correlation between the resistome and the metagenomics data (Ju et al., 2016). Therefore the transfer of ARGs from natural microbiota to human pathogenic bacteria could pose an increased health risk to humans. Standard methods for assessing water quality has been applied to marine sediment close to aquaculture farms and found that the native resistome has been enriched by antibiotic use at the fish farm (Muziasari et al., 2016). However, those methods are limited to a set of genes. Metaproteomic analysis, the large-scale characterization of the entire protein complement of an environmental sample at a given time, could solve that limitation (Rodriguez-Valera, 2004). The application of this methodology to bacterial communities in freshwater sediments has revealed new insights into the adaptation of the community structure to high metal levels (Gillan et al., 2015). Finally, metaproteomics coupled to stable isotope probing (SIP) from those environmental communities enables the characterization of microbial assemblage through identifying and quantifying the enrichment of isotopically labeled proteins (Jehmlich et al., 2008). Environmental metaproteomics offers opportunities to improve our understanding and management of microbial ecosystems and their global impacts (Fig. 17.1).

17.3.2 Proteomics in Fish Safety

Fish safety is related to the potential human health hazards caused either by fraudulent practices like fish substitution, fish diseases or contamination, and fish allergens. Several regulations related to fishery products aimed to protect consumers against some of these health hazards have been released. Among those, European Union, Directives EC No 104/2000 and 2065/2001 regulate labeling characteristics and EC No 2073/2005 focus on regulation of microbiological contamination. Labeling has become an important feature of a seafood product and it must include the commercial designation, scientific species name, production method (wild or farmed), and geographical origin. In order to invigilate the information stated, suitable detection methods must be put into place. Proteomics can play an important role in providing an accurate, reliable, sensitive, and specific control method. In the case of fish authentication, there are several works reporting the use of proteomics-based methods (Mazzeo and Siciliano, 2016; Nessen et al., 2016). The identification of unique species biomarkers has been conducted by Carrera et al. (2010) (described in detail in Chapter 21), where authors carried out the de novo sequencing of 25 new parvalbumins

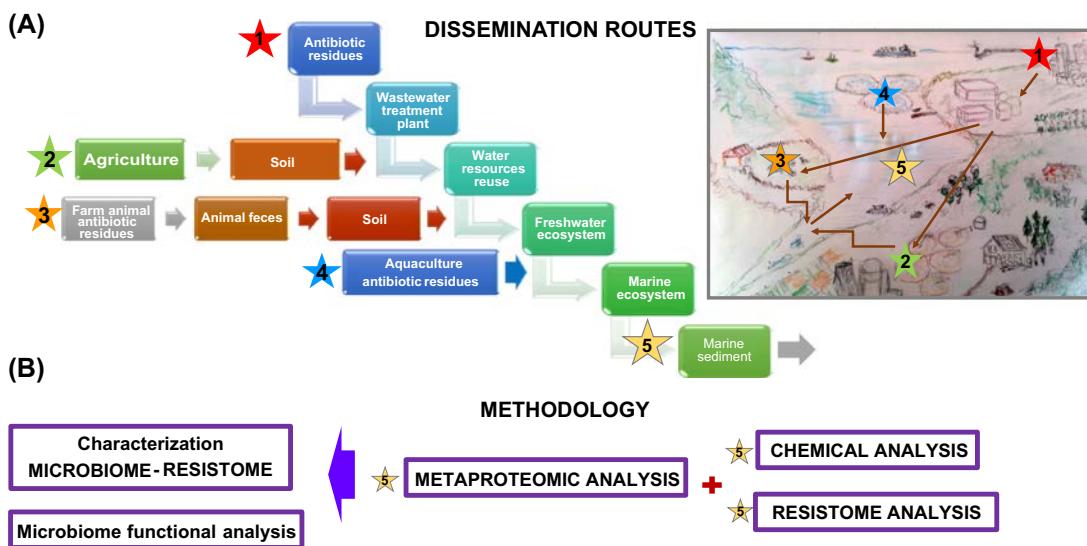


Figure 17.1

Workflow for tracking antibiotic resistance through the dissemination routes from water to aquatic sediments by metaproteomic analysis.

(PRVBs) discriminating 11 different species from the Merlucciidae family and two subspecies belonging to *Macruronus novaezelandiae*. The methodology described in the paper was validated in 10 commercial hake products (Carrera et al., 2011) and also provides an alternative method for fish parvalbumin detection (Carrera et al., 2012). Similarly Mazzeo et al. (2008) successfully applied proteomics to identify 25 different fish species from three different orders (Gadiformes, Perciformes, and Pleuronectiformes) describing specific PRVB peptide masses for each species. More recently, a shotgun proteomics approach was used to characterize the edible muscle of gilthead seabream, revealing quantitative differences in PRVB between farmed and wild animals, with this protein being overexpressed in farmed fish samples (Piovesana et al., 2016).

Microbial activities in fish can cause a process denominated fish spoilage resulting in biochemical changes and are one of the main reasons for fish food poisoning. As mentioned before, MALDI-TOF MS methods have been developed to detect and identify bacterial pathogens present in seafood and those methods are also applicable to fish safety. Gram-negative bacteria, including *Aeromonas hydrophila*, *Acinetobacter baumanii*, *Pseudomonas* spp., and *Enterobacter* spp. (Bohme et al., 2010, 2011a), and gram-positive bacteria *Bacillus* spp., *Listeria* spp., *Clostridium* spp., *Staphylococcus* spp., and *S. parauberis* (Bohme et al., 2011b; Fernandez-No et al., 2012) were identified by MALDI-TOF MS as the main species in seafood and fish spoilage, responsible for poisoning. Available online is a library containing the mass spectral fingerprints of the main spoilage and pathogenic bacteria species from seafood (<http://www.spectrabank.org>).

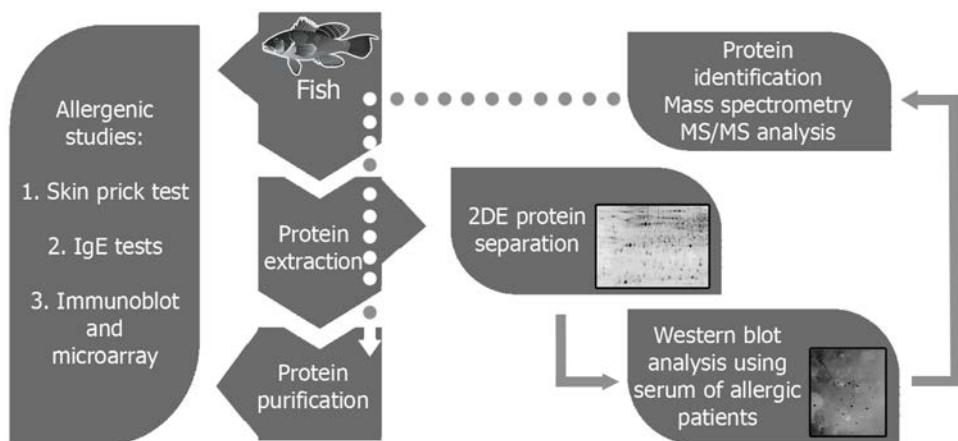


Figure 17.2
Workflow for fish allergen characterization with proteomic tools.

Proteomics can be an important tool with regard to the purification and identification of fish allergens. A possible workflow using this technique for fish allergen characterization is shown in [Fig. 17.2](#). SDS-PAGE can be used to obtain purified allergens, while individual isoforms can be detected by 2-DE, HPLC, or capillary electrophoresis and molecular masses accessed by mass spectrometry using MALDI or ESI as the ion source. A fast detection method for fish allergen detection using SMIM in a linear ion trap (LIT) mass spectrometer was proposed by [Carrera et al. \(2012\)](#). In recent years, proteomics-based approaches have been applied to the identification of novel fish allergens ([Tomm et al., 2013](#)) and the characterization of the potential allergenicity of transgenic and nontransgenic fish ([Nakamura et al., 2009](#)).

17.4 Future Perspectives

Proteomics-based strategies have demonstrated their potential to offer a holistic view of welfare, safety, and traceability of species of interest for aquaculture. However, for species of interest to aquaculture only a few fully sequenced genomes are available. The technical developments in the genomics have been associated with development of high-throughput sequencing facilities and reduction of sequencing costs. Therefore it is anticipated that shotgun proteomic studies combined with genomic sequencing attempts will facilitate in-depth studies of aquaculture species. In particular, the development of noninvasive methodologies using fish plasma or serum and seafood hemolymph that could combine high-resolution and high-throughput methods to monitor diverse parameters associated with quality and/or safety is a direction of high interest. Recently, exploration of the earth microbiome has started and it is becoming more accessible ([Alivisatos et al., 2015](#)). Several methodological platforms have been developed ([Mesuere et al., 2015](#); [Muth et al., 2015](#); [Sachsenberg et al., 2015](#)) that could facilitate a deeper understanding of the dynamics of the microbial communities from water, sediments, and gut microbial communities as a tool to evaluate

aquaculture welfare and safety. Proteomic research in aquaculture with environmental sustainability as a key goal is actively seeking methodologies that will enable the implementation of sector management strategies and preserve environmental quality thus protecting human health.

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Proteomics to Assess Fish Quality and Bioactivity

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18.1 Introduction

Fish is considered an important part of the diet due to its content of proteins, vitamins, and minerals, but also provides a source for the intake of the long-chain polyunsaturated fatty acids (PUFAs) eicosapentaenoic (EPA; 20:5 n-3) and docosahexaenoic (DHA; 22:6, n-3) and has proven bioactive effects in the prevention and treatment of coronary heart diseases (Venugopal and Shahidi, 1996). The healthy effect of fish and marine long-chain PUFA lipids consumption was firstly pointed out when Bang and Dyerberg observed the low incidence of cardiovascular diseases and diabetes mellitus among Greenland Eskimo population, despite the high content of fat in their diet (Bang et al., 1976, 1971). The fatty acid pattern of the consumed lipids by Greenlanders was characteristically enriched in marine long-chain n-3 PUFAs as a result of the intake of high amounts of fish and marine animals. Different experiments with animal models and clinical interventions have also evidenced that marine long-chain n-3 PUFAs have antiinflammatory properties and, therefore, might be useful in the management of inflammatory and autoimmune diseases (Simopoulos, 2002). In addition to the long-chain n-3 PUFAs, seafood (fish and shellfish) is also recognized to be particularly enriched in other bioactive constituents: such as peptides with antihypertensive and hypoglycemic action (Lassoued et al., 2015); selenium that has been linked to cancer protection (Garcia-Rodriguez et al., 2012); and taurine that is suggested to have potential protective effects on cardiovascular diseases (Wójcik et al., 2010).

Global production of seafood products has grown significantly in the last decades, mainly due to the pronounced geographical expansion of aquaculture (FAO, 2014). In fact, world fish aquaculture expansion outpaces that of the other food-producing industries with a production volume more than doubled from 32.4 million tons in 2000 to 66.6 million tons in 2012. Moreover, fish production used for direct human consumption has been growing up to 73%, 81%, and 86% in the 1990s, the 2000s, and 2012, respectively (FAO, 2014). This impressive development of fish production has been driven by a combination of population growth, major technological innovations that facilitate fish production, product integrity, and more efficient

distribution channels, and new scientific evidence of the beneficial role of fish products to maintain normal cellular metabolism and prevent the prevalence of certain diseases.

Fish and derived products are highly perishable leading to faster deterioration rates of quality compared to meat from terrestrial animals. The key differences between fish and terrestrial animals that directly translate into the quality issues are the high content of fish in very unsaturated lipids and heat-labile muscle proteins, the abundance of proteolytic enzymes, and the psychrophilic ability of the fish microbiota to survive and proliferate at low temperatures (Jacek et al., 2005). In this sense, appropriate production, handling, processing, preservation, packaging, and storage are essential to maintain quality, safety, and nutritional attributes in fish products and avoid waste and losses.

The term “proteomics” was firstly coined in 1994 by Marc Wilkins to refer to protein expression in a holistic manner (Wilkins and Appel, 2007). Nowadays, proteomics has extended its definition to “the large-scale analysis of the proteome” and the field of application covers from protein identification and quantification to the analysis of posttranslational modifications, protein–protein interactions, and protein function (Pandey and Mann, 2000). The progress of proteomics has been technology-driven by a combination of conceptual breakthroughs and technical advances in separations techniques, mass spectrometry, protein chemistry, and bioinformatics, which have transformed protein chemistry into the discipline of proteomics (Wilkins and Appel, 2007). Recently, proteomics has emerged as a reliable and sensitive analytical methodology to monitor specific issues that endanger quality and safety in fish and also to understand the mechanistic basis behind the beneficial effect of fish and its constituents to maintain the homeostatic balance and prevent diseases. The present chapter will give an overview of the diverse and distinctive information that proteomic strategies provide to evaluate and understand important issues concerning quality and bioactivity of food systems based on fish and seafood.

18.2 Proteomics to Evaluate Fish Quality

Resolution of rigor mortis in fish muscle is much faster than that of mammalian animals, and unlike meat, postmortem proteolytic tenderization provokes deteriorative changes in fish muscle that involve loss of firmness and elasticity, and increase in softness (Lougovois and Kyrana, 2005). As a result, postmortem tenderization is considered one of the most adverse alterations related to fish quality and freshness. Postmortem tenderization in fish muscle is linked to the activity of endogenous proteases on myofibrillar and structural proteins. The main factors affecting postmortem proteolytic tenderization in fish species are physical stress during capture and slaughtering, primary processing operations, and storage temperature.

Proteomics has been applied to characterize the effect of postmortem metabolism on muscle proteome. Kjaersgard and Jessen (2003) evaluated protein changes in cod (*Gadus morhua*)

muscle during ice storage by two-dimensional gel electrophoresis (2-DE). Results evidenced significant changes in 11 unidentified protein spots. In nine protein spots the intensity increased, and for eight of these the increases were significant within the first 2 h postmortem. In contrast, two protein spots showed significant decreases in intensity. The postmortem proteome of cod muscle was also evaluated during frozen storage by 2-DE and tandem mass spectrometry (MS/MS) analysis, revealing change in abundance of myosin light chains, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, aldolase A, two α -actin fragments, and a nuclelease diphosphate kinase B fragment (Kjaersgard et al., 2006b). In a two-dimensional (2D) difference gel electrophoresis (DIGE) and mass spectrometry study in sea bass (*Dicentrarchus labrax*) muscle, nucleoside diphosphate kinase B and phosphoglycerate mutase 2 were also significantly reduced under chilled storage (Terova et al., 2011). Furthermore, a proteomic approach based on 2-DE protein separation and MALDI-TOF/TOF identification revealed that arginine kinase, phosphopyruvate hydratase, and actin were downregulated in Pacific white shrimp (*Litopenaeus vannamei*) with the increase of storage time (Fu et al., 2014). The application of gel electrophoresis, combined with immunological protein detection, has also allowed identifying myofibrillar myosin heavy chain and α -actinin as relevant targets for postmortem alteration in shrimp species during ice storage (Martinez et al., 2001).

The study of muscle proteomes during postmortem aging has also been comprehensively used to evaluate the effect of preslaughter stress and physical activity on the quality of farmed fish. Silva et al. (2012) tracked muscle soluble proteome expression in postmortem gilthead seabream subjected to distinct levels of preslaughter stress by 2-DE and protein identification by MALDI-TOF/TOF mass spectrometry (MS). Analysis of the results indicated postmortem changes on several cellular pathways, including oxidative and proteolytic activity on sarcoplasmic proteins and degradation of myofibrillar proteins, processes that were hastened by conditions of preslaughter stress. A 2D DIGE approach was also applied to investigate the impact of three different slaughtering techniques (asphyxia in air, asphyxia in ice, and spinal cord severance) on the postmortem integrity of muscle tissue proteins in European sea bass (*D. labrax*) (Addis et al., 2012). This investigation concluded that slaughtering by severing the spinal cord preserves protein integrity better than death by asphyxia, either in ice or in air. In terms of single proteins, the myofibrillar myosin heavy chain and myosin binding protein C, the cytosolic nucleoside diphosphate kinase B, fructose biphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, and enolase 1 were significantly better preserved upon slaughtering by spinal cord severance as compared to asphyxia. Recently, an approach based on 1-DE Western blot combined with MS was able to identify creatine kinase a potential marker of burnt tuna, or yake-niku, which is a quality problem of the muscle characterized by a pale color, and exudative and softer texture, in large tropical tunas, particularly associated to exhausting exercise prior to death (Erdaide et al., 2016).

High hydrostatic pressure has shown to enhance shelf life and food safety in fish species by inhibiting microbial development, and to maintain quality by inactivating deteriorative hydrolytic (lipases and phospholipases) and oxidative (peroxidases and lipoxygenases) endogenous enzymes (Teixeira et al., 2013). 1-DE and mass spectrometry analysis of Coho salmon (*Oncorhynchus kisutch*) treated under 170–200 MPa for 30 s showed a partial loss of a band corresponding to 29 kDa, that was identified by MS/MS analysis as phosphoglycerate mutase (Ortea et al., 2010). Phosphoglycerate mutase was also pressure sensitive in frozen Atlantic mackerel (*Scomber scombrus*) previously treated at 150–200 MPa (Pazos et al., 2015a). The effect of a broad range of high-pressure pretreatments (150, 300, and 450 MPa for 0, 2.5, and 5 min) on myofibrillar and sarcoplasmic protein fractions was evaluated in frozen Atlantic mackerel (Pazos et al., 2014). Myofibrillar proteins were stable under pressurization treatment in terms of 1-DE and 2-DE profiles. However, high pressure exerted a selective effect on particular sarcoplasmic proteins depending on processing conditions. Protein identification by MS/MS showed that stability of creatine kinase, fructose-bisphosphate aldolase A, glycogen phosphorylase, and β -enolase was pressure sensitive from 300 MPa, whereas triosephosphate isomerase B, phosphoglucomutase, and phosphoglycerate kinase 1 were decomposed at 450 MPa. High pressure also induced the generation of a protein product of pyruvate kinase at 300 MPa and two products derived from tropomyosin at 450 MPa. A similar proteomics approach was used to evaluate the same high pressure processing conditions in horse mackerel (*Trachurus trachurus*) (Pazos et al., 2015b). As described previously for mackerel, high pressure processing changed the electrophoretic profiles for horse mackerel sarcoplasmic proteins when treated at 300 MPa or above, meanwhile, those from myofibrillar proteins remained mainly unaltered. The results highlighted that high pressure may provide degradation of specific sarcoplasmic proteins depending on pressure intensity and holding time conditions. Importantly, high pressure at 450 MPa also induced the formation of new protein bands assigned to tropomyosin α -1 chain, fast myotomal muscle troponin T, and parvalbumin beta 2.

Fish muscle is a matrix very susceptible to develop oxidative reactions of lipids, proteins, and vitamins that compromise quality and nutritional value during processing and storage. Until recently, most of the attention has been focused on lipid oxidation and its deleterious effects in the generation of rancid off-flavors and the loss of PUFAs and vitamins, although protein oxidation may cause adverse textural changes in muscle-based foods (Xiong, 2000). In fact, protein oxidation has shown negative impact on water-holding capacity, tenderness, and juiciness in meat-based foods (Clausen et al., 2009). However, the precise effect of protein oxidation on muscle protein functionality and texture depends on the extent of this posttranslational modification, and so, conditions of mild protein oxidation have shown improvements on certain functional properties such as hydration (Liu et al., 2010) and water-holding capacity (Xiong et al., 2010). In the beginning, the lack of suitable and specific methodologies to assess protein oxidation severely curtailed the study of the oxidative reactions of proteins in muscle foods. Among posttranslational oxidative modifications, protein carbonylation is the

most conventional marker of protein oxidation. Moreover, protein carbonylation has attracted a great deal of attention due to its irreversible and unrepairable nature, and its association with a large number of metabolic diseases and age-related disorders (Nystrom, 2005). Carbonylated proteins are targeted for proteolysis by the proteasome and the Lon protease, but can escape degradation and form cytotoxic high-molecular-weight aggregates that accumulate with certain diseases and age.

Protein carbonyls can be generated either by oxidation of the side chains of certain amino acids or by reaction of proteins with lipid oxidation products and advanced glycation end products (Hawkins and Davies, 2001; Miyata et al., 1998). The variety of oxidative mechanisms, combined with the elevated oxidative predisposition of fish meat and the huge number of proteins that can be targeted by oxidation, confers additional complexity to the development of protein carbonylation studies in fish. Traditionally, protein oxidation was assessed in muscle foods by a colorimetric measurement of the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone (DNP) (Lund et al., 2011). The direct measurement of the optical absorbance of DNP has reduced sensitivity and reproducibility, and more importantly, it provides a global averaged value of protein oxidation rather than individual levels of oxidation for those proteins with potential relevance on quality and bioactivity. In the last decade, the implementation of the technical advances in mass spectrometry and proteomics has overcome many of the limitations in the analysis of protein carbonyls. The main proteomic approaches employed to monitor this posttranslational oxidative modification in fish are based on Western blots directed to protein-DNPH derivatives or labeling protein carbonyls with a fluorescent probe, fluorescein-5-thiosemicarbazide (FTSC). In both proteomic procedures, the first step involves tagging the set of carbonylated proteins with the respective reagent, DNPH or FTSC. Then, proteins are separated on 1D or 2D polyacrylamide gel electrophoresis (PAGE) gels. The main methodological difference among both methods lies in the visualization step of protein carbonyls. Western-blotting on gels is required to visualize DNPH-labeled protein carbonyls, meanwhile FTSC-labeled protein carbonyls are directly visualized on PAGE gels by exposure to an ultraviolet light beam. FTSC exhibits an excitation maximum at a wavelength of 492 nm and an emission maximum at 516 nm. The fluorescent-labeling approach on 2D gels is found to be advantageous compared to the Western blotting alternative (Pazos et al., 2011). In the fluorescence-based proteomic procedure, gels used to monitor carbonylation can be later employed for protein digestion and identification; meanwhile the procedure based on the DNPH probe has need of exclusive gels for the immunoblot-based monitoring of carbonylation and separate gels for protein identification by MS. Thus, the determination of protein carbonylation by the fluorescence-based approach is less costly and time-consuming, and avoids the loss of proteins during the transfer from the electrophoretic gel to the membrane (Pazos et al., 2011). In both proteomic approaches, a semiquantitative determination of carbonylation on individual protein spots can be achieved by image processing with a densitometry system (Chaudhuri et al., 2006; Kjaersgaard et al., 2006a). The next step is the identification of the protein spots of

interest by in-gel digestion with proteolytic enzymes such as trypsin or chymotrypsin, followed by peptide analysis by MS/MS, and database searching against known protein sequences such as those present in the NCBIInr protein database. The mass spectrometers of choice allow MS/MS fragmentation, thus revealing the amino acid sequence of peptides is typically configured with soft ionization techniques matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI).

The immunoblotting-based procedure targeted to protein carbonyls was first applied to monitor these posttranslational modifications in fresh and tainted rainbow trout muscle (Kjaersgard and Jessen, 2004). Protein carbonylation increased in trout muscle kept at room temperature for 48 h compared to fresh muscle, showing in the myofibrillar protein fraction the biggest increase in carbonylation during fish tainting. However, identifications of individual proteins with the highest exposure to oxidation were not provided in this investigation. Later, the combination 2D immunoblotting and liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to monitor protein carbonyls in trout fillets during frozen storage (Kjaersgard et al., 2006a). The storage at -20°C resulted in a twofold increase in protein carbonylation in trout muscle, and more importantly, proteome analysis gave a description of the protein carbonylation pattern. Protein spots identified as nucleoside diphosphate kinase, adenylate kinase, pyruvate kinase, actin, creatine kinase, tropomyosin, myosin light chains 1 and 2, and myosin heavy chain were found carbonylated in trout muscle. 1-DE immunoblotting targeted to DNP-labeled protein carbonyls was also performed in salted herring during the ripening period (Andersen et al., 2007). Results revealed that a significant level of protein oxidation occurred in fish muscle during the ripening period, but no lipid oxidation was detected. Thus, these authors suggested that protein oxidation might play a principal role in the development of the characteristic organoleptic properties of salted herring, although mass spectrometry was not performed to identify proteins bearing carbonyl groups. A similar immunoblotting approach was applied to evaluate protein oxidation in horse mackerel mince during surimi processing and chilled storage (Eymard et al., 2009). The consecutive washing steps included in the surimi production process increased protein carbonylation; however, unprocessed raw fish mince developed faster protein carbonylation during chilled storage compared to processed mince.

A proteomics approach based on the fluorescent-tagging with FTSC and tandem mass spectrometry has been successfully employed to track protein carbonylation in fish muscle during storage or metal-induced oxidation. This methodology allowed the identification of proteins like enolase 3, aldolase, and L-lactate dehydrogenase A as the main targets for carbonylation in bonito (*Katsuwonus pelamis*) muscle during storage (Kinoshita et al., 2007). A different oxidative susceptibility was also corroborated in cod proteins treated by ferrous-catalyzed oxidation that is a relevant prooxidant system in fish muscle (Pazos et al., 2011). The cumulative findings revealed that structural proteins, mainly actin and myosin,

had significantly increased carbonylation by metal-mediated oxidation. Sarcoplasmic proteins such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), nucleoside diphosphate kinase B (NDK), triosephosphate isomerase, phosphoglycerate mutase, lactate dehydrogenase, creatine kinase, and enolase were also found highly vulnerable to oxidation. Importantly, isoforms of NDK, phosphoglycerate mutase, and GAPDH showed diverse susceptibilities to metal-catalyzed oxidation, indicating that posttranslational modifications as phosphorylation may change the resistance of proteins to metal-mediated oxidative damage. Later, the same fluorescence-based proteomic methodology was applied to investigate myofibrillar proteins from mackerel mince to undergo oxidation during chilled storage, and the capacity of the polyphenolic catechin to prevent these oxidative processes of proteins (Pazos et al., 2013). Catechin showed an inefficiency to prevent carbonylation of the myofibrillar protein actin, in contrast to the extremely high efficiency of catechin in inhibiting oxidation of lipids and other proteins. These results highlight that both the magnitude of protein carbonylation and its inhibition by polyphenolic antioxidants are highly protein dependent and that more research is needed to fully understand oxidative reactions of proteins in fish and its real contribution to quality deterioration. In this aspect, proteomics has shown an incomparable performance to comprehend and evaluate oxidative modifications in fish during processing and storage, but also to achieve a new generation of antioxidant procedures directed to protect the integrity and functionality of proteins in foods, and particularly in fish (Table 18.1).

Table 18.1: Summary of proteomic approaches used to evaluate protein carbonylation in fish and its relationship with quality.

| Species | Treatment | Carbonylation Labeling | Protein Identification | References |
|--|---|---|------------------------|------------------------------|
| Rainbow trout (<i>O. mykiss</i>) | Storage at RT for 48 h | 2-DE DNP-immunoblotting on gels | No | Kjaersgard and Jessen (2004) |
| Rainbow trout (<i>O. mykiss</i>) | Frozen storage | DNP-immunoblotting on 2-DE gels | LC-MS/MS | Kjaersgard et al. (2006a,b) |
| Herring (<i>C. harengus</i>) | Repining of salted herring | DNP-immunoblotting on 1-DE gels | No | Andersen et al. (2007) |
| Horse mackerel (<i>T. trachurus</i>) | Surimi processing and storage | DNP-immunoblotting on 1-DE gels | No | Eymard et al. (2009) |
| Bonito (<i>K. pelamis</i>) | Storage at 15°C | FTSC-fluorescence labeling on 2-DE gels | MALDI-TOF/ TOF MS | Kinoshita et al. (2007) |
| Cod (<i>G. morhua</i>) | Metal-mediated oxidation | FTSC-fluorescence labeling on 2-DE gels | MALDI-TOF/ TOF MS | Pazos et al. (2011) |
| Mackerel (<i>S. scombrus</i>) | Storage at 4°C and incorporation of a polyphenolic antioxidant (catechin) | FTSC-fluorescence labeling on 2-DE gels | MALDI-TOF/ TOF MS | Pazos et al. (2013) |

18.3 Proteomics to Evaluate the Bioactivity of Fish

In general terms, bioactive compounds can be described as “essential and nonessential compounds (e.g., vitamins or polyphenols) that occur in nature, are part of the food chain, and can be shown to have an effect on human health” (Biesalski et al., 2009). In other words, there are natural constituents of food that provide health benefits beyond the basic nutritional value of the product and whose presence determine its classification as a functional food. Bioactive compounds are also referred to as nutraceuticals, a term coined in 1979 by Stephan DeFelice, which results of the union of “nutrition” and “pharmaceutical,” reflecting their existence in the human diet and their biological activity (Brower, 1998).

Seafood is considered an important functional food that exerts numerous benefits in human health. On one hand, fish and shellfish are rich in proteins containing all the essential amino acids, polyunsaturated fatty acids (especially EPA and DHA), calcium, iodine, vitamins, and many other nutrients (Venugopal and Shahidi, 1996). On the other hand, marine organisms, in general, are considered the best potential reservoir for novel bioactive compounds, since they constitute nearly half of the worldwide biodiversity and more than 90% of all living classes of organisms are found in the marine environment (Lazcano-Pérez et al., 2012).

As a consequence, food science has focused on the development of sensitive technological tools to assay the ability of seafood to prevent or cure diseases, to find novel bioactive compounds with attractive properties for human health promotion as well as to seek new application areas. The introduction of -omics methodologies allows large-scale analyses of cellular components by combining techniques of mass spectrometry, bioinformatics, or chemometrics which are postulated as promising tools to deal with food science demands. Specifically, proteomic tools are emerging as an important part of this holistic approach because they allow large-scale characterizations of food proteins to study their functional, nutritional, and biological relevance, as well as, to study their conformation and interactions.

Among the different functional compounds present in seafood, proteins and peptides are one of the main groups that influence the bioactive role of fish and related food systems. Bioactive peptides have been defined as “food-derived components (genuine or generated) that, in addition to their nutritional value exert a physiological effect in the body” (Vermeirissen et al., 2004). They are usually 2–20 amino acid residues in length, although some larger polypeptides have been reported. Curiously, many of the peptides are inactive within the parent protein sequence and thus must be released to exert an effect by enzymatic activity during intestinal digestion or by fermentation processes during food processing or ripening (Kim and Wijesekara, 2010). These peptides may be absorbed through the intestine where they subsequently enter intact the circulatory system to exert various physiological effects. A huge range of bioactivities have been described for food peptides which include opiate, antithrombotic, antihypertensive, immunomodulating, antilipemic, osteoprotective, antioxidative, antimicrobial, ileum contracting, anticariogenic, and growth promoting (Moller et al., 2008).

Bioactive peptide discovery traditionally consists of a stepwise approach, typically including (Panchaud et al., 2012):

1. Selection of an appropriate protein source of plant or animal origin.
2. Release of encrypted peptides: proteolysis by enzymatic processing, fermentation, or gastrointestinal digestion.
3. Initial in vitro screening for targeted bioactivity.
4. Fractionation of the peptide pool into more defined subfractions by chromatography or membrane separation.
5. Further bioactivity screening followed by identification of bioactive peptide(s) by Edman degradation and mass spectrometry, although amino acid analysis and protein sequencers are traditional tools that remain in use.
6. Production of synthetic peptide analogs to validate bioactivity in vitro and in vivo.

By following this traditional workflow, several bioactive peptides in seafood have been identified, some of them from fish, although marine vertebrates have not been extensively studied as a source of peptides and proteins with biological activity.

Antihypertensive peptides, principally angiotensin I converting enzyme (ACE) inhibitory peptides, are the most extensively bioactive peptides researched in fish. In tuna (*Thunnus obesus*), Qian et al. (2007) have identified a peptide with antihypertensive effect. After enzymatic hydrolysis of tuna dark muscle performed using a mixture of several proteases (alcalase, R-chymotrypsin, neutrase, papain, pepsin, and trypsin) and purification of ACE inhibitory peptide by prefractionation of the hydrolysate by two subsequent chromatographic separations (ion-exchange and reversed-phase high-performance liquid chromatography, RP-HPLC), the purified peptide was identified using TOF-MS/MS with an ESI source (ESI-TOF-MS/MS). Peptide sequence was obtained with the PepSeq de novo sequencing algorithm to be WPEAAELMMEVDP. The authors also proved that this peptide was an effective hypotensor in vivo, after its administration to spontaneously hypertensive (SHR) rats (Qian et al., 2007). In tuna, Lee et al. (2010) also identified another bioactive peptide with ACE inhibitory effect. In this work, enzymatic hydrolysis was performed using six commercial enzymes (alcalase, a-chymotrypsin, papain, pepsin, neutrase, and trypsin) and peptide purification was achieved after two chromatographic separation steps (ion-exchange and RP-HPLC). Amino acid sequence analysis was performed by ESI-TOF-MS/MS which revealed a peptide of 21 amino acids, GDLGKTTTVSNWSPPKYKDTP, with an important hypotensive activity noted when administered to SHR rats (Lee et al., 2010).

Nine novel ACE inhibitory peptides (SFHPYFSY, AFVGYVLP, LAGDGY, STHGVW, ASILGF, GS, GIHETTY, ELSYELP, and VELYP) able to exert an antihypertensive effect in SHR rats have been described in the muscle of cuttlefish (*Sepia officinalis*). To release bioactive peptide, Balti et al. (2015) employed crude enzyme preparations from *Bacillus mojavensis A21* and cuttlefish hepatopancreas. The isolation of active peptides was then

achieved after various types of chromatographic separation (gel filtration and RP-HPLC). Finally, their molecular masses and amino acid sequences were determined using ESI-MS/MS. Interpretations of spectra of MS/MS were made using BioAnalyst software (Balti et al., 2015). ACE inhibitory peptides were also found in hydrolysate from shark meat by Wu et al. (2008). In this case, shark meat hydrolysate was obtained with protease SM98011 digestion and by ultrafiltration, gel filtration, and RP-HPLC, and four peptides (CF, GT, MF, and FG) with high ACE inhibitory activity were purified. Their sequences were identified by LC-ESI-MS/MS (Wu et al., 2008).

Peptides with antioxidant properties were also described in fish. For instance, Bougatef et al. (2010) identified seven peptides with antioxidant properties from enzymatic hydrolysates of sardinelle (*Sardinella aurita*). The hydrolysate, generated with crude enzyme extract from sardine (*Sardina pilchardus*), was fractionated by size exclusion chromatography and RP-HPLC. As a result, seven antioxidant peptides (LHY, LARL, GGE, GAH, GAWA, PHYL, and GALAAH) were isolated whose molecular masses and amino acid sequences were determined by ESI-MS and ESI-MS/MS, respectively, using the BioAnalyst software to spectra interpretations (Bougatef et al., 2010). These and other peptides with similar biological function, as well as, bioactive peptides displaying antimicrobial or antiproliferative effects were also identified in fish products. In these studies, peptide sequences were mainly determined by a combination of Edman degradation, MS, and HPLC analysis of the native peptides (Senevirathne and Kim, 2012).

The most traditional peptide discovery strategy is undoubtedly efficient and most of the food-derived bioactive peptides currently known have been identified by this approach. Nevertheless, this rather empirical workflow is an expensive and time-consuming process which requires substantial resources and even the extensive fractionation and purification do not always enable unambiguous identification of single bioactive peptides (Panchaud et al., 2012).

To cope with these inconveniences, new analytical approaches combine MS, bioinformatics, and database searching for a deep screening of potential bioactivity in protein/peptides from raw or processed products. The high-throughput proteomic approaches termed shotgun (or bottom-up) allow the identification of peptide profiles after in vitro protein digestion and prediction of possible bioactive sequences by database screening (Mamone et al., 2009). The proteomics approach applied to the classical workflow includes aspects such as high-resolution separation techniques (2-DE, nano-HPLC, multidimensional liquid chromatography, capillary electrophoresis) in order to separate and characterize protein profiles that can be analyzed in databases; protein characterization by mass spectrometry or Western blotting; quantitation of the interest peptide by ELISA or more recently by MS-based methods; the search for bioactive peptides for all protein sequences reported for the food source by databases (virtual screening) and simulation of the binding between the peptide to a specific receptor (Carrasco-Castilla et al., 2012). The newest in silico analytical approach, also called “reverse genome engineering,” employs computational and bioinformatics tools to predict functional peptides from

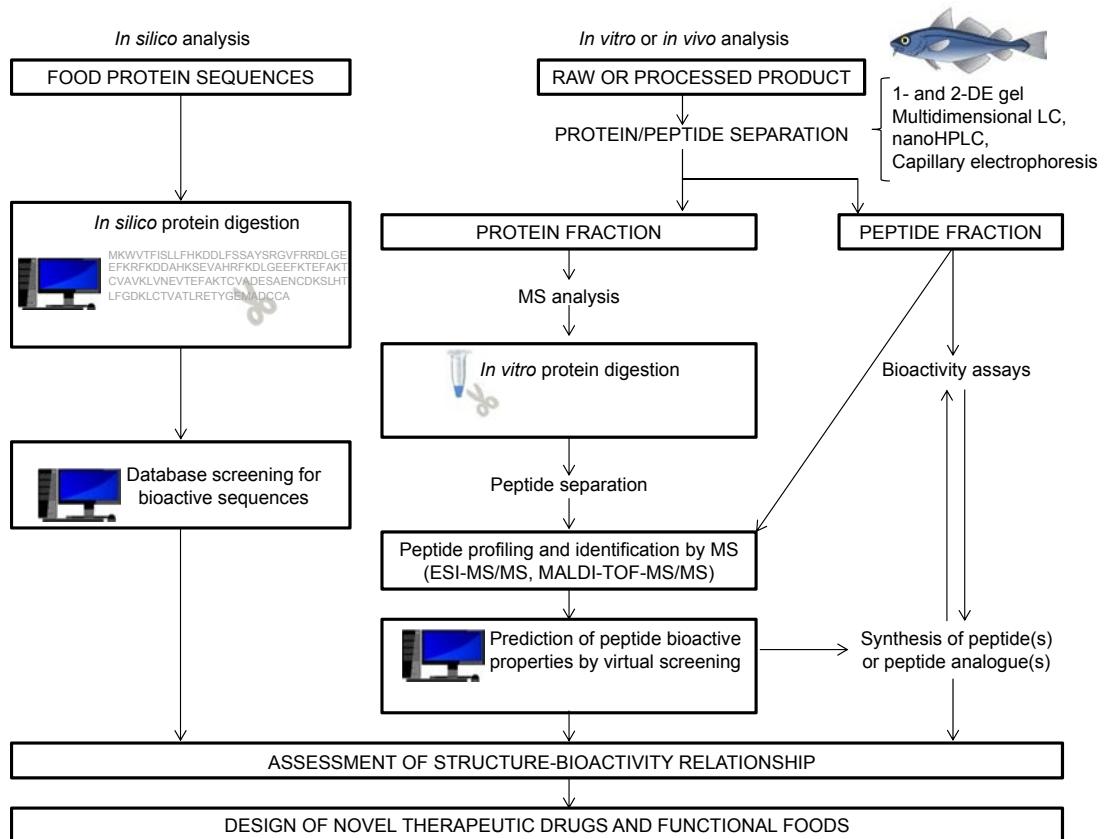


Figure 18.1
Proteomics workflows for discovering and characterization of food bioactive peptides.

proteome sequence information by simulating the *in silico* protein digestion and modeling the docking between the receptor molecule to test its biological activity. Therefore, *in silico* analysis can be used as fast initial screening tool on a large scale to look for high-potential precursor proteins of bioactive peptides, reducing cost and time. However, although *in silico* digestion can help to select an enzyme for the hydrolysis of a given protein, an exact description of peptides released and thus, their possible bioactivities cannot be given; this part of the procedure will remain largely empirical (Verheyen et al., 2009). Fig. 18.1 shows a schematic representation of the different workflows used in the discovery and characterization of bioactive peptides in food.

For marine food science, the use of this *in silico* proteomic approach for bioactive discovery is still scarce. A major limitation is the lack of available annotated genomes and proteomes for most marine organisms, including several “model species” (Slattery et al., 2012). Carrera et al. (2013) have recently predicted potential valuable bioactive peptides (i.e., antimicrobial, bioactive collagen peptides, and antihypertensive peptides) from the sarcoplasmic proteome of 15 commercial fish

species (6 demersal and 9 pelagic) after an in silico simulated human gastrointestinal digestion and database screening for bioactive sequences. Later, Capriotti et al. (2015) identified more than 200 potential antimicrobial bioactive peptides in sarcoplasmic and myofibrillar protein fraction from seabass (*D. labrax*). These proteomics studies underlined how the appropriate use of bioinformatic tools can speed up the research into potential target peptides without incurring major costs, even if further studies are needed to confirm the results.

The creation of several bioactive peptide databases has been essential in the development of these new approaches. Some of these databases available online are shown in Table 18.2. Moreover, the table also includes several programs to simulate in silico proteolysis and to predict the secondary structure and physicochemical properties of peptides and amino acids.

It is well recognized that understanding the effect of diet on health requires the study of the mechanisms of nutrients and other bioactive food constituents at the molecular level. In this sense, the application of -omics techniques (namely genomics, transcriptomics, proteomics, and metabolomics) to nutritional research constitutes a promising strategy to study how diet affects the balance between health and disease. High-throughput omics might also be ideal for elucidating the effects of novel functional foods and nutraceuticals on global expression of genetic information and cell function without making assumptions about what to look for in terms of risk and providing new means for discovering biomarkers for efficacy testing of bioactive functional food ingredients.

Table 18.2: Bioinformatic tools used in bioactive peptide discovery.

| Database/Program | Website | References |
|--|---|--------------------------|
| Bioactive Peptide Database | | |
| BioPep | http://www.uwm.edu.pl/biochemia/ | Iwaniak et al. (2005) |
| PepBank | http://www.pepbank.mgh.harvard.edu/ | Shtatland et al. (2007) |
| EROP-Moscow | http://www.rop.inbi.ras.ru/ | Zamyatnin et al. (2006) |
| PeptideDB | http://www.peptides.be/ | Liu et al. (2008) |
| Peptidome | http://www.peptidome.jp/ | Slotta et al. (2009) |
| APD2 | http://aps.unmc.edu/AP/main.php/ | Wang et al. (2009) |
| CAMP | http://www.bicnirrh.res.in/antimicrobial/ | Thomas et al. (2010) |
| In Silico Digestion | | |
| BioPep | http://www.uwm.edu.pl/biochemia/ | Iwaniak et al. (2005) |
| PeptideCutter | http://web.expasy.org/peptide_cutter/ | Gasteiger et al. (2005) |
| POPS | http://pops.csse.monash.edu.au/ | Boyd et al. (2005) |
| MS-Digest | http://prospector.ucsf.edu/prospector/mshome.htm | |
| Secondary Structure and Physicochemical Properties of Peptides and Amino Acids | | |
| Uniprot | http://www.uniprot.org/ | Consortium (2010) |
| GOR V | http://gor.bb.iastate.edu/ | Sen et al. (2005) |
| PreSSApPro | http://www.bioinformatica.isa.cnr.it/PRESSAPRO/ | Costantini et al. (2007) |
| AAindex | http://genome.jp/aaindex/ | Kawashima et al. (2008) |

In spite of this consideration, genomics and transcriptomics tools continue to be the routine analysis to test the effect of food on life system and only a few publications in nutritional research have applied the other -omics tools in their studies (Moore and Weeks, 2011). In the field of marine food science, the application of proteomics approaches is consequently scarce and mainly used to study the effect of marine/fish bioactive compounds against cancer or chronic diseases closely related to diet, such as obesity or cardiovascular diseases. Likewise, the main bioactive compounds studied are the omega-3 fatty acids from marine source because the nutritional quality of fish, seafood products, and most marine oils is often associated with their high content of these essential fatty acids, especially EPA and DHA.

In cancer research, the role of fish oil against prostate cancer cell growth was investigated by using proteomic tools. Through a shotgun proteomic analysis based on a label-free protein quantification and bioinformatics analysis, Zhao et al. (2016) suggested that fish oil suppressed prostate cancer cell viability by activating molecular pathways independently of the downregulation of the fatty acid synthase by fish oil. Narayanan et al. (2006) evaluated the effects of low doses of docosahexaenoic acid (DHA) in combination with celecoxib (a chemopreventive agent) on the molecular targets at the proteins level in rat prostate cancer cells. In this case, a bottom-up strategy (2D gel electrophoresis and protein identification by MALDI-TOF-MS/MS) allowed the identification of 12 proteins differently regulated by the combination of DHA and celecoxib. Among those, DHA and celecoxib significantly increased proteins involved in protein folding and synthesis, such as dnaK-type molecular chaperone grp78 (GRP78), heat shock 70 kDa proteins 5 and 8 (HSP70), and protein disulfide isomerase precursor (PDI) (Narayanan et al., 2006).

The effect of fish oil in reducing breast cancer risk was also evaluated. Fabian et al. (2015) found that the intake of 1860 mg EPA + 1500 mg DHA ethyl esters/day for 6 months exerted favorable effects in a proteomics array for several proteins associated with mitogen signaling and cell-cycle arrest, evidencing a promising use of high-dose EPA and DHA ethyl esters for primary prevention of breast cancer. Moreover, Blanckaert and coworkers investigated the effect of only DHA in reducing the invasion of the triple-negative breast cancer cell line MDA-MB-231 (Blanckaert et al., 2015). A bottom-up proteomics based on 2-DE gels and identification of proteins differentially regulated by MALDI-TOF-MS/MS revealed that DHA increased the levels of three membrane proteins (Keratin, type II cytoskeletal 1 (KRT1), catalase, and lamin-A/C): one of them (KRT1) resulted directly involved in the antiinvasive activity of DHA in breast cancer cells (Blanckaert et al., 2015).

The health effect of fish oils in preventing lung cancer induced by tobacco carcinogens were evaluated by Chang et al. (2007) in a proteomics approach based on the separation of rat lung proteins by 2D liquid chromatography and identification of proteins differently regulated by MALDI-TOF-MS/MS. These authors found that the consumption of fish oils decreased levels of apolipoprotein A-I and Clara cell 17-kDa, which can be potential biomarkers of lung cancer induced by tobacco carcinogens (Chang et al., 2007).

Several marine bioactive compounds with potential anticancer properties were also studied by proteomic approaches. For instance, [Dyshlovoy et al. \(2012, 2016, 2014\)](#) assayed the antiproliferative activity of some marine alkaloids (two analogues of rigidins, aaptamine, and monanchocidin A) by following bottom-up proteomics based on quantification in 2-DE gels and identification by MALDI-TOF-MS/MS. A similar proteomic methodology was used to understand how hemocyanin obtained from the marine mollusc *Megathura crenulate* is able to reduce the growth of Barrett's cancer cells ([Vona-Davis et al., 2004](#)).

Besides cancer research, just a few studies used proteomics to investigate the influence of fish/marine compounds, mainly EPA and DHA, on several chronic human diseases.

Bottom-up proteomics based on 2-DE gels and MALDI-TOF-MS/MS analysis allowed to analyze the influence of acute ([Camargo et al., 2013](#)) or prolonged ([Rangel-Zuniga et al., 2015](#)) omega-3 fatty acid consumption on the proteome of peripheral blood mononuclear cells from patients suffering metabolic syndrome. As a result, omega-3 intake induced changes in five and seven proteins that, respectively, led to improve insulin signaling and reduce inflammation and oxidative stress.

By two different proteomics techniques (a protein-centric approach based on 2-DE gel electrophoresis and MALDI-TOF-MS/MS analysis and a peptide-centric LC-MS-based approach), [Wrzesinski et al. \(2013\)](#) characterized the changes induced by fish oils on mitochondrial subproteome of rat livers into the context of diet-induced metabolic syndrome. Fish oils were able to alter 54 proteins mainly involved in fatty acid and amino acid metabolism, lipid oxidation, and oxidative phosphorylation. 2-DE gels and LC-ESI-MS/MS analysis were also successfully used to distinguish the effect of fish oil, or only DHA, on the liver proteome against the changes induced by high fat and high cholesterol diet. [Mavrommatis et al. \(2010\)](#) found 35 liver proteins, related to lipoprotein metabolism and oxidative stress, modulated by DHA and fish oil, but interestingly, four proteins responded differently to fish oil or DHA supplementation. Finally, bottom-up proteomic tools (2-DE gels and MALDI-TOF-MS/MS analysis) were used to identify serum proteins modulated by the consumption of fish oils in healthy adult humans. The data obtained showed the effectiveness of the intake of fish oils to reduce the level of 10 serum protein modulators of inflammation and/or lipid and lipoprotein metabolisms ([de Roos et al., 2005](#)).

Since protein oxidation, particularly protein carbonylation, has been associated with the development and progression of several diseases such as cancer, cardiovascular diseases, and diabetes, redox proteomics constitutes a promising tool to understand the underlying mechanisms of fish bioactivity against these pathologies.

Redox proteomics is a novel subfield of proteomics aimed at identifying and quantifying redox-based changes within the proteome both in redox signaling and under oxidative stress conditions ([Butterfield and Dalle-Donne, 2012](#)). However, the application of redox proteomics to the discovery of protein targets of the fish consumption is extremely limited. To

date, only one publication has assayed the effect of different EPA and DHA ratios on the redox proteome. This investigation evaluated the impact of these dietary lipids from marine sources on the redox proteome of plasma, liver, and skeletal muscle in a healthy rat model. Using a bottom-up proteomic approach consisting of FTSC labeling of protein carbonyls, FTSC intensity visualization on 1-DE or 2-DE gels, and protein identification by LC-ESI-MS/MS, [Méndez et al. \(2013\)](#) demonstrated that dietary supplementation with an equal proportion of EPA and DHA was able to specifically decrease the carbonylation level of albumin in plasma, actin in skeletal muscle and albumin, and argininosuccinate synthetase and 3- α -hydroxysteroid dehydrogenase in liver.

18.4 Conclusions and Final Considerations

Today's consumers demand food with high organoleptic quality, increased functional and nutritional properties, guaranteed safety, but with less processing, fewer additives as well as fewer technological interventions. These requirements are even higher in the case of seafood products because of their lower stability and higher susceptibility to adverse changes under technological processing. The demand for seafood that is perceived to be healthier and safer has increased despite the existing caveat of a lack of scientific evidence. In this sense, proteomics provides excellent opportunities to scientifically assure the integrity and safety of seafood, but also warranting their nutritional value. Therefore, proteomic tools have been successfully applied in monitoring the changes occurring in fish postmortem, in evaluating the influence of slaughtering and protein oxidation during storage on muscle fish quality. Also, proteomics allowed testing new technological interventions to enlarge shelf life such as the use of antioxidants in preventing protein oxidation or high hydrostatic pressure treatments.

Besides aspects related to seafood quality, proteomics have also proved to be one of the best strategies to test the functional and bioactivity properties of seafood. Proteomics has been recently applied in the discovery, characterization, and production of bioactive peptides in fish/marine products. Proteomic approaches have accelerated almost every step of the discovery workflow and increased the capacity of research aimed at detecting potential sources of bioactive peptides through the holistic view of proteomics, all in relatively cost-saving and time-saving processes. In fact, proteomics has proven helpful in discovering peptides with potential antihypertensive, antimicrobial, antioxidant, or antiproliferative effects in both fish and other marine organisms.

Conversely, proteomics constitutes an important strategy to uncover the biological mechanisms that govern the health effect of seafood and their bioactive compounds. The effect of fish oil consumption to prevent and/or improve several types of cancer and chronic diseases closely related to the uptake of unhealthy diets were the most commonly studied processes. The findings of these investigations provide the basis to identify suitable biomarkers of disease, design high-specificity drugs with minimal side effects and personalized nutraceuticals and functional foods.

Despite its promise, current proteomics applied to seafood science has to cope with a variety of difficulties at levels ranging from experimental design and sample processing and optimization strategies to data analysis and identification. Advances in the characterization of marine proteomes and in high-throughput proteomic technologies can help to deal with some of these inconveniences. Over time, the integration of proteomics with other -omics such as metabolomics will undoubtedly provide more robust information in order to guarantee the high quality, safety, and nutritional value of seafood from farm to table as well as to contribute to the design of new nutraceuticals and functional foods.

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Proteomic Identification of Commercial Fish Species

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19.1 Introduction

Fishery markets have grown rapidly in recent decades. In developing countries, the average annual per capita fish consumption is 9.0–17.8 kg (FAO, SOFIA, 2014). This high consumption is because fish are considered to be one of the main human nutritional sources and because of the strong evidence regarding the positive health benefits of its consumption. Its high ω-3 fatty acid content has been demonstrated to be beneficial for the treatment and prevention of inflammatory, cardiovascular, and neurological illnesses (Hooper et al., 2006). Fig. 19.1 shows the main commercial fish species intended for human consumption (FAO, SOFIA, 2014). However, the globalization of fishery markets presents potential risks. A common fraudulent practice is fish species substitution, which can occur unintentionally but more frequently is done to avoid tax or to sell lower-priced fish species at a premium price. Moreover, potential human health risks exist because the substituted fraudulent species can be harmful and may aggravate symptoms in people with allergies (Sicherer and Sampson, 2010).

In this context, consumers have been demanding clear and reliable information about the species that they consume, necessitating the establishment of new standardized labeling norms and inspection control regulations. In the European Union, the need for labeling regulations has led to [Council Regulations \(EC\) Nos. 104/2000, 2065/2001](#) and by the [EU Regulation 1379/2013](#) covering the common organization of the markets for fishery and aquaculture products. This regulation directs that fishes should be correctly labeled, indicating (1) the catch area, (2) the commercial designation of the species, and (3) the production method (caught or farmed). To meet these regulations, each member state is asked to draw up and publish a list of the commercial designations that are accepted in their territory, indicating the scientific name for each species and the name(s) in the language(s) of the member state. The designation of the catch area described in (1) previously is normalized in [Council Regulation \(EC\) No. 2065/2001](#) in the Annex following the FAO yearbook (FAO yearbook, 2000). Regulation (EC) No. 104/2000 also indicates the importance of labeling seafood products with the scientific name of the included species to ensure traceability.

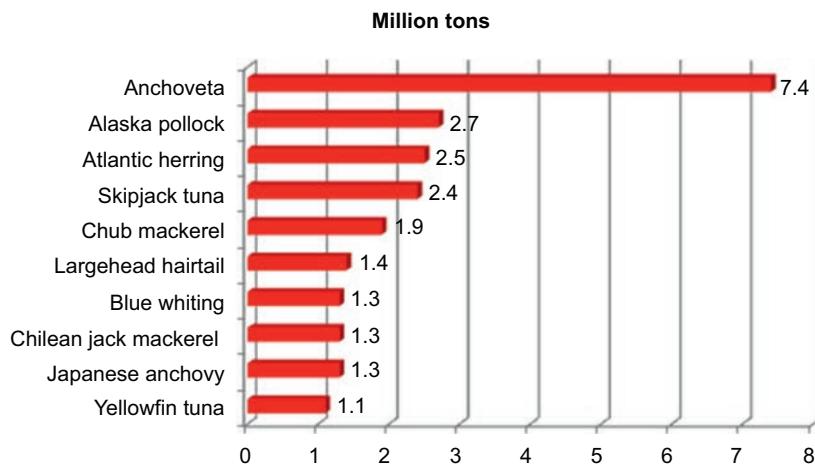


Figure 19.1

Marine capture fisheries production: the top 10 species in 2014. FAO, SOFIA, 2014. *The state of world fisheries and aquaculture. In: Opportunities and Challenges. ISBN: 978-92-5-108275-1.*

These requirements have been implemented in each of the European States, including Spain, where several regulations have been promulgated to ensure the correct labeling and identification of seafood products ([Royal Decree 1380/2002](#); [Royal Decree 121/2004](#); [Royal Decree 1702/2004](#)).

To comply with these regulations, accurate, sensitive, and rapid detection methods that permit the direct identification of commercial fish in any food product are highly recommended. Unprocessed fish are conventionally identified based on an examination of their anatomical and morphological features. However, even for marine expert biologists, it is difficult to distinguish very closely related fish species that coexist in the same catch area. Due to overlapping distributions, the species are caught and managed jointly, and no distinction is made in stock management. Additionally, due to the development of the fishing industry, seafood products can be processed (beheaded, eviscerated, skinned, filleted, smoked, cooked, or canned), often rendering the appreciation of their external anatomical or morphological features impossible.

For these reasons, the use of molecular tools represents a suitable strategy for circumventing such problems with species identification. Although several DNA and protein molecular markers have been developed over the last two decades, the recent successes enjoyed by proteomic methodologies renders them promising alternatives for fish identification purposes ([Gallardo et al., 2013](#)). In this context, a comprehensive overview of state-of-the-art proteomic approaches and their possible future use for the identification of commercial fish species is given in this chapter.

19.2 Traditional Molecular Strategies for the Identification of Commercial Fish Species

Electrophoretic analysis, immunological assays, and DNA-based methods have been extensively exploited for the authentication of fish species. Isoelectric focusing (IEF) is the most commonly used protein-based technique for species identification (Piñeiro et al., 2000; Tepedino et al., 2001). Indeed, IEF was adopted by the Association of Official Analytical Chemists (AOAC) as the officially validated method for species identification (AOAC, 1990). In addition, the FDA offers a library of IEF patterns for the sarcoplasmic proteins of various fish species (Regulatory Fish Encyclopedia, FDA). Several reports concerning the application of two-dimensional gel electrophoresis (2-DE) have described potential species-specific proteins that could be used for the discrimination of closely related fish species, such as hakes (Piñeiro et al., 2001; Carrera et al., 2006), puffer fish (Chen et al., 2004), commercial flatfish (Piñeiro et al., 1999), and perch (Berrini et al., 2006) species. The following 2-DE database servers covering various fish species are available on the Internet: the World-2DPAGE List (<http://world-2dpage.expasy.org/list/>) and the FishProm database (<http://www.abdn.ac.uk/fishprom/>).

Immunological methods combine specificity, sensitivity, and simplicity. Several polyclonal antibodies (pAbs) against certain sarcoplasmic proteins have been developed for the discrimination of fish species such as salmon, trout, halibut, haddock, and Nile perch (Domínguez et al., 1997; Céspedes et al., 1999a; Asensio et al., 2003a). The production of pAbs is simple and economical but presents the following drawbacks: (1) the method is of low specificity resulting in cross-reactivity problems, (2) a limited amount of Ab is available, and (3) the method requires continuous immunizations of new animals, leading to issues with nonreproducible batches. These problems can be solved by generating monoclonal Abs (mAbs). Several mAbs have been generated against species of red snapper (Huang et al., 1995), grouper (Asensio et al., 2003b), and catfish (McNulty and Klesius, 2005; Gajewski et al., 2009). The main immunological techniques using pAbs or mAbs are immunodiffusion (Domínguez et al., 1997), ELISA (Huang et al., 1995; Cespedes et al., 1999a; Asensio et al., 2003a,b; McNulty and Klesius, 2005), and Western blot (Zhang and Rasco, 1996; Domínguez et al., 1997). Immunoassays are advantageous in that, once developed, they are easy to use and exhibit high sensitivity and throughput, thus enabling a large number of samples to be processed in a short time.

DNA-based procedures have become popular for the unambiguous identification of fish species, including closely related species (Sotelo and Pérez-Martín, 2006; Rasmussen and Morrissey, 2009). Among the DNA targets used, mitochondrial DNA (mtDNA) is generally preferred because of its maternal inheritance, rapid evolutionary rate, and lack of intermolecular genetic recombination. The most commonly used mtDNA markers include the cytochrome b gene (Rehbein et al., 1997; Sotelo et al., 2001; Calo-Mata et al., 2003; Chapela

et al., 2007), the mtDNA control region (Quinteiro et al., 2001), and the 12S rRNA region (Comesaña et al., 2003; Zhang et al., 2006). Today, most DNA-based methods that are used for species identification in foods involve the highly specific amplification of one or more DNA fragments using the polymerase chain reaction (PCR). This technique is potentially valuable due to its simplicity, sensitivity, and specificity. Nuclear genes such as 5S ribosomal DNA, 5S ribosomal RNA, internal transcribed spacer 1 (ITS1), and certain microsatellite loci have been considered for the study of phylogenetic relationships among fishes (Céspedes et al., 1999b; Castillo et al., 2003; Asensio et al., 2004; Pérez and García-Vázquez, 2004). Several methods have been developed to perform polymorphism searches such as searches for restriction fragment length polymorphisms (using PCR-RFLP), which are used for the identification of various species of salmon, gadoids, flatfishes, and hakes (Russell et al., 2000; Quinteiro et al., 2001; Sotelo et al., 2001; Pérez et al., 2004; Aranishi et al., 2005). Other techniques include those based on amplified fragment length polymorphisms (PCR-AFLP) (Maldini et al., 2006); single-stranded conformational polymorphisms (PCR-SSCP), which are used to identify tuna, salmon, flatfish, Nile perch, and hakes (Colombo et al., 2005; Chapela et al., 2007); random amplified polymorphic DNA (RAPD), which is used to discriminate between tilapia species, Nile perch, and grouper (Partis and Wells, 1996; Asensio et al., 2002); and multiplex PCR, which has been used to identify grouper fillets (Trotta et al., 2005). In addition, some sequencing techniques, such as forensically informative nucleotide sequencing (PCR-FINS), have been used to identify anchovies, sardines, and hake species (Jérôme et al., 2003; Santaclara et al., 2006). The latest and more fashionable methods for fish species identification are those based on real-time PCR (Sánchez et al., 2009), microarrays (Kochzus et al., 2008), and lab-on-a-chip systems (Chen et al., 2011), which can identify and quantify seafood species on a large scale. Recently, DNA-barcoding has been proposed as a reliable method for fish authentication, and DNA barcode data have been included in the Regulatory Fish Encyclopedia of the FDA (Di Pinto et al., 2015; Khaksar et al., 2015).

Although several DNA and protein molecular markers have been developed over the past two decades, proteomic methodologies are emerging as a promising strategy for fish identification.

19.3 Proteomic Methodologies for the Identification of Commercial Fish Species

Proteins are central molecules in all biological systems. In this context, the recent success of proteomic methodologies shows their promise for use in food science. As a discipline, proteomics is defined as the large-scale analysis of proteins in a particular biological system at a particular moment. Proteomics includes not only the study of the structure and function of proteins but also the analysis of protein modifications, interactions between proteins, their intracellular location, and the quantification of their abundance. The recent

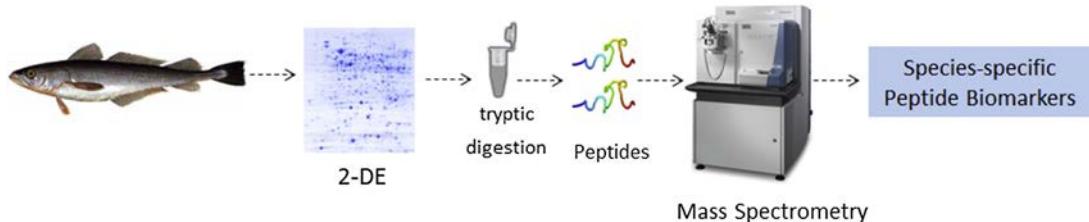
successes enjoyed by proteomic and bioinformatic methodologies demonstrate their promise for use in food science studies; thus, research institutions, industries, agencies, and regulatory laboratories are combining their efforts to acquire the needed knowledge on food composition, quality, and safety. Very useful reviews on proteomic applications in food science have recently been published (Carrera et al., 2013). Proteomics have been used for the identification of several seafood species, such as mussels (López et al., 2002) and shrimp (Ortea et al., 2009); however, their application to the authentication of *Teleostei* species remains rare. The limited impact of proteomics in any type of research involving the *Teleostei* group at present can be appreciated if one considers that protein databases include 997,848 amino acid sequences from *Teleostei*, but more than 2,383,025 amino acid sequences have been reported for the Tetrapoda group (UniProtKB, June 2016).

Fig. 19.2 shows the proteomic pipeline and tools that are currently being applied by our laboratory for fish identification. Two consecutive phases, the discovery phase (Carrera et al., 2006, 2010) and the target-driven phase (Carrera et al., 2011), are described in detail in the following sections.

19.3.1 Discovery Phase

In this phase, we use a classical bottom-up proteomic approach to identify novel peptide biomarkers for the identification of fish species (i.e., Merlucciidae species) whose genomes have not yet been sequenced (Fig. 19.2).

(A) Discovery phase



(B) Target-driven phase

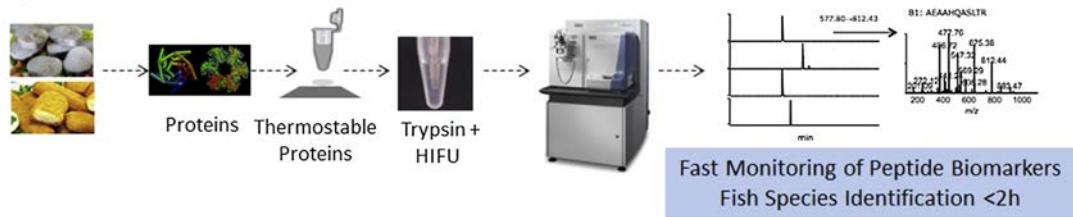


Figure 19.2

The proteomic pipeline used for the identification, characterization, and detection of species-specific peptide biomarkers for fish authentication purposes: (A) discovery phase and (B) target-drive phase.

Candidate species-specific proteins (based on 2-DE analysis) are selected and subjected to tryptic digestion; the recovered peptides are then ionized and analyzed using mass spectrometry (MS). As mentioned previously, different spots or clusters of spots corresponding to parvalbumin (PRVB) isoforms (M_r , 11.20–11.55 kDa; pI , 3.75–4.57) (Piñeiro et al., 2001; Carrera et al., 2006, 2010), nucleoside-diphosphate kinase (NDK) proteins (M_r , 16.80–18.60 kDa; pI , 5.04–5.47) (Piñeiro et al., 2001; Carrera et al., 2007), and aldolase proteins (M_r , 42–43 kDa; pI , 6.5) (Carrera et al., 2009) showed noticeable qualitative interspecific differences in 2-DE experiments and were further investigated by MS.

Recent successes illustrate the role of mass spectrometry (MS; mainly matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and electrospray-ion trap (ESI-IT) mass spectrometry) as an indispensable tool in proteomic studies (Aebersold and Mann, 2003). The usefulness of the peptide mass fingerprinting (PMF) method involving MALDI-TOF MS was ascertained by analyzing 10 commercially valuable closely related species of the Merlucciidae family (Carrera et al., 2006). MALDI-TOF mass fingerprints of the sarcoplasmic protein PRVB were used to define a set of molecular fish authentication markers; based on the presence or absence of species-specific peptide masses, the method aided in (1) selective differentiation between the genus *Merluccius* and *Macruronus*, (2) classification of hake species into two groups according to their geographic provenance (as American or Euro-African hakes), and (3) the unequivocal identification of several hake species: *M. bilinearis*, *M. australis polylepsis*, *M. australis australis*, *M. productus*, *M. paradoxus*, and *M. pollie*; the remaining hake species can be grouped in two clusters, comprising *M. hubbsi* and *M. gayi* in one and *M. merluccius* and *M. capensis* in the other. Due to the interspecific variability of PRVB and its high concentration in fish muscle, this protein is expected to be a good biomarker for fish species identification. The selection of PRVB as a target protein has additional importance in that it is highly thermostable (Kawai et al., 1992; Carrera et al., 2010). Thus, the monitoring of peptide masses can be equally applied in fish authentication to both fresh and processed seafood products. A similar approach was successfully used to identify 25 fish species (Mazzeo et al., 2008). Using the same strategy previously reported by our group, the authors characterized specific PRVB peptide masses that enabled a large number of fish species belonging to Perciformes, Gadiformes, and Pleuronectiformes to be differentiated using MALDI-TOF MS.

In a subsequent study (Carrera et al., 2010), we proposed a novel strategy for the extensive characterization of all PRVB isoforms in all commercial species in the Merlucciidae family. This strategy combined a classical bottom-up proteomic approach with the accurate M_r determination by Fourier-transform ion-cyclotron resonance (FTICR)-MS of intact proteins and the selected tandem mass spectrometry (MS/MS) ion monitoring (SMIM) of peptide mass gaps. For each PRVB, mass spectra were obtained using LC-ESI-IT-MS/MS from two digests (trypsin and Glu-C), subjected to database searches using Sequest (Eng et al., 1994),

and peptides were de novo sequenced manually with the aid of PEAKS (Ma et al., 2003) and DeNovoX (Thermo Electron Co.) software (Scigelova et al., 2007). The deduced peptide sequences were aligned and the theoretical M_r values for the resulting sequences were calculated. Experimental M_r values for each PRVB were measured with high mass accuracy using FTICR-MS. The masses of several missing peptide gaps were estimated by comparing the theoretical and experimental M_r values, and the MS/MS spectra corresponding to these ions were obtained using LC-ESI-IT-MS/MS in the SMIM scanning mode. Finally, all peptide sequences were combined to generate the final protein sequences. This approach allowed the complete de novo MS-sequencing of 25 new PRVB isoforms. This study contributes complete sequences of a larger number of new proteins than were previously reported, making use of MS-based techniques alone.

Several species-specific peptide biomarkers were selected to effectively identify all species within the Merlucciidae family. PRVB peptide sequences with high interspecies variability, which were obtained after the extensive de novo sequencing of previously published PRVBs (Carrera et al., 2010), were used for this purpose. Eleven tryptic peptides were selected based on the information that their combined presence or absence could provide to confidently identify all of the studied species. A flow diagram for unambiguous systematic discrimination was also prepared (Carrera et al., 2011). According to this scheme, the presence/absence of several peptide biomarkers achieves the following: (1) the identification of any member of the Merlucciidae family that is present in the sample, (2) the discrimination between the *Merluccius* genera, (3) the classification of hake species into two groups according to their geographic distribution (American hakes and Euro-African hakes), and (4) the combined presence/absence of eight other peptide biomarkers allows the unambiguous identification of any species of the Merlucciidae family.

Moreover, particular attention has focused on the characterization and identification by MS-based biomarker discovery of several other peptide markers that can discriminate among all commercial species in the Merlucciidae family. In fact, using the same classical bottom-up proteomic approach, the characterization by de novo peptide sequencing of the NDK (Carrera et al., 2007) and aldolase protein variants (Carrera et al., 2009) allowed the characterization of additional species-specific peptides that also can be used for fish authentication purposes. These peptide biomarkers could be used to develop easy-to-use kits based on antibodies or could be monitored by MS using an innovative strategy, which is described in the following for the next phase of our pipeline.

19.3.2 Target-Driven Phase

In the second phase of the proteomic pipeline used in our laboratory for fish authentication (Fig. 19.2), we developed a new targeted MS-based strategy for the rapid monitoring of the species-specific peptide biomarkers that were found in the discovery phase (Carrera et al., 2011).

The performance of this target-driven method was established in terms of the unequivocal identification of all commercial fish species belonging to the Merlucciidae family. The method is based on (1) the purification of PRVBs by heat treatment (time required, 45 min), (2) their accelerated tryptic digestion using high-intensity focused ultrasound (HIFU) (time required, 2 min), and (3) the monitoring of 11 PRVB peptide biomarkers by selected tandem mass spectrometry MS/MS ion monitoring (SMIM) using a linear ion trap (LIT) mass spectrometer (time required, 60 min). Each step was individually adjusted to minimize the time of analysis. Thus, PRVBs, considered the best protein biomarker for the authentication of Merlucciidae species, were purified from the sarcoplasmic extracts by exploiting their thermostability (Kawai et al., 1992). After treatment with heat (70°C, 5 min), most of the identified peptides corresponded to PRVBs (77.87%). These results demonstrated that heat treatment is a simple, rapid, and effective procedure to purify and selectively enrich the samples in PRVBs. The purified PRVBs were digested with trypsin using a fast procedure involving HIFU. Accelerated HIFU-tryptic digestions produced results comparable to those obtained using conventional overnight incubation methods. Thus, the combination of a rapid and easy protein purification procedure with the use of HIFU for protein digestion considerably simplified and reduced the time needed for sample preparation, as was reflected in the overall time needed for monitoring. Then, a particular combination of only 11 specific transitions (m/z precursor ion → m/z fragment ion) (Fig. 19.3) resulting from the HIFU-assisted tryptic digestion of the thermostable protein PRVBs were subjected to SMIM analysis in an LIT mass spectrometer, focusing the MS/MS events on the corresponding precursor ions. Once MS/MS spectra were recorded, virtual chromatograms for all of the fragments were obtained. Tracing the highly sensitive transitions (m/z precursor ion → m/z fragment ion) for each peptide biomarker enables the unequivocal identification of all Merlucciidae species (Fig. 19.3). In addition, use of the SMIM mode for scanning allowed us to obtain all of the MS/MS information necessary to validate the sequence of the peptide biomarkers. An additional validation step using commercial fish products successfully demonstrated the applicability of this new targeted strategy for the rapid detection of mislabeling practices in both fresh and processed fish products.

Using this new strategy, we demonstrated that all relevant fish species belonging to the Merlucciidae family that are present in any seafood product can be unequivocally identified in less than 2 h.

19.4 Conclusions and Future Outlook

This chapter summarizes the power of proteomic methodologies in the identification of commercial fish species. The two consecutive steps described here (the discovery phase and the target-driven phase) enable the identification and characterization of species-specific peptides that can be monitored by MS, thus allowing the unequivocal and rapid identification of the fish species present in any seafood product. Currently, this pipeline is also being

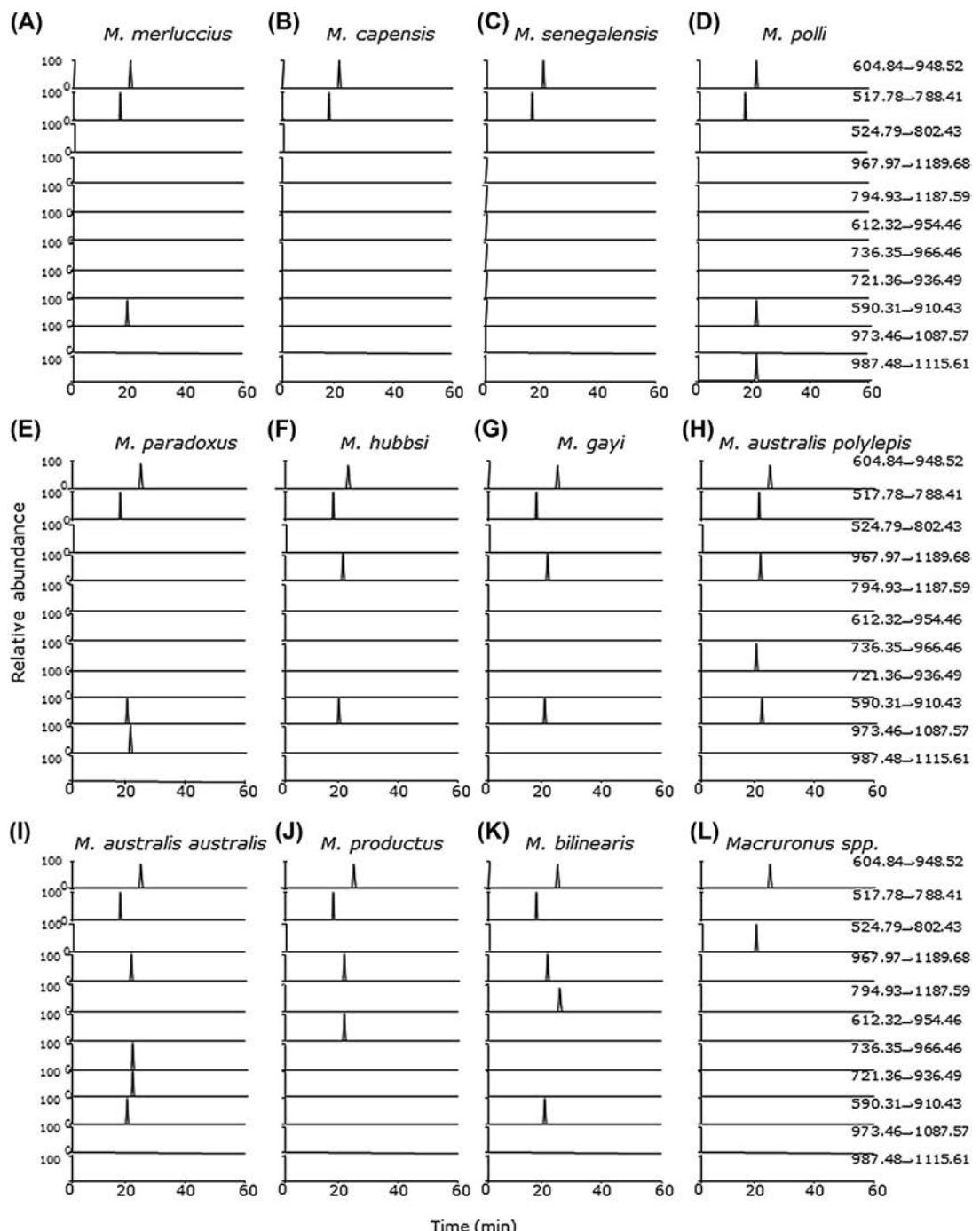


Figure 19.3

Reference SMIM traces for each Merlucciidae species; the corresponding canonical transition is plotted for each PRVB tryptic peptide biomarker. *Reprinted with permission from Carrera, M., Cañas, B., López-Ferrer, D., Piñeiro, C., Vázquez, J., Gallardo, J.M., 2011. Fast monitoring of species-specific peptide biomarkers using high-intensity-focused-ultrasound-assisted tryptic digestion and selected MS/MS ion monitoring. Anal. Chem. 83, 5688–5695. Copyright 2011 American Chemical Society.*

successfully applied in our laboratory for the identification of species belonging to Order Decapoda (Ortea et al., 2009) and described in *Chapter 20*. Moreover, the same approach is being used in order to develop a rapid and reliable method for bacterial identification in foodstuffs (Böhme et al., 2011) and for the rapid monitoring and detection of Anisakids in fish products (Carrera et al., 2016). Nevertheless, we consider that the application of this method is not restricted to food authentication purposes but may offer new opportunities for the food science sector, for example, in the detection of allergens, the characterization of bioactive peptides, and in studies of the effects caused by processing and storing on food proteins.

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Food Authentication of Seafood Species

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20.1 Introduction

The authentication of food products is currently demanding great attention from consumers, the food industry, and authorities. Consumers demand complete and truthful information about the products they eat. Food components are regularly adulterated, either deliberately or unintentionally. This may occur in the form of substitution of a species by a similar but lower-quality counterpart or mislabeling of products and therefore leading to commercial fraud (Moore et al., 2012). Assuring the authenticity of food products is a primary concern for preventing economic fraud, which affects both consumers and the industry. Additionally it is critical for health and safety reasons, since a nondeclared food ingredient may represent a potential public health risk to a specific group of consumers, such as the case for an allergenic product (Spink and Moyer, 2011).

The substitution of a high-quality species by a cheaper and often less appreciated counterpart is especially frequent in seafood products such as fish and shellfish, where the identification of the species is complex (Pascoal et al., 2008). Seafood products, including fish and shellfish species, are one of the food commodities most commonly traded at an international level. In these species, the wide biological diversity, the close phylogenetical relationships among them, and the removal of external features during processing steps render the morphological identification extremely difficult if not impossible.

In order to avoid food adulteration and food fraud, many regulations have been promulgated all over the world for food products in general (U.S. Food and Drug Administration, 2016a; European Parliament and European Council, 2002) and for seafood products in particular. For instance, the Council Regulation (EC) No 104/2000 (European Council, 1999) on the common organization of the markets in fishery and aquaculture products requires that seafood products must be labeled with the commercial name of the species at every step of the marketing chain.

For all of the previously mentioned circumstances, conclusive and accurate analytical methods are demanded in order to assure that the components in a food product are of the nature and

quality as declared by the seller, thus guaranteeing a correct and complete labeling and the authenticity of foodstuffs. Many classical instrumental techniques have been used for food authentication. For instance, high-performance liquid chromatography, gas chromatography, nuclear magnetic resonance, infrared and fluorescence spectroscopy, capillary electrophoresis, and more recently, immunological technologies (e.g., ELISA) and DNA approaches (e.g., polymerase chain reaction, restriction fragment length polymorphism, DNA barcoding) have been applied to species authentication and/or geographical origin determination (Rasmussen and Morrissey, 2008; Drivelos and Georgiou, 2012). Over the last years, omics-based techniques (metabolomics, DNA-based, and proteomics) are emerging as alternative technologies. In this sense, proteomics approaches can help to overcome the drawbacks of the classical techniques, improving accuracy, sensitivity, throughput, and multiplexing capacity (Gallardo et al., 2013). Proteomics, the large-scale analysis of proteins in a particular biological system at a particular time (Pandey and Mann, 2000), has been proposed as a fast and sensitive mean for food authenticity assessment (Piñeiro et al., 2003). Proteins can act as markers for many features of the food products from farm to fork. Proteomics-based tools can be applied for the discovery of new protein markers, such as species-specific polypeptides. In a second step, accurate and sensitive analytical assays targeting the previously identified markers can be developed and validated in order to detect and even quantify food adulteration and deceptive practices (Gallardo et al., 2013) (Fig. 20.1). In a similar manner to that used in DNA-based analyses, MS/MS-based methods enable discrimination at the sequence level, making it possible to differentiate between closely related species. Proteomics-based methods can overcome one of the major drawbacks of DNA methods, that is the degradation of DNA in highly processed samples, since (1) marker peptides would be quite stable against processing (Buckley et al., 2013); (2) modifications in amino acid sequence due to food processing (nonenzymatic PTMs) can be monitored; and (3) heat stable proteins can be selected as targets. These facts should encourage researchers to develop standard proteomics-based protocols to assess food authenticity.

This chapter is a comprehensive and updated overview of the specific applications, drawbacks, and challenges regarding the study of proteins for the species authentication of seafood species by means of proteomics approaches.

20.2 *Gel-Based Methods*

Although currently MS is the method of choice for the analysis of proteins, gel-based electrophoretic methods are still frequently used and reported. Since the late 1980s, SDS-PAGE and isoelectric focusing (IEF) have showed their potential for differentiating fish and shellfish species using raw or cooked material. In SDS-PAGE, the proteins are separated according to molecular weight, and therefore the protein profiles from different samples can be compared. In fact IEF was adopted by the Association of Official Analytical Chemists as the only official

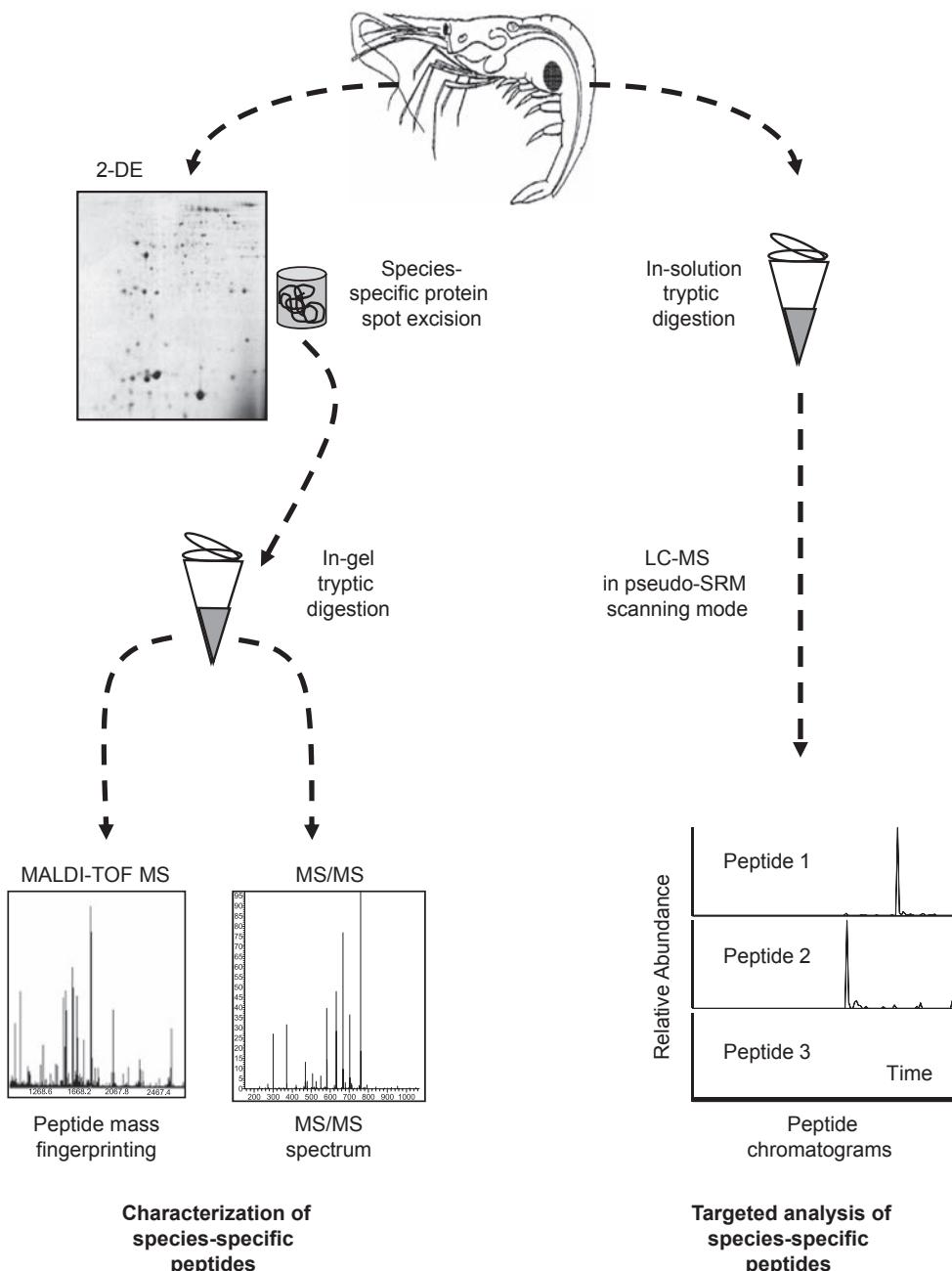


Figure 20.1

Proteomics workflows used in our laboratory for the discovery and characterization of species-specific markers and for the subsequent development of fast methods for species authentication. Reprinted from Ortea, I., Pascoal, A., Cañas, B., Gallardo, J.M., Barros-Velázquez, J., Calo-Mata, P., 2012. *Food authentication of commercially-relevant shrimp and prawn species: From classical methods to foodomics*.

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validated method for species identification (AOAC, 1984), which consists on the separation of proteins in a polyacrylamide gel according to their isoelectric points (pI). An et al. (1988, 1989) demonstrated that electrophoretic profiles of muscle proteins could be used to differentiate very closely related species, even when samples had been heated, although the studies were limited to only three shrimp species. SDS-PAGE and IEF also succeed in differentiating shrimp from crab (Civera and Parisi, 1991) and from fish, lobster, and cephalopod meat (Rehbein, 1995). These studies also demonstrated that no single universal protein extraction method and electrophoretic system was appropriate for all cases, since SDS extracts worked better for cooked samples while the water-soluble extract (sarcoplasmic proteins) performed better for nonheated samples. In an interlaboratory study, the performance of standardized SDS-PAGE and IEF in order to identify 10 commercial fish species after cooking was evaluated (Etienne et al., 2000). Both methods gave different performance depending on the species, and therefore the complementary use of both methods was recommended. One of the main issues identified for the electrophoretic approaches was the need for appropriate reference samples to be run in parallel with the unknown sample. Alternatively, the use of reference gel images stored at public repositories has been proposed, for example, the Gel Library from the US Food and Drug Administration (U.S. Food and Drug Administration, 2016b). In this sense, Bossier and Cooreman (2000), built a databank of IEF protein profiles from 17 flatfish species and used it for the authentication of 17 commercial fish fillets. Up to three specimens were analyzed for each species, and interspecies differences were found to be larger than intraspecies differences. By separating the sarcoplasmic proteins in IEF gels, Renon et al. (2005) were able to differentiate between swordfish, blue marlin, and Mediterranean spearfish in fresh, frozen, and smoked fillets, although few biological replicates were used as references. Fourteen shrimp and prawn species of commercial interest were identified using native IEF analysis of the water-soluble muscular proteins (Ortea et al., 2010). The pI of the marker protein bands were measured by image analysis and reported, providing a reference dataset enabling the identification of unknown samples. These marker proteins were subsequently identified by MS as sarcoplasmic calcium-binding proteins, a class of heat stable proteins found in fish and crustacean muscle, and reported as allergens (Shiomi et al., 2008).

Two-dimensional electrophoresis (2-DE) has also been demonstrated to be a valuable tool for the assessment of species authentication. In 2-DE, proteins are consecutively separated according to pI and molecular weight, and therefore it can differentiate species that show an identical IEF profile. Moreover, using 2-DE, individual proteins can be isolated and subsequently identified by MS. Since the late 1990s, 2-DE profiles of sarcoplasmic proteins have been used for discriminating fish species. Differences in the amino acid sequences of the parvalbumins, a group of heat-resistant sarcoplasmic proteins, were utilized in the differentiation of gadoid fishes (Piñeiro et al., 1998), flatfishes (Piñeiro et al., 1999), and hake species (Piñeiro et al., 2001). The analysis of the 2-DE protein profiles from two marine mussel species led to the identification of 37 protein spots that were differentially expressed, 15 of which were identified by means of MS analysis

(López et al., 2002a). In a subsequent study combining 2-DE protein isolation and MS protein identification, species-specific proteins for the three most traded European mussel species were reported (López et al., 2002b). Myosin light chains isoforms were reported to differentiate between cod, haddock, mackerel, saithe, and capelin (Martinez and Friis, 2004), and triose phosphate isomerase was proposed as marker for the tuna species *Thunnus thynnus* (Pepe et al., 2010). Chen et al. (2004) were also able to differentiate between five puffer fish species using 2-DE, although in this case the species-specific protein spots were not subjected to MS analysis and therefore were not identified. 2-DE profiles of sarcoplasmic proteins were used to investigate differences in hake species (Carrera et al., 2006, 2007) and Decapoda species (Ortea et al., 2009a). Gel-based applications of proteomics to seafood species authentication are compiled in Table 20.1.

20.3 MS-Based Methods

Electrophoretic approaches show some limitations, including the challenges with variation in protein gel spot abundance arising from protein modification and/or degradation that can occur during food processing. The low resolving power of electrophoresis can be challenging

Table 20.1: Summary of the gel-based methods applied to food authentication of seafood species.

| Technique | Food Products (Number of Species) | Target Proteins | Reference(s) |
|---------------|--|---|---|
| SDS-PAGE | Shrimp species ($n=3$) Shrimp and crab meat | Muscular proteins Muscular proteins | An et al. (1988) Civera and Parisi (1991) |
| IEF | Shrimp species ($n=3$) Flatfish species ($n=17$) | Muscular proteins Muscular proteins | An et al. (1989) Bossier and Cooreman (2000) |
| | Swordfish, blue marlin, and spearfish | Sarcoplasmic proteins | Renon et al. (2005) |
| | Shrimp and prawn species ($n=14$) | Sarcoplasmic calcium-binding proteins | Ortea et al. (2010) |
| SDS-PAGE, IEF | Fish species ($n=10$) Cod, redfish, lobster, shrimp, mussel, squid, salmon, trout | Muscular proteins Sarcoplasmic and myofibrillar proteins | Etienne et al. (2000) Rehbein (1995) |
| 2-DE | Gadoid fishes ($n=8$) Flatfish species ($n=9$) Hake species | Parvalbumins | Piñeiro et al. (1998) Piñeiro et al. (1999) Piñeiro et al. (2001) |
| | Mussel species ($n=2$) Cod, saithe, haddock, mackerel, and capelin | Muscular proteins Myosin light chains | López et al. (2002a) Martinez and Friis (2004) |
| | Puffer fish species ($n=5$) Tuna species ($n=3$) | Muscle proteins Triose phosphate isomerase | Chen et al. (2004) Pepe et al. (2010) |

2-DE, two-dimensional electrophoresis; IEF, isoelectric focusing.

when examining protein orthologues from closely related species and moreover when examining mixtures of several species. Implicit is the need for reference samples to enable accurate identification. For these reasons, authentication methods using MS have gained much attention recently. MS is a robust and powerful technique for protein detection, identification, and characterization, and therefore a great advantage of MS-based techniques over 2-DE is that the identification of the analyzed proteins is obtained. Moreover, the MS spectra can be used as species-specific fingerprints, and therefore they are helpful for the direct comparison of the samples. Two main MS approaches have been used for species authentication, both of them based on the MS analysis of the peptides resulting from tryptic digestion of the protein(s) of interest. These two approaches are peptide mass fingerprinting (PMF), where the masses of the peptides are measured and act as a fingerprint for that protein (Pappin et al., 1993), and tandem mass spectrometry (MS/MS), where the spectra obtained reflect the amino acid sequence of the peptides (Eng et al., 1994).

PMF and MS/MS analyses of proteins previously isolated by 2-DE were assessed in the classification of hake species, and the parvalbumins and the protein NDK B were proposed as species-specific markers (Carrera et al., 2006, 2007). Likewise, PMF and MS/MS spectra of the protein arginine kinase were proposed as authentication markers for the seven most commercially important shrimp and prawn species (Ortea et al., 2009a,b; Pascoal et al., 2012). The sequences of the species-specific peptides were reported and served as the foundation for the identification of unknown samples. Using a similar approach, combining PMF and MS/MS of previously 2-DE isolated proteins, differences in the muscular proteins from two commercial fish species from the genus *Sperata* were analyzed (Barik et al., 2013). Eleven species-specific proteins were identified, and of these triosephosphate isomerase isoforms were proposed as species-specific markers. Using MS/MS, Wulff et al. (2013) were able to correctly identify the species present in both fresh and heavily processed samples. They first generated a reference MS/MS spectral library from 22 fish species and then obtained the spectra for unknown samples and matched them to the library.

In addition to PMF and MS/MS, other types of MS analysis have been applied to food authentication, including protein profiling. Using this method, the masses of the intact proteins are obtained and compared between samples. Mazzeo et al. (2008) developed a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) protein profiling method able to obtain specific profiles for 25 different fish species. Masses around 11 kDa, which were subsequently identified as parvalbumins, were again proposed as specific biomarkers. The same method allowed the discrimination of three commercial fish species common in Italian lakes using muscle and liver protein extracts (Volta et al., 2012). Salla and Murray (2013) reported a similar method, employing computer comparison of the MALDI-TOF protein profiles of unknown samples with a profile library built from reference specimens from six shrimp samples. The protein profiling approach is simple and rapid due to (1) fast sample preparation steps employed in order to avoid protein digestion and (2) the high-throughput nature of MALDI analysis.

Once marker peptides or proteins are identified, sensitive, fast, and easy-to-use targeted MS methods for the detection of the species-specific markers may be developed. Examples include selected ion monitoring (SIM), where only the peptides or proteins with selected m/z values are detected, and selected reaction monitoring (SRM) or pseudo-SRM, where only those peptides of interest are fragmented and recorded. In this sense, López et al. (2002b) reported an SIM method for the monitoring of the tropomyosin peptides that had been previously identified and characterized, specific to three commercial mussel species. Ortea et al. (2011) described a pseudo-SRM methodology, combining fast sample preparation and monitoring previously characterized peptide markers, in which they were able to identify the seven most commercially important shrimp species in less than 90 min (Fig. 20.2). A similar method was also reported for differentiating several commercial hake species (Carrera et al., 2011), as described in Chapter 19, in both raw and cooked products. MS-based applications of proteomics to seafood species authentication are compiled in Table 20.2.

20.4 Future Trends

The power of MS-based proteomics as an alternative method for assessing food authenticity lies in its sensitivity, discriminating power, robust nature, high-throughput nature, and multiplexing capacity. However, there are several key considerations that most of the studies published to date lack, such as the standardization of sample treatment and analytical procedures, the use of certified reference materials, a careful reporting of technical details, the inclusion of a proper number of representative samples (biological replicates and technical replicates), and the use of adequate statistical analysis. To assess that a change is produced by interspecies variability and not by intraspecies differences, all possible sources of variability, for example, changes caused by food processing, population variability, stational changes over the year, development status, or pathological conditions, should be addressed.

The development and implementation of more powerful MS instrumentation is continually increasing the sensitivity and specificity of the assays. The requirements of a food authentication assay for routine use should be fast, easy-to-use, and cheap. The assay should be able to detect small fragments that are present in both raw and highly processed products. It is highly desirable to be able to multiplex several authenticity markers in a single assay and to be able to detect, but also to perform quantification of the markers present in a foodstuff. Targeted MS/MS acquisition methods, such as SRM and pseudo-SRM, can help to meet all of these challenges, as they allow the high-throughput, sensitive multiplexed identification, and quantification of marker peptides in complex matrices (Gallien et al., 2011). Data independent acquisition MS modes promise much for the future, since they improve the analytical coverage and allow the quantitation of low abundance peptides in the same run that is used for protein identification, therefore merging the initial marker discovery step and the subsequent hypothesis-driven validation in a single platform (Geromanos et al., 2009; Gillet et al., 2012). Furthermore, future improvements in high-resolution MS instruments and MS/MS fragmentation techniques will make possible the

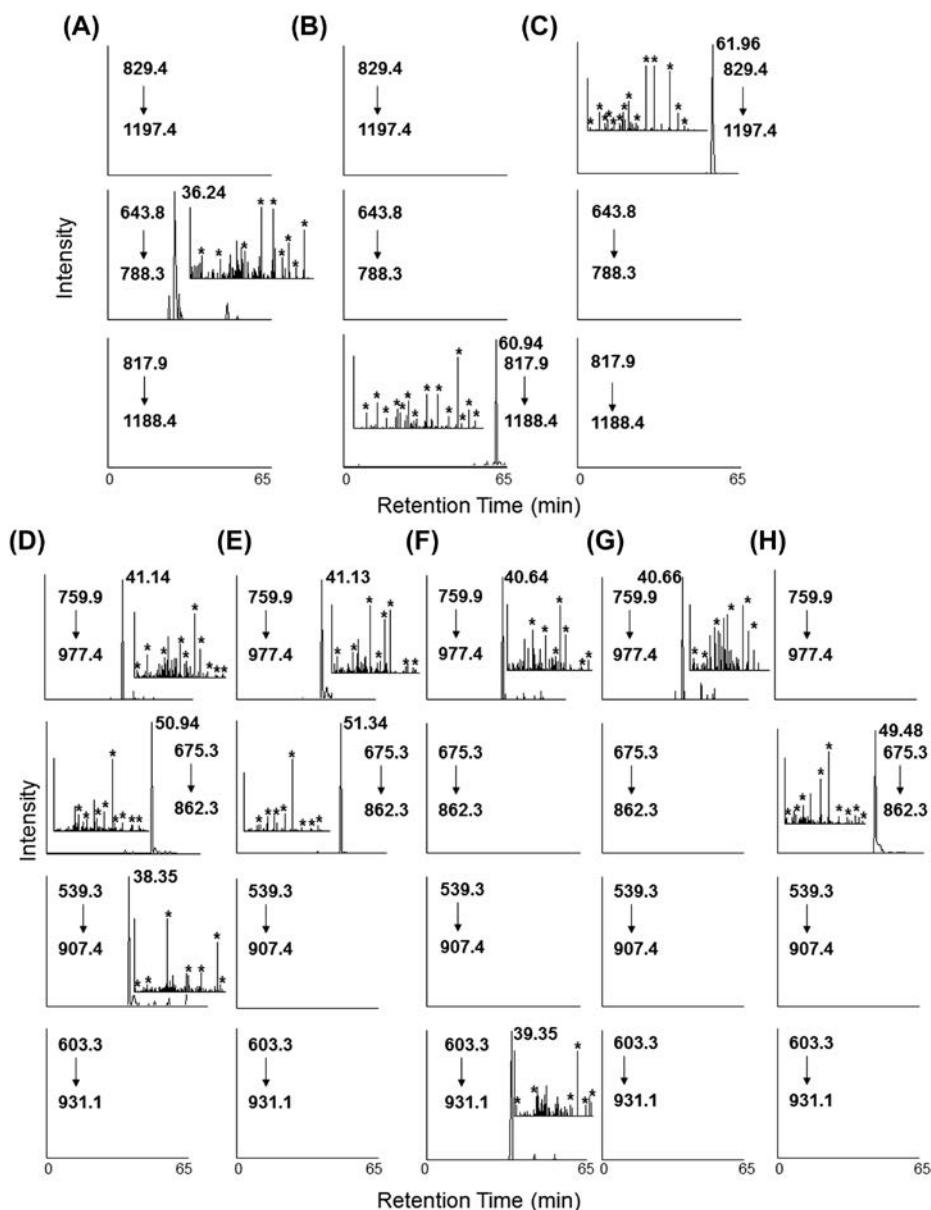


Figure 20.2

Discrimination of seven closely related commercial shrimp species by pseudo-SRM of seven AK tryptic peptides. The protein extracts were subjected to trypsin digestion followed by LC-MS/MS. The mass spectrometer was set to perform a continuous fragmentation of the ions at m/z 829.4, 643.8, 817.9, 759.9, 675.3, 539.3, and 603.3. The traces of the selected product ions as a function of retention time are plotted in the chromatograms for the discrimination of (A) *Pleoticus muelleri*, (B) *Pandalus borealis*, (C) family Penaeidae species, (D) *Penaeus monodon*, (E) *Litopenaeus vannamei*, (F) *Fenneropenaeus merguiensis*, (G) *Fenneropenaeus indicus*, and (H) *Farfantepenaeus notialis*. Insets show the corresponding averaged MS/MS spectra (relative intensity vs. m/z) around the peak apex; asterisks mark fragment peaks matching theoretical ion masses from y- or b-series. Reprinted from Ortea, I., Cañas, B., Gallardo, J.M., 2011. Selected tandem mass spectrometry ion monitoring for the fast identification of seafood species. *J. Chromatogr. A* 1218 (28), 4445–4451, Copyright (2011), with permission from Elsevier.

Table 20.2: Summary of the MS-based methods applied to food authentication of seafood species.

| Technique | Food Products (Number of Species) | Target Proteins | Reference(s) |
|-----------------------------|--|--|-------------------------|
| MALDI-TOF protein profiling | Commercial fish species ($n=25$) | Parvalbumins | Mazzeo et al. (2008) |
| | Commercial freshwater fish species ($n=3$) | Muscle and liver proteins | Volta et al. (2012) |
| | Shrimp samples ($n=6$) | Muscle proteins | Salla and Murray (2013) |
| | Hake species ($n=5$) | Parvalbumins and nucleoside diphosphate kinase A | Piñeiro et al. (2001) |
| | European mussels ($n=3$) | Tropomyosin | López et al. (2002b) |
| | Merlucciidae hake and grenadier species ($n=10$) | Parvalbumins | Carrera et al. (2006) |
| | Merlucciidae hakes and grenadiers ($n=11$) | Nucleoside diphosphate kinase B | Carrera et al. (2007) |
| | Shrimp and prawn species ($n=6$) | Arginine kinase | Ortea et al. (2009a) |
| | Northern shrimp | Arginine kinase | Pascoal et al. (2012) |
| | River fish species ($n=2$) | Triosephosphate isomerase | Barik et al. (2013) |
| MS/MS | Hake species ($n=5$) | Parvalbumins and nucleoside diphosphate kinase A | Piñeiro et al. (2001) |
| | European mussels ($n=3$) | Tropomyosin | López et al. (2002b) |
| | Merlucciidae hakes and grenadiers ($n=11$) | Nucleoside diphosphate kinase B | Carrera et al. (2007) |
| | Shrimp and prawn species ($n=17$) | Arginine kinase | Ortea et al. (2009b) |
| | Northern shrimp | Arginine kinase | Pascoal et al. (2012) |
| | Fish species ($n=22$) | Muscle proteins | Wulff et al. (2013) |
| | River fish species ($n=2$) | Triose phosphate isomerase | Barik et al. (2013) |
| SIM Pseudo-SRM | European mussels ($n=3$) | tropomyosin | López et al. (2002b) |
| | Shrimp and prawn species ($n=7$) | Arginine kinase | Ortea et al. (2011) |
| | Merlucciidae hakes and grenadiers ($n=11$) | Parvalbumins | Carrera et al. (2011) |

MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS/MS, tandem mass spectrometry; PMF, peptide mass fingerprinting; pseudo-SRM, pseudo-selected reaction monitoring; SIM, selected ion monitoring.

application of top-down proteomics (McLafferty et al., 2007) to the high-throughput analysis of food products at the intact protein level. Finally, the application of new bioinformatics tools to food proteomes and matrices will play an important role in the implementation of integrative biology and systems biology in food technology and nutrition areas.

The demonstrated potential of proteomics-related approaches indicates that they will play a key role in the authentication assessment of seafood products, from the discovery of species-specific markers to the development and implementation of standardized methods for the routine testing of food products, therefore helping to comply with labeling regulations and fighting against food adulteration and fraud, ultimately improving consumer confidence and reducing potential health risks.

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Proteomic Analysis of Disease in Sydney Rock Oysters

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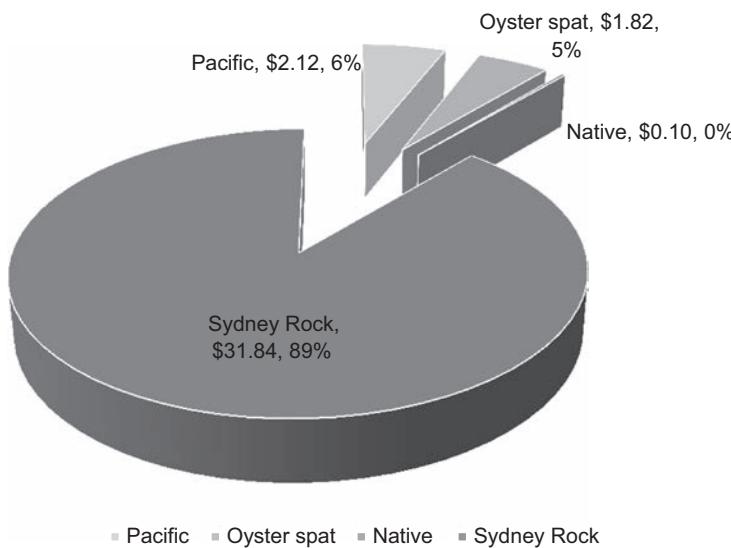
21.1 Introduction

21.1.1 Oysters and the Aquaculture Industry

The aquaculture industry is responsible for ensuring the sustainable use of aquaculture resources which includes crustaceans, freshwater and marine fish, hatcheries, and oysters. For the period 2014/15 the total value of the NSW aquaculture industry was estimated to be worth more than \$60 million (Livingstone, 2016).

More than half of the NSW aquaculture production is contributed by the oyster industry. The oyster industry includes the farming of three different species of oysters—Sydney rock oysters (SRO), pacific oysters, and native oysters. According to the aquaculture production report of 2014/15 the oyster industry contributed more than \$40 million to the total production value of the aquaculture industry. To bring the importance of the research in perspective for the NSW industry the SROs contributed \$32 million to the oyster industry. The breakdown of production value of different oyster species according to 2015 figures is shown in Fig. 21.1.

Environmental factors such as temperature and salinity play a key role in determining the yield of oyster farming. Like any other crop, oyster farming is greatly dependent on the quality of water. The quality of water is one of the most important factors which determines the production value of oysters. There are many ways in which water quality is assessed: in terms of the water quantity, the amount of nutrients, the types of nutrients, and/or the suitability for the organism including the pH and temperature. With global climate changing as a result of human activities the estuaries are changing too and the production of oysters is affected. Environmental changes such as seasonal temperature and rainfall variations also play a role in enhancing the effect of certain disease such as Queensland Unknown (QX) disease (Rubio et al., 2013) and Winter Mortality (WM) disease (Spiers et al., 2014) where temperature and rainfall have been shown to exacerbate the consequences.

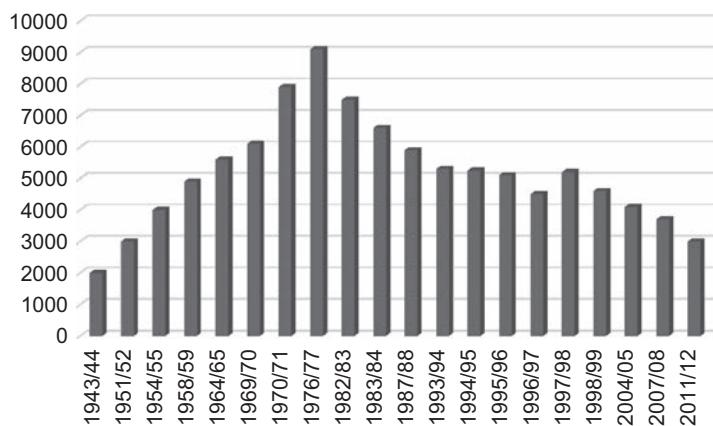
**Figure 21.1**

Sydney rock oyster production annual value in millions. *Modified from Livingstone, S., 2016. Aquaculture Production Report 2014–2015, NSW Department of Primary Industries.*

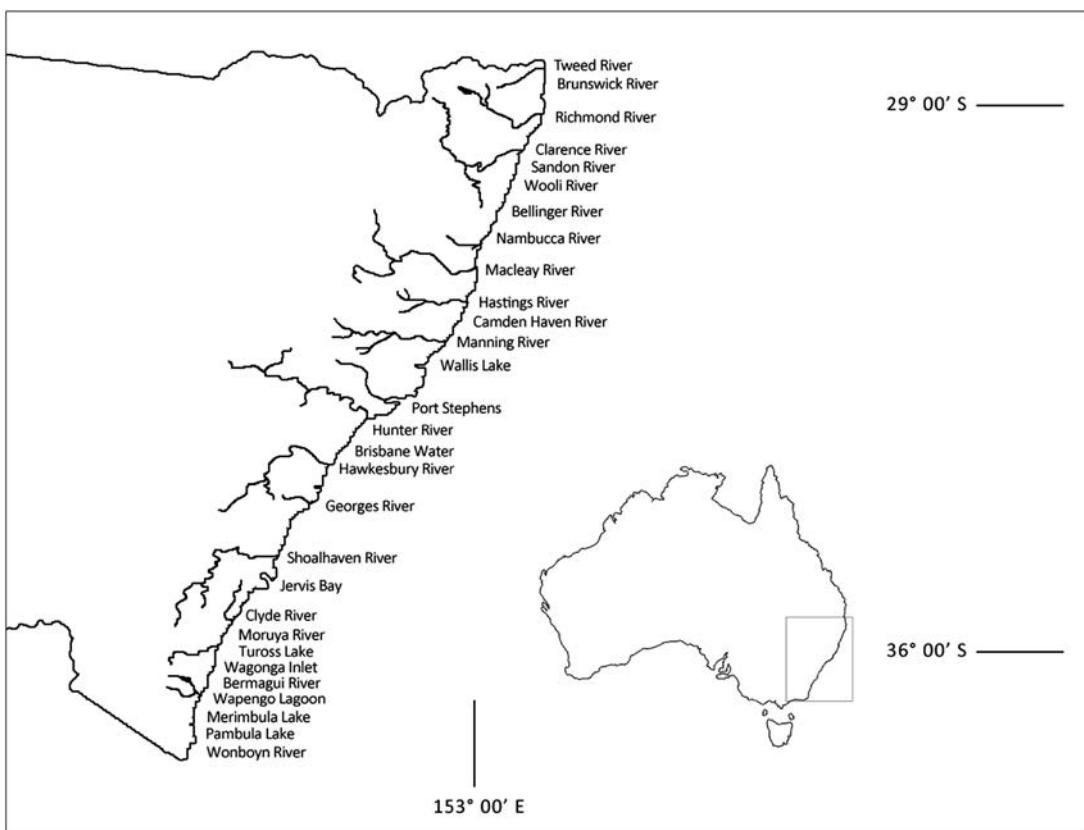
Apart from the environment, human activities are quite damaging to oyster production. As a result of urbanization and industrialization the water in surrounding areas becomes contaminated with various chemical species. Studies have focused in detail on how prolonged exposure to these contaminants affect the oysters (Melwani et al., 2016; Muralidharan et al., 2012; Thompson et al., 2011).

Fig. 21.2 shows the annual production of oysters in NSW over the last 70 years. SRO commercial production in Australia has an even longer history, dating back to 1870, when they were first used for commercial production (Nell, 1993). Since then the industry has increased greatly, up to a peak in the mid-1970s. However, this was followed by a gradual decline in the production of oysters, which has been attributed to disease outbreaks resulting in the mass destruction of farmed oysters in the estuaries. Two major diseases, QX and WM, were the major reason for the losses, with QX disease responsible for more damage.

The first major outbreak of QX disease in NSW was reported in Georges River in 1994 (Adlard and Ernst, 1995). This outbreak almost wiped out the SRO farming in this estuary, along with the previously established breeding colonies which were established as resistant lines against WM. As a result of which, in 1997, the selective breeding program was forced to include the development of QX-resistant lines essential for the sustainable growth and maintenance of the SRO industry (Nell et al., 2000). A map showing the major oyster farming estuaries in NSW is presented in Fig. 21.3.

**Figure 21.2**

Historical NSW annual oyster production in tons. Modified from Livingstone, S., 2014. NSW Oyster Industry Sustainable Aquaculture Strategy, second ed. NSW Department of Primary Industries.

**Figure 21.3**

Major oyster farming estuaries in NSW. Modified from Livingstone, S., 2014. NSW Oyster Industry Sustainable Aquaculture Strategy, second ed. NSW Department of Primary Industries.

The NSW oyster industry has stipulated a production target of 7500 tons of premium oyster products. The policy which defines the rules and guidelines to help achieve this target is known as the NSW Oyster Industry Sustainable Aquaculture Strategy (OISAS). This strategy defines a set of guidelines to ensure the sustainable usage of the natural resources, as well as identifying estuaries suitable for oyster farming ([Livingstone, 2014](#)).

21.1.2 Proteomics in Oysters

Proteomic analysis is widely used in assessing environmental impact in marine fauna and flora. Marine organisms, such as bivalve mollusks, have been used as a biomonitoring tool to assess the impact of heavy metal pollutants, changes in water temperature, and variation in carbon dioxide levels. The ability of bivalve mollusks to bioaccumulate contaminants and to display a time-dependent and dosage-dependent relationship with respect to the contaminants means that they can be used to monitor the degree of pollutants ([Thompson et al., 2011](#)).

Proteomics relies largely on availability of the sequenced genome of the organism under consideration. Until 2012, oyster proteomics studies were limited by the lack of a sequenced genome for oysters. [Zhang et al. \(2012\)](#) successfully sequenced the genome of *Crassostrea gigas* which has accelerated oyster proteomics research. One major focus of proteomics study in oysters has been to study the effect of different types of stressors. Stressor can be defined as any phenomenon imposed externally which affects the normal functionality of the organisms. It could be a change in environmental conditions such as ocean acidification, change in temperature of water, or it could be an anthropogenic factor such as metal contamination from industry effluents. Additionally, it could be a disease caused by a parasite. In recent times oyster production has been threatened by a combination of all these stressors. The increasing human interference with the environment resulting in the global warming, ocean acidification, and metal deposition in the estuaries has deteriorated things further.

21.1.3 Environmental Stressors

A high-throughput quantitative proteomic analysis, employing iTRAQ and LC-MS/MS, of *C. gigas* larvae exposed to multiple climate stressors (temperature and acidity) revealed significant change in proteins involved in different biological functions: calcification, metabolic processes and oxidative stress ([Dineshram et al., 2016](#)). Another work investigating the nonadditive effect of ocean acidification and warming on the larval proteome of *C. gigas* identified 12 different proteins using classical 2-DE in conjunction with nano-LC-MS/MS ([Harney et al., 2016](#)). The effect of three different stressors (high temperature, low salinity, and aerial exposure) on the proteome of oyster gills revealed 98 differentially regulated gill proteins. These proteins were reported to be involved in many biological pathways such as metabolism, ion transportation, immune responses, DNA duplication, and protein synthesis ([Zhang et al., 2015](#)).

Among the stressors, the effect of increased CO₂ on the proteome of oysters has been extensively studied. In a work studying the effect of elevated CO₂ on *C. gigas* proteome using 2-DE and MALDI-TOF/TOF, it was found that an increase in CO₂ levels affects the energy metabolism and cytoskeleton-related processes (Wei et al., 2015a, 2015b). This work was supported by another study where the elevated CO₂ affected the antioxidant response, carbohydrate metabolism, and transcription and translation pathways (Timmins-Schiffman et al., 2014).

A comparative study using 2-DE analysis of two populations of oysters (selected for fast growth and disease resistance, as opposed to wild SRO) to study the effect of elevated CO₂ revealed that shell length of the wild population was affected more as compared to selected oysters. On the other hand, the selected oyster populations were more resilient toward change in shell length in response to increased CO₂ (375–1000 ppm) (Parker et al., 2011). A recent proteomics study using a combination of 2-DE and LC-MS/MS on the same populations revealed substantial but opposite responses of selected and wild oysters in response to elevated CO₂, with potential cellular dysfunction in the select population (Thompson et al., 2015).

21.1.4 Anthropogenic Impacts

Muralidharan et al. (2012) and Thompson et al. (2011, 2012) studied the effect of metal (cadmium, copper, lead, and zinc) contamination on SRO using both 2-DE and label-free shotgun proteomics. In an attempt to develop protein biomarkers, they identified a unique set of proteins indicative of different types of metal contamination. This proteomics study was corroborated by two transcriptomics studies using quantitative PCR on SRO (Taylor et al., 2013, 2015). Apart from edible oysters, some notable work has also been performed on nonedible oysters such as pearl oysters (*Pinctada martensii*). This included a proteomic evaluation performed on the toxicological effect of benzo[a]pyrene (BaP) using 2-DE and MALDI-TOF/TOF. The proteins that were affected as a result of BaP were involved in biological functions including energy metabolism, cytoskeleton, cell injury, oxidative stress, and signal transduction (Chen et al., 2016). It is evident that use of 2-DE and different mass spectrometry techniques in oyster proteomics research have contributed a great deal to the knowledge of the disease and stress responses. Stress studies relies heavily on comparative study of organismal proteome response to different stressors. In large-scale comparative studies, high-throughput technologies such as shotgun proteomics have proved very efficient. With continuous evolution in proteomics research, our understanding of oyster biology in the context of disease and stress response will continue to improve.

Fig. 21.4 presents the most common genes identified in oysters in response to 10 different kind of environmental stress (Anderson et al., 2015). This meta-analysis identified over 400 genes differentially regulated in oysters in response to various environmental stressors.

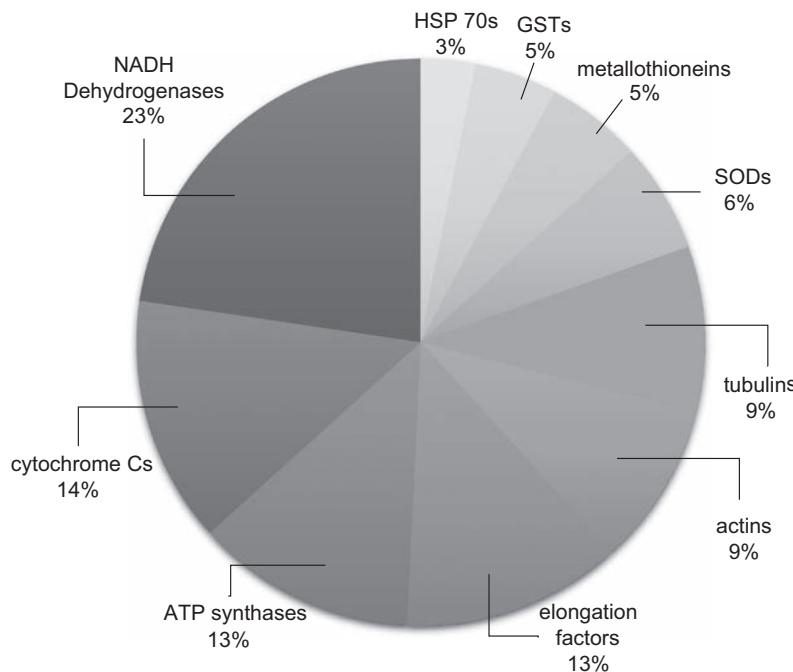


Figure 21.4

Top 10 differentially regulated genes in oysters exposed to 10 different environmental stressors. Modified from Anderson, K., Taylor, D., Thompson, E., Melwani, A., Nair, S., Raftos, D.A., 2015. Meta-analysis of studies using suppression subtractive hybridization and microarrays to investigate the effects of environmental stress on gene transcription in oysters. *PLoS One* 10, e0118839.

21.2 Proteomic Analysis of Diseases in Oysters

21.2.1 Proteomics in Biomarker Discovery

The expressed proteome of an organism reflects the genome sequence, but the protein expression is not solely dependent on genomic information (Anderson and Seilhamer, 1997), it may vary depending on many molecular or cellular conditions. Hence, the genome and proteome share a complex relationship (Rogers et al., 2008). Proteomics has contributed to the discovery of biomarkers addressing a variety of concerns in aquaculture, or other production systems, such as disease diagnosis and environmental effects.

A biomarker can be defined as a quantifiable unit, which represents a particular biological state concerning the study under consideration (Anderson and Anderson, 1998; Rifai et al., 2006). Proteomic examination has broad applications in biomonitoring of the stress response of organisms, as any changes in the level of protein present in body fluids in response to stress can be easily monitored (Blackstock and Weir, 1999). It should be noted that the biological system under investigation can be monitored using mRNA (transcriptomics), proteins

(proteomics), or metabolites (metabolomics). However, the results of each, though interrelated, do not necessarily correlate with each other.

21.2.2 *Important Diseases in Oysters*

Apart from the anthropogenic and environmental factors affecting the well-being of oysters there are certain diseases responsible for mass mortalities. European flat oysters (*Ostrea edulis*) were affected by two different pathogens. The protozoan *Marteilia refringens* was responsible for confining the production of flat oysters to the subtidal areas and in 1979 another protozoan parasite, *Bonamia ostreae* infection affected the farming (Naciri-Graven et al., 1998). American oysters (*Crassostrea virginica*) were affected by a haplosporidian parasite, *Minchinia nelsoni* (MSX). In 1958–59 more than 90% of the native oysters were killed in the high-salinity regions and 50%–70% in the lower salinity regions (Ford and Haskin, 1987).

Simonian et al. (2009a, 2009b) looked into the QX disease of SRO which is caused by *Marteilia sydneyi*. The proteomes of two oyster populations, one selected against the disease and the other unselected, were compared to look for differential protein expression. The study was, however, limited by the unavailability of a complete annotated genome sequence. It was shown in an earlier study that selective breeding of SRO for QX disease negatively selects one of the isoforms of a defensive enzyme, phenoloxidase (Newton et al., 2004). Previous studies have shown that selected SRO have heightened phagolysosomal activity against the protozoan *M. sydneyi*, which is why they are more resistant to the QX disease (Kuchel et al., 2010).

In a transcriptomics study performed on SRO comparing oysters selected for QX resistance versus unselected oysters, it was revealed that certain proteins showed increased expression in resistant oysters, specifically extracellular superoxide dismutase (ecSOD) and a small heat shock protein (sHSP), while the expression of a few proteins (peroxiredoxin 6, Prx6; and interferon inhibiting cytokine factor) were significantly decreased. Results of this study helped to understand the underlying mechanism of resistance (Green et al., 2009).

As discussed above, SRO are susceptible to QX while *C. gigas* are completely unaffected by the disease. However, there are other instances where SRO are unaffected by a disease causing agent while *C. gigas* are susceptible to it. Pacific oysters (*C. gigas*) are endangered by the infection of ostreid herpesviruses, while SRO are naturally immune to the infection. A proteome analysis using iTRAQ to study the response of these two species to generic dsRNA proved that there were substantial differences between the different oyster species. For example, a unique set of proteins involved in TLR signaling pathway were identified in *Saccostrea glomerata* which were absent in *C. gigas*. These results at the molecular level help to explain the different susceptibility of the two oyster species to the herpesvirus infection (Masood et al., 2016). To understand the pathogenesis of the infection of herpesvirus better, a

comparative study using 2-DE and nano-LC-MS/MS was performed on Pacific oysters. A comparison of two populations of *C. gigas*, with or without disease challenge, revealed a significant change in proteins associated with many important biological pathways, such as cytoskeleton organization, stress response, protein processes, signaling pathways, and energy metabolism (Corporeau et al., 2014).

21.2.3 Winter Mortality (WM) Disease and Queensland Unknown (QX) Disease

WM is a parasite-borne disease of SRO. The pathogen *Bonamia roughleyi* is responsible for WM and was first characterized by Farley et al. (1988) in a histological study of tissue samples from WM-infected oysters. The presence of *B. roughleyi* in SRO is characterized by the presence of pus-filled blisters and ulcers widespread in various tissues, including gills, palps, gonads, and the digestive system (Bower et al., 1997).

The protozoan *M. sydneyi* is responsible for QX disease in SRO. Oysters, being a filter-feeder organism, take in the free-floating parasites through gills and palps (Roubal et al., 1989). The parasite undergoes extrasporogonic development in the epithelia of the gills and palps, which is preceded by complicated cell within cell multiplication of the parasite. Following the extrasporogonic development, the parasite spreads into connective tissue and hemolymph spaces. From gills and palps, the parasite further sporulates in the digestive gland of oyster (Anderson et al., 1995; Kleeman et al., 2002; Roubal et al., 1989). Before the oyster dies the parasite passes out into the environment through the alimentary canal, ready for the next cycle of infection as sporonts (Roubal et al., 1989). The infected oysters show gross signs of infection such as thin and translucent body parts, slow growth, and yellowish digestive glands because of the presence of spores. In a recent development, the secondary host of the parasite was identified as *Nephtys australiensis* (Adlard and Nolan, 2015). This will certainly help to understand the biology of the parasite and help to curb the infection to some extent.

21.3 Case Study—Recent Work in Our Laboratory on Winter Mortality Disease

A recent proteomic study from our own laboratory on oysters selected for resistance to WM disease as compared to the unselected oyster has revealed a differential response of the oyster proteome. We compared the gills tissue proteome to gain insights into the effect of disease stress, because doing so is likely to shed light on the mechanisms of resistance. The observed changes ranged from twofold downregulation of proteins to 11.5 upregulation of proteins.

We have identified proteins involved in various biological pathways, which follows a cytoskeletal breakdown pattern similar to that observed in previous studies with different stressors. As pointed out, this breakdown is a result of the organisms attempting to cope with the stressors by increasing energy production, leading to an increase in reactive oxygen species (ROS)

(Thompson et al., 2015). In our study, there was a clear upregulation of cytoskeletal proteins, confirming the hypothesis. We have also identified other unique proteins commonly found in previous studies with the potential to be used as stress response biomarkers. Identification of stress response and heat shock proteins in resistant populations suggests a higher level of adaptability under the disease stress (Vaibhav et al., 2016).

21.3.1 Oyster Growth and Protein Extraction

Oyster proteomics is a relatively new area of study and with limited literature available, hence sample processing is still context dependent. In previous studies, the body fluid of oyster, i.e., hemolymph or gills have been used to study the proteome. Gills and hemolymph are important to study the stress response of oysters as hemolymph is the major carrier of immune cells, gills are infiltrated with hemolymph. In the oyster gill proteomics study in our laboratory, we used 20 oysters of each population (selected and control). The oysters, after collection from the hatchery, were transferred to the aquarium facility and kept there for 10 days to acclimatize (Thompson et al., 2012). The oysters were regularly fed with aquasonic invertebrate food supplement (5 mL/200L). After 10 days of acclimatization, the oysters' gills were excised and protein was extracted from the gills following the procedure described in Thompson et al. (2011). The extracted protein was quantified using Bradford reagent, following the manufacturers' instructions. Quantification was followed by pooling of five oyster gill samples to make one study sample, which is done to account for high variability in oysters. Gill protein samples at this stage divided into two aliquots for mass spectrometry and 2-DE study. A schematic of the work flow for the study is shown in Fig. 21.5.

21.3.2 Analysis of Protein Extracts and Identification of Differentially Expressed Proteins

Oyster proteomics is limited by the amount of protein per sample of oyster and because of this it is difficult to comprehensively characterize a single oyster. In our study, we chose to pool protein from five oysters. This has the advantage of allowing the 2-DE and shotgun proteome studies to be undertaken on the same sample; however, on the other hand, information concerning biological variation was potentially lost with pooling. Following the protein extraction, one set of protein samples were washed in guanidine hydrochloride, ethanol, and glycerol solution. Resuspension buffer consisting of urea, CHAPS, and bromophenol blue was used to suspend the protein pellet. This step was necessary to get rid of all the contaminants such as salt (oyster protein extract is expected to have high-salt content) and other interfering agents. High-salt content is very likely to cause problems in the first dimension separation (Rabilloud and Lelong, 2011). Once the 2-DE gels were successfully run, the gel images were analyzed by PD Quest analysis software (Non Linear Dynamics, Newcastle-upon-Tyne, UK). A representative gel image showing differentially expressed proteins in

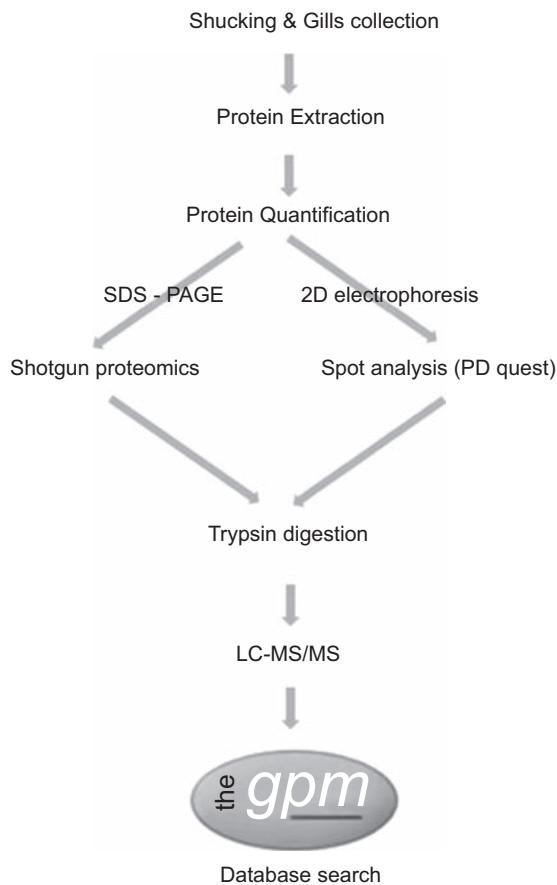


Figure 21.5
Proteomics workflow to study protein biomarkers of disease in SRO.

oysters selected for WM resistance is shown in [Fig. 21.6](#). The differential protein spots were then excised and digested with trypsin. The peptides were then identified using LC-MS/MS.

2-DE is not the only label-free way to study the proteome and is well complemented by other approaches such as bottom-up or shotgun proteomics. To corroborate the findings of 2-DE we used shotgun proteomics on the same batch of previously extracted protein samples. This is a simple, but relatively high-throughput label-free quantitative proteomics technique used to study the changes in the proteome of the organism. Our shotgun proteomics workflow starts with protein fractionation using one of the simplest and most robust biochemistry techniques, SDS-PAGE gel electrophoresis. In our hands, protein fractionation by this approach works well as it concentrates and desalts the proteins, and allows for good peptide recovery by in-gel trypsin digestion. For both shotgun experiments and 2-DE protein spot identifications, we used nanoflow liquid chromatography tandem mass spectrometry on a linear ion trap mass

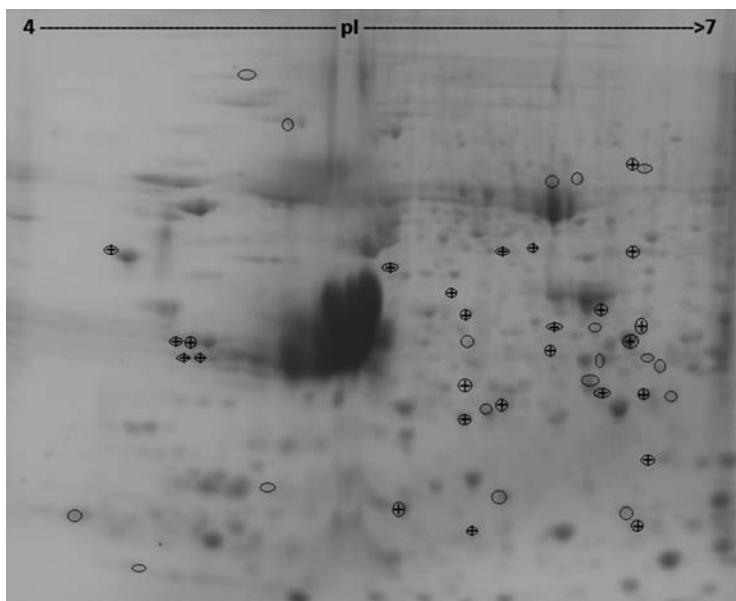


Figure 21.6

2-DE gel image with differentially expressed proteins from gills of QX disease resistance selected oysters when compared to unselected oysters. Spots marked with a circle and plus sign were more intense, spots marked with a circle were less intense, the remainder were unchanged.

spectrometer. Spectra collected from peptides produced from *S. glomerata* gill protein extracts were searched against the *C. gigas* database containing 29,000 protein sequences (Zhang et al., 2012) using the Global Proteome Machine XE software. Using the approaches outlined above, we were able to produce large amounts of detailed quantitative information regarding the changes in expression of different proteins in oyster gills caused by selection for WM disease resistance.

21.4 Oyster Selective Breeding Programs

The first oyster selective breeding program in New South Wales was established in 1990 mainly to reduce the time it takes to reach to market (Nell et al., 2000). This program was a big success in terms of production of fast growing oysters. Time to reach marketable size was reduced from 38 months (diploid control) to 31 months.

However, when the QX disease outbreak occurred in 1994 in the Georges River (Adlard and Ernst, 1995) the earlier selection program was devastated (Nell, 2001). As a result of which, in 1997 the selective breeding program was rebooted with the inclusion of selection for QX disease along with the previous selection interests (Nell et al., 2000). The survivors of the outbreak were used for feeding into the selection program. Since then, a steady improvement

has been observed in subsequent generations of the oysters. It has also been observed that selection for one disease does not impart or help selection for the other disease; rather they appear to be completely independent of each other (Nell and Perkins, 2006).

When the mortality was compared with the nonselected oysters grown at the three sites during the same period of time and exposure, only 28% mortality rate was observed in QXr lines as compared to 97% in the control, nonselected oysters. Individually, the lines grown at different locations displayed very good resistance for both QX disease and WM. However, there was no evidence that resistance to one disease conferred any resistance to the other (Dove et al., 2013).

21.4.1 Marker-Assisted Selection

Since the advent of farming and agriculture, humans have displayed an unquenchable desire to improve the crops by selecting desirable traits. For example, commercial corn varieties now contain sweet yellow kernels which are relatively soft, whereas the American forerunners of approximately 800 years ago were dark blue and so hard that they caused damage to human teeth. This change has occurred over time with selective breeding for desirable quantities, without any intentional genetic modification. This has led to the introduction of many cross-breed varieties, especially when we consider plants. However, the traditional way of doing this was often very time-consuming, and often subject to unexpected outcomes.

Recent advancements in biotechnology have helped greatly in development of disease-resistant lines of plants and animals. Marker-assisted selection has also allowed us to select for better yielding plants and quicker growth of commercial plants and animals to reach market in a shorter time frame. This has allowed farmers to select for many commercially beneficial traits. Marker-assisted selection also incorporates the idea of selection of certain desirable genes which can be used in other genome to transfer certain traits it carries, which is of interest for different purposes. When the marker-assisted selection encompasses single gene transfer, the success rate is very good. However, in case of multiple gene transfer, this mode of selection has proven to be less efficient (Ribaut and Hoisington, 1998).

For the success of marker-assisted selection, it is important to identify the target genes, which may be achieved either by laboratory observation or based on field testing. Once the target gene is identified, it is important to know if a single gene controls the trait or it is a polygenic trait, or quantitative trait loci (QTL) (Ribaut and Hoisington, 1998). In polygenic traits, several genes control one trait that is of interest and each gene in the loci their individual contribution toward the phenotype of that particular trait, and hence each requires equal attention. Therefore, in order to get the desired outcome, it is necessary to study and manipulate each of the contributory genes in the same way, keeping all the other constraints as close to each other as possible so as to introduce minimal variation. To test this successfully, many field experiments are required to be conducted to accurately define the contribution of each gene in QTL. Furthermore, there is one additional complication in the selection process of

traits involving QTL; one should always take into consideration the presence of gene interaction, which, if present, may cause an aberrant result.

21.5 Conclusions and Future Outlook

The goals of this chapter were to discuss the importance of the oyster industry, with emphasis on SRO, and the proteomic advancements employed to address the environment and disease-related problems. The oyster industry of Australia is of great economic importance with more than \$30 million contribution from the SRO industry alone. It is evident that the environmental stresses and diseases have had a major impact on the production of oysters since the 1970s. We have focused our discussion on two major diseases of SRO, QX, and WM, since both of these diseases are major concerns for oyster farmers in NSW. In this chapter we have discussed proteomics-based investigations on SRO studying different stressors. We have also provided a brief case study account of recent proteomics experiments we have performed on gill tissue of QX-selected and WM-selected oysters.

Researchers at NSW Department of Primary Industries have initiated a selective breeding program to tackle the disease by selecting the survivors of disease outbreak; however, the selection was done without reference to any molecular information. Why did the organism survive in the first place: was it just chance, or some phenotypic trait that made them resistant? Why are some oysters resistant and not others? What traits are the breeders selecting for? These questions remain largely unanswered, and to address them studies are underway to understand the biology behind the selection. It is clear that much progress has been made, but even more progress will be needed in future to ensure the long-term sustainability and viability of the oyster industry.

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SECTION 4

Processed Foods

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Proteomics of Fermented Milk Products

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22.1 Introduction

Fermentation has been an integral process applied to products constituting the human diet since ancient times and fermentation processes passed from sacred ceremonies of community sharing to everyday life meals, regardless of the cultural society concerned (Garine et al., 2001). However, fermented foods remain a partial mystery that tools such as proteomics can investigate deeper. Proteomics has recently helped archeologists to decipher the ancient human dairy activities from at least the 2nd century BC and to show that they were close to those used at the present time namely for Kefir making (Hong et al., 2012; Yang et al., 2014).

Fermented food products represent up to 30% of our daily diet and are the major source of ingested living bacteria (Olivares et al., 2006). Dietary deprivation of fermented food products causes a decline in the innate immune response that can be prevented by the consumption of yogurt (Olivares et al., 2006), highlighting the important role of fermented milk products in the human diet. This is supported by the fact that yogurt is one of the very few alleged fermented products with an EFSA-accepted functional claim, regarding lactose intolerance alleviation (EFSA Panel on Dietetic Products, 2010).

Milk fermentation was a prerequisite for its conservation in the absence of the cold chain. Hundreds of fermented milk products have evolved throughout the world and these products possess varied appearance, texture, flavor, taste, and health benefits, thanks to the association of various microorganisms with milk (and its derived product the whey) and with human knowledge. Fermented milk products, that is, yogurts, fermented milks used as beverages such as Kefir and Koumiss, and a huge number of cheeses, harbor ecosystems ranging from simple to highly complex. They consist of (1) milk matrices arising from various mammals (cow, goat, ewe, mare, camel, yak, etc.) having a variable composition in proteins, lipids, carbohydrates, minerals and (2) microorganisms belonging to various phylogenetic groups (firmicutes, eukaryotes, proteobacteria, and actinobacteria) present within and at the surface of the product, most notably on some cheese varieties. Proteomic investigations are particularly relevant for such food products, as proteins act both as the supplier and substrate of enzymes, during milk proteolysis and degradation of bacterial proteins, a point that is addressed by peptidomics. Furthermore, bacteria in these products also have dual functionality as producers of bioactive compounds and as probiotic

agents. The increase in the number of sequenced genomes facilitates greater knowledge of the bacterial machinery. Only proteomic tools are able to show definitively the end products of the enzymatic machinery and thus assess its efficiency within the milk environment and through the technological processes applied. However, hedonic quality and health properties cannot be extrapolated to all fermented milk products. The remarkable biodiversity in substrates and microorganisms requires fine characterization of each fermentation process and/or functional fermented foods.

In this chapter, we will give an overview of the major breakthroughs performed thanks to proteomics analyses: from pure culture to complex fermented products such as cheeses, with a particular interest in fermented milk product quality and probiotic effects.

22.2 Qualitative and Quantitative Proteomic Tools Used to Study Milk Fermented Products

Numerous approaches have been developed in proteomics to allow a qualitative and quantitative view of the food proteome. They have been regularly reviewed (Bantscheff et al., 2012; Carrasco-Castilla et al., 2012; Gagnaire et al., 2009; Ibáñez et al., 2013; Ndoye et al., 2011; Oudenhoove and Devreese, 2013; Zhang et al., 2010). Two-dimensional (2D) gel approaches were the basis of pioneering work in proteome analysis. Electrophoretic analysis was firstly based on self-forming pH gradients as described by O'Farrell (1975). It then improved in terms of both reproducibility and ease of use thanks to the implementation of immobilized pH gradients developed by Görg et al. (1999). This was accompanied by the development of differential protein quantitation directly in-gel via differential gel electrophoresis (DIGE) labeling allowing comparative proteomic analyses of microorganisms under various environmental conditions (Karp et al., 2008; Missous et al., 2012; Wu et al., 2006). Identification, firstly based on biochemistry tools such as N-terminal and internal de novo sequencing experienced a major breakthrough thanks to the dramatic increase in bacterial genome sequencing and the ever-improving tandem mass spectrometry methods. Moreover, the coupling of different mass spectrometry analyses offers the possibility to further increase proteome coverage of milk products (Mollé et al., 2009). Hence, the reference maps by 2D gel electrophoresis thoroughly used in the 2000s have been losing favor when compared to the 1D-liquid chromatography (LC) and 2D-LC approaches directly coupled with tandem mass spectrometry (MS) or even 1D-gel and LC MS/MS. The 2D gel drawbacks were actually insuperable with regards to the limited dynamic range and molecular mass range, as well as the difficulty in directly identifying the proteins in the gels. A proteomic profiling by gel-free technique has been shown for *Streptococcus thermophilus* (Salzano et al., 2007) and for *Lactobacillus plantarum* (Heunis et al., 2014).

Besides the exploration of proteomes, quantitation of proteins present in the sample by mass spectrometry can be performed by using stable isotope tags (Albaum et al., 2011; Bantscheff et al., 2012). In most cases, this quantification is only relative and based on a comparison of

quantity of the same peptide present in two or more experimental conditions. This strategy has been successfully applied to cheese in order to quantify the bacterial proteins released during Emmental cheese ripening with isobaric tags for relative and absolute quantitation (iTRAQ) isotope labeling peptides (Jardin et al., 2012). For absolute quantification of some peptides, an internal standard has to be used. Such applications have been developed to quantify food biomarkers in a multiplex fashion and at high sensitivity level (10 ppb) (Agrawal et al., 2013) and among them food allergens (Ahsan et al., 2016) and food toxins (Giacometti et al., 2013) were assayed. Absolute quantification is based on multiple-reaction monitoring (MRM) mass spectrometry (MS) coupled with isotope-labeled internal standard. The target precursor ions corresponding to the mass of the targeted peptides are selectively isolated as well as the peptide-specific fragment ions and their intensity are compared to the precursor ion of the synthetic isotope-labeled internal standard of known abundance (AQUA peptide for absolute quantification) (Ahsan et al., 2016). The MRM method has been recently applied to quantify, in whole milk and in yogurt, two branched chain amino acid leucine metabolites that have beneficial health activity on skeleton fortification (Ehling and Reddy, 2014). The two metabolites, β -hydroxy- β -methylbutyric acid (HMB) and α -hydroxy-isocaproic acid (HICA) were mainly produced during fermentation through the leucine catabolism by lactic acid bacteria up to a concentration range 3.0–15.2 mg/L, while both HMB and HICA concentrations were <20 μ g/L in milk. This technique was also used as a way to detect bitter peptides from β -casein in cheddar cheeses (Karametsi et al., 2014) and the fraudulent presence of cow whey in water buffalo, sheep, or goat Italian ricotta cheese (Camerini et al., 2016) or in other various types of Italian cheeses (Bernardi et al., 2015).

22.3 Techno-Functionalities of Dairy Microorganisms Through the Prism of Proteomics

During fermentation process, microorganisms have to cope with various technological stresses, that is, temperature either cold or heat, osmotic with salting during cheese manufacture, and acid during lactic acid bacteria growth. The adaptation of the bacteria has been tested under various environment conditions from culture media, to milk or cheeselike medium and *in situ*, as shown in Table 22.1.

The first proteomic works were dedicated to establish reference maps of the main bacterial species, that is, lactic acid bacteria that are traditionally used in the manufacture of dairy products according to various culture conditions. Thus, various proteomes were established: *S. thermophilus* strain PB18 in M17 or strain LMG18311 in milk (Derzelle et al., 2005; Perrin et al., 2000), *Lactococcus lactis* NCDO763 in milk (Gitton et al., 2005), or five strains of *Lactobacillus rhamnosus* cultured on MRS broth and under cheeselike conditions (Bove et al., 2012) and *Lactobacillus helveticus* H9 in milk (Chen et al., 2014). 2D-gel electrophoresis-based approaches formed the basis of this pioneering work evidencing acid adaptation in lactobacilli (Lee et al., 2008b), in bifidobacteria (Sánchez et al., 2007a), and in propionibacteria (Jan et al., 2001).

Table 22.1: Proteomic investigations on dairy bacteria.

| Genera/Species | Strain | Growth Conditions | Studied Parameter | Electrophoretic Separation | MS/MS Equipment | Reference |
|---|--|---|---|----------------------------|---|----------------------------|
| <i>Bifidobacterium</i> | | | | | | |
| <i>B. animalis</i> subsp. <i>lactis</i> | BB-12 | MRS-rich medium + cysteine | Extracellular proteome | 2-DE | MALDI TOF MS | Gilad et al. (2011) |
| <i>B. animalis</i> subsp. <i>lactis</i> | KLDS 2.0603 | MRS-rich medium + cysteine | Surface proteins | SDS-PAGE | Nano-LC MS/MS | Zhu et al. (2016) |
| <i>B. animalis</i> subsp. <i>lactis</i> | IPLA 4549 | MRS-rich medium + cysteine | Bile salts adaptation | 2-DE | MALDI TOF MS | Sánchez et al. (2007b) |
| <i>B. bifidum</i> | MIMBb75 | MRS-rich medium + cysteine | Surface proteins | SDS-PAGE | N-terminal sequencing | Guglielmetti et al. (2008) |
| <i>B. bifidum</i> | S17 | MRS-rich medium + cysteine | General proteome | SDS-PAGE | Nano-LC coupled online ESI orbitrap MS/MS | Wei et al. (2016) |
| <i>B. infantis</i> | BI107 | MRS-rich medium + cysteine | General proteome | - | LC ESI Q-TOF MS/MS | Vitali et al. (2005) |
| <i>B. longum</i> | NCC2705 | MRS-rich medium + cysteine or modified Garches medium | General proteome | 2-DE | MALDI-MS + ESI-MS/MS | Yuan et al. (2006) |
| <i>B. longum</i> | NCC2705 | MRS-rich medium + cysteine or modified Garches medium versus dialysis bag implanted in rabbit intestine | Physiological changes during growth under various media | 2-DE | MALDI-MS + ESI-MS/MS | Yuan et al. (2008) |
| <i>B. longum</i> biotype <i>longum</i> | NCIMB 8809 | MRS-rich medium + cysteine | Acid stress adaptation | 2-DE | | Sánchez et al. (2007a) |
| <i>Lactobacillus</i> | | | | | | |
| <i>L. acidophilus</i> | NCFM 150B, FloraFIT probiotics; DuPont | Chemically defined medium | Adhesive and proteome changes to expand molecular insight of the symbiotic-host interplay | CyDye labeling + 2D-DIGE | MALDI-TOF MS + MS/MS | Celebioglu et al. (2016) |
| <i>L. brevis</i> | ATCC8287 | Chemically defined medium | Heat stress conditions | 2-DE + immunolabeling | - | Savijoki et al. (2006) |

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|--|------------|--|--|--------------------------|--|-------------------------|
| <i>L. casei</i> | Zhang | MRS-rich medium | Acid stress adaptation | CyDye labeling + 2D-DIGE | iTRAQ + nano-LC coupled online MALDI TOF/TOF | Wu et al. (2011) |
| <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> | NCFB 2772 | Skim milk | Early synthesis protein during growth | Radiolabeling + 2-DE | N-terminal sequencing | Rechinger et al. (2000) |
| <i>L. delbrueckii</i> subsp. <i>lactis</i> | 200 | MRS-rich medium | Bile adaptation | 2-DE | MALDI TOF MS | Burns et al. (2010) |
| <i>L. delbrueckii</i> | ATCC11842 | MRS-rich medium | Acid tolerance response | 2-DE | MALDI TOF MS | Fernandez et al. (2008) |
| <i>L. delbrueckii</i> subsp. <i>lactis</i> | ATCC15808 | MRS-rich medium | Surface proteins | - | Nano-LC coupled online ESI Q-TOF MS/MS | Espino et al. (2014) |
| <i>L. helveticus</i> | H9 | Milk | Milk adaptation | 2-DE | MALDI TOF MS | Chen et al. (2014) |
| <i>L. helveticus</i> | ITG LH1 | MRS-rich medium | Peptidase subproteome | 2-DE | MALDI TOF MS | Manso et al. (2005) |
| <i>L. plantarum</i> | 299v | MRS-rich medium | Surface proteins | 1D | Q-TOF MS/MS | Beck et al. (2009) |
| <i>L. plantarum</i> | | MRS-rich medium | Surface proteins | - | Nano-LC coupled online ESI orbitrap MS/MS | Remus et al. (2013) |
| <i>L. plantarum</i> | | MRS-rich medium, wheat flour hydrolyzed, whey milk, tomato juice | Adaptation to various food conditions | 2-DE | MALDI TOF/TOF MS/MS | Siragusa et al. (2014) |
| <i>L. plantarum</i> | 423 | MRS-rich medium | Acid tolerance response | - | Nano-LC coupled online ESI orbitrap MS/MS | Heunis et al. (2014) |
| <i>L. reuteri</i> | E97849 | Chemically defined medium | Efficient protein radiolabeling under heat stress conditions | 2-DE + immuno-labeling | - | Savijoki et al. (2006) |
| <i>L. reuteri</i> | ATCC 23272 | MRS-rich medium | Bile salts adaptation | 2-DE | MALDI TOF MS | Lee et al. (2008a) |
| <i>L. reuteri</i> | ATCC 23272 | MRS-rich medium | Acid tolerance response | 2-DE | MALDI TOF MS | Lee et al. (2008b) |
| <i>L. rhamnosus</i> | E97800 | Chemically defined medium | Efficient protein radiolabeling under heat stress conditions | 2-DE + immunolabeling | - | Savijoki et al. (2006) |

Continued

Table 22.1: Proteomic investigations on dairy bacteria—cont'd

| Genera/Species | Strain | Growth Conditions | Studied Parameter | Electrophoretic Separation | MS/MS Equipment | Reference |
|---|-------------------|---|---|----------------------------|---|------------------------------|
| <i>L. rhamnosus</i> | LC705 | MRS-rich medium | Surface proteins | - | Nano-LC coupled online ESI Q-TOF MS/MS | Espino et al. (2014) |
| <i>L. rhamnosus</i> | | Cheese conditions versus MRS | Adaptation to cheese conditions | 2-DE | MALDI TOF MS/MS | Bove et al. (2012) |
| <i>Lactococcus</i> | | | | | | |
| <i>L. lactis</i> | M4 | M17-rich medium | Physiological changes during growth phases | - | UPLC-MS/MS | Yap et al. (2014) |
| <i>L. lactis</i> | NCDO763 | Skim milk versus chemically defined medium | Physiological changes during growth under various media | 2-DE | MALDI TOF MS | Gitton et al. (2005) |
| <i>L. lactis</i> | | M17-rich medium | Surface proteins | - | Nano-LC coupled online ESI orbitrap MS/MS | Meyrand et al. (2013) |
| <i>L. lactis</i> | NZ9000 and MG1363 | M17-rich medium | Cold stress | 2-DE | N-terminal sequencing | Wouters et al. (2001) |
| <i>L. lactis</i> | LD61 and UCMA5713 | Ultrafiltrate model cheese | Physiological changes during growth under various media | 2-DE | MALDI TOF MS ESI-ion trap MS/MS | Yvon et al. (2011) |
| <i>L. lactis</i> subsp. <i>lactis</i> | IL2661 | M17-rich medium | Physiology in the digestive environment | 2-DE | MALDI TOF MS | Roy et al. (2008) |
| <i>L. lactis</i> subsp. <i>cremoris</i> | MG1363 | Chemically defined medium | Purine starvation | Radiolabeling + 2-DE | MALDI-MS + ESI-Q-TOF MS/MS | Beyer et al. (2003) |
| <i>L. lactis</i> subsp. <i>cremoris</i> | MG1363 | M17-rich medium | Acid tolerance response | Radiolabeling + 2-DE | ESI MS/MS | Budin-Verneuil et al. (2005) |
| <i>L. lactis</i> subsp. <i>cremoris</i> | MG1363 | M17-rich medium | Acid tolerance mutants | 2-DE | MALDI TOF MS | Budin-Verneuil et al. (2007) |
| <i>L. lactis</i> subsp. <i>cremoris</i> | MG1363 | M17-rich medium or chemically modified medium | GAPDH metabolism | Radiolabeling + 2-DE | MALDI-MS + ESI-MS/MS | Willemoës et al. (2002) |
| <i>L. lactis</i> subsp. <i>lactis</i> | IL1403 | M17-rich medium | Copper stress response | 2-DE | MALDI TOF MS | Barré et al. (2007) |

| | | | | | | |
|--|-----------------------|---|---|-----------------------------------|---|-------------------------------|
| <i>L. lactis</i> subsp. <i>lactis</i> | IL1403 | Chemically defined medium | Protein stability | 2-DE | MALDI TOF MS | Dressaire et al. (2009) |
| <i>L. lactis</i> subsp. <i>lactis</i> | IL1403 | Chemically defined medium | Isoleucine starvation | 2-DE | MALDI TOF MS | Dressaire et al. (2011) |
| <i>L. lactis</i> subsp. <i>lactis</i> | IL1403 | Chemically defined medium | Amino acid metabolism | - | iTRAQ + nano-LC coupled online ESI orbitrap MS/MS | Lahtvee et al. (2011) |
| <i>L. lactis</i> subsp. <i>lactis</i> | IL1403 | Chemically defined medium | Sugar metabolism | 2-DE | MALDI TOF MS | Palmfeldt et al. (2004) |
| <i>L. lactis</i> subsp. <i>lactis</i> bv. <i>diacetylactis</i> | CRL264 | M17-rich medium | Acid tolerance response | 2-DE | MALDI TOF/TOF MS/MS | García-Quintáns et al. (2008) |
| <i>Propionibacterium</i> | | | | | | |
| <i>P. acidipropionici</i> | CGMCC 1.2230 | YEL-rich medium | Acid tolerance response wild strain versus mutant acid tolerant | 2-DE | MALDI TOF/TOF MS/MS | Guan et al. (2014) |
| <i>P. freudenreichii</i> | CIRM-BIA1 | Cheese juice versus chemically defined medium | Physiological changes during growth under various media | Radiolabeling + 2-DE | nano-LC coupled online ESI Q-TOF MS/MS | Gagnaire et al. (2015) |
| <i>P. freudenreichii</i> | CIRM-BIA1 | YEL-rich medium | Cold storage | 2-DE | ESI MS/MS | Dalmasso et al. (2012) |
| <i>P. freudenreichii</i> | ITGP20 (CIRM-BIA 129) | Sweet whey media supplemented with casein peptone | Stress tolerance before drying | 2-DE | nano-LC coupled online ESI Q-TOF MS/MS | Huang et al. (2016) |
| <i>P. freudenreichii</i> | ITGP20 | Milk ultrafiltrate | Surface proteins | SDS-PAGE/CyDye labeling + 2D-DIGE | nano-LC coupled online ESI Q-TOF MS/MS | Le Maréchal et al. (2015) |
| <i>P. freudenreichii</i> | SI41 | Chemically defined medium | Acid tolerance response | Radiolabeling + 2-DE | | Jan et al. (2001) |
| <i>P. freudenreichii</i> | SI41 | Chemically defined medium | Bile salts adaptation | Radiolabeling + 2-DE | | Leverrier et al. (2003) |
| <i>P. freudenreichii</i> | SI41 | Chemically defined medium | Multitolerance | Radiolabeling + 2-DE | Q-TOF MS/MS | Leverrier et al. (2004) |
| <i>P. freudenreichii</i> | | Chemically defined medium | Thermotolerance | Radiolabeling + 2-DE | MALDI TOF MS | Anastasiou et al. (2006) |

Continued

Table 22.1: Proteomic investigations on dairy bacteria—cont'd

| Genera/Species | Strain | Growth Conditions | Studied Parameter | Electrophoretic Separation | MS/MS Equipment | Reference |
|----------------------------|-------------|---------------------------|---|----------------------------|--|-----------------------------|
| <i>Streptococcus</i> | | | | | | |
| <i>S. thermophilus</i> | LMG18311 | Milk | Milk adaptation | 2-DE | MALDI TOF MS | Derzelle et al. (2005) |
| <i>S. thermophilus</i> | LMG18311 | Skim milk | Physiological changes during late stage of growth | 2-DE | MALDI TOF MS | Herve-Jimenez et al. (2008) |
| <i>S. thermophilus</i> | PB18 | M17-rich medium | General proteome | SDS-PAGE + 2-DE | N-terminal sequencing | Perrin et al. (2000) |
| <i>S. thermophilus</i> | ATCC19258 | M17-rich medium | General proteome | SDS-PAGE + 2-DE | Micro-LC coupled online ESI ion trap MS/MS | Salzano et al. (2007) |
| Fermented dairy products | | | | | | |
| | | Cheese | Bacterial protein release | SEC + 2-DE | ESI MS/MS | Gagnaire et al. (2004) |
| | | Cheese | Quantification of bacterial proteins released in cheese aqueous phase | - | iTRAQ + nano-LC coupled online ESI Q-TOF MS/MS | Jardin et al. (2012) |
| | | Grana Padano cheese | Alteration by clostridia | - | 2D-LC ESI ion trap MS/MS | Soggiu et al. (2016) |
| | | Ancient Kefir grains | Archeology of dairy technology | SDS-PAGE | LC MS/MS | Yang et al. (2014) |
| Yeast and mold | | | | | | |
| <i>Geotrichum candidum</i> | ATCC 204307 | Chemically defined medium | Cold stress | CyDye labeling + 2D-DIGE | Micro-LC coupled online ESI MS/MS | Missous et al. (2012) |
| <i>Yarrowia lipolytica</i> | 1E07 | Chemically defined medium | Amino acid metabolism | 2-DE | MALDI TOF MS | Mansour et al. (2009) |

2-DE, two-dimensional electrophoresis; CyDye, fluorescent dye used in gel for protein differential quantification; DIGE, differential gel electrophoresis; ESI, electrospray ionization; iTRAQ, isobaric tags for relative and absolute quantitation; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; Q-TOF, quadrupole time-of-flight.

Fractionation of the cytoplasmic protein content was also performed to complete the proteome view in terms of peptidases present (Begonovic et al., 2013; Manso et al., 2005), differentiation of the cytosolic and the membrane proteins (Salzano et al., 2007), the alkaline proteome (Drews et al., 2004), or the surfaceome (Beck et al., 2009; Le Maréchal et al., 2015, 2014). Protein fractionation, highlighting specific parts of the cell, gives crucial information on the global physiology of the cell enabling the reconstruction of metabolic pathways and notably the stress tolerance pathway. It addresses the management of the interface between bacteria and between bacteria and human host, through immune modulating properties (Mangiapane et al., 2015) and between bacteria and matrices in the fermented dairy products (Gagnaire et al., 2009).

Various technological stresses have been deeply explored to determine the effectors induced and how the bacterial cells can increase resistance to one applied stress such as acid conditions and other environmental challenges to adapt and to survive within various dairy products as reviewed (De Angelis and Gobbetti, 2004; Hussain et al., 2013; van de Guchte et al., 2002). Actually, bacteria in the fermented dairy products have to cope with acid, thermal (heat or cold), osmotic (NaCl in cheese manufacture), nutritional starvation including carbon and nitrogen, and oxidative stresses. Among the mechanisms of stress tolerance developed by bacteria, some of them were generic and other specific to one stress and some examples are presented in the following. Thus, *Propionibacterium freudenreichii* developed strategies based on both common and distinct pathways (Leverrier et al., 2004). For example, under storage conditions in the cold (4°C), *P. freudenreichii* remains metabolically active and induces pathways able to maintain its long-term survival notably by rerouting its carbon metabolism to gluconeogenesis and by using alternative energy storage compounds like polyphosphates (Dalmasso et al., 2012). For *L. lactis* subsp. *cremoris*, five proteins seem to play a crucial role in the constitutive acid stress tolerance of the strain MG1363: RecA DNA recombination protein implied in the regulation of the SOS response, pyruvate carboxylase, and PyrG CTP synthase implied in the biosynthesis of aspartate and CTp, respectively, glutamyl tRNA synthetase and 30S ribosomal protein S1 both implied in translation (Budin-Verneuil et al., 2007). *Lactobacillus delbrueckii* subsp. *bulgaricus*, among all changes during acid adaptation, rerouted pyruvate metabolism to favor fatty acid biosynthesis and changes to membrane fluidity (Fernandez et al., 2008).

Changing the composition of the medium was used to test the adaptive capability of the dairy microorganisms. Thus, changing the availability of amino acid in the culture medium induced a reorganization of the *Yarrowia lipolytica* metabolism toward amino acid catabolism, that is the first step of aroma compound production and also generated a higher induction of proteins related to carbon metabolism and protein biosynthesis (Mansour et al., 2009). In the case of leucine starvation, *L. lactis* was also able to (1) activate the pathway of carbon and nitrogen metabolism to supply leucine via a CodY regulation; (2) limit its growth by downregulating processes of transcription, translation, carbon metabolism and transport, pyrimidine and fatty

acid metabolism; and (3) unexpectedly overexpress oxidative stress response (Dressaire et al., 2011). The availability of micronutrients is also essential for bacterial adaptation, and among them the incorporation of copper, an essential micronutrient used as a cofactor for many enzymes that has to be controlled by the cell to avoid detrimental effect. The addition of copper to *L. lactis* IL1403 cells showed, under semiaerobic condition, a strong induction of the NAD-independent lactate oxidase that catalyzes the conversion of lactate to pyruvate, which can be used as a way to scavenge molecular oxygen or to protect the cell against hydrogen peroxide toxicity in this organism (Barré et al., 2007).

The use of milk and cheeselike media give insights into the overall bacterial metabolism under conditions that more accurately reflect the environmental conditions encountered *in situ* than the usual cultivation medium conditions. Indeed, most of the pathways were changed: protein biosynthesis, nucleotide, carbohydrate metabolisms, glycolytic pathway, proteolytic activity, cell wall and exopolysaccharide biosynthesis, cell regulation, amino acid and citrate metabolism, oxidation/reduction processes, and stress responses (Bove et al., 2012; Gagnaire et al., 2015; Yvon et al., 2011). Changes in these metabolic pathways give new adaptive capability to bacteria to other kinds of stressful environments such as the digestive tract (Gagnaire et al., 2015), which is discussed in detail in Section 5 of this chapter. In this sense, proteomics allowed deciphering the protocooperation at the molecular level between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, during growth in milk for making yogurt (Herve-Jimenez et al., 2009, 2008). Nutritional exchanges, iron metabolism were shown in a more complex view than the usual amino acid and peptide supply between both bacteria. Moreover, it appears that *S. thermophilus* was able to detect its yogurt partner and set up regulatory responses to its presence (Thevenard et al., 2011).

Even if cheese constitutes a good example of a fermented food matrix, within which bacteria are responsible for the main changes in milk proteins, carbohydrates, and fat during ripening, the proteomic studies are far less numerous. In fact, it requires the development of new methodologies to extract bacteria and/or bacterial proteins from the cheese matrix, to separate them from the major milk proteins prior to mass spectrometry analyses, which is a daunting task (Gagnaire et al., 2004). However, proteomics allowed the identification of the enzymes that are released throughout the ripening process, namely the proteolytic ones from *S. thermophilus* and *L. helveticus* strains that are implicated in casein degradation mechanisms. Stress responses triggered by thermophilic lactic acid bacteria and by the propionibacterium strain were also highlighted at the end of Emmental cheese ripening (Gagnaire et al., 2004). The quantification of these proteins in the cheese matrix at different stages of ripening was performed in order to gain a dynamic view of the sequential release of the bacterial proteins throughout ripening (Jardin et al., 2012). Through a combined metagenomic and metaproteomic approach, alteration of Grana Padano cheese by spores of clostridia was assayed (Soggiu et al., 2016). Addition of lysozyme to the cheese actually limited this late blowing that led to undesirable formation of “eyes,” cracks, and shredding in the cheeses. These

authors followed several enzymes directly modulated by lysozyme treatment and associated to the level of clostridia spores added during manufacture.

Moreover, cheese favors the implantation of biodiverse bacterial communities. Thus, a subset of propionibacteria strains with a higher thermotolerance compared to reference strains were observed by studying wild propionibacteria strains collected from traditional Graviera Kritis cheese (Anastasiou et al., 2006). These bacteria had a constitutive overexpression of a specific subset of proteins, including heat shock proteins, chaperonins, ATP-dependent proteinases, and enzymes involved in the transcarboxylase (Wood–Werkman) cycle, specific of propionic fermentation in propionibacteria. An adaptive thermotolerance response was also evidenced, depending on the strain, and involved signal transduction, biosynthetic pathways and cell wall maintenance, in addition to protein turnover. No cross-protection of salt-adapted cells against heat stress was observed regardless of the strains.

22.4 Peptidomics of Fermented Milk Products

A complementary approach to proteomics consists of evaluating the peptides present in the various fermented milk products that are produced through a continuous concomitant and/or sequential proteolytic process. These peptides contribute to the final texture, flavor, and beneficial health properties of fermented milk products (Lacou et al., 2016; Nongonierma and FitzGerald, 2015; Sánchez-Rivera et al., 2014b). They are produced by the numerous proteolytic enzymes with various substrate specificity, arising from the milk, added during the process of manufacture as coagulant for cheeses, or those arising from the various microorganisms present intentionally as starter or not as in raw milk cheeses. The multitude of proteolytic enzymes leads to a diverse peptide length distribution from 5 to over 40 amino acid residues and to a high dynamic range of 12 orders of magnitude (Panchaud et al., 2012). This also gave rise to various methodologies to extract them from the products and to analyze them as well as development of algorithms and both analytical and computational techniques to process all the peptide data sets (Dallas et al., 2015; Urfer et al., 2006).

Peptidomics has been used to characterize the hydrolysis of the main milk proteins, that is, caseins during elaboration, of various fermented milk products, for example, in yogurt (Kunda et al., 2012) and in Kefir grains (Dallas et al., 2016) and in the aging of many cheeses. Numerous peptides have actually been identified by tandem mass spectrometry and many of these quantified in Cheddar (Singh et al., 1997, 1995), Grana Padano (Fernandez et al., 1998; Ferranti et al., 1997), Serra (Sousa and Malcata, 1998), Emmental (Gagnaire et al., 2001), Ragusano (Gagnaire et al., 2011), 24-month-old Parmigiano Reggiano (Sforza et al., 2012), Spanish blue cheese (Sánchez-Rivera et al., 2014a), and Coalho, a Brazilian cheese (Silva et al., 2016). This, in turn, gives information on the types of peptides involved in textural properties as stretchability of Swiss type cheeses that is dependent on the proteolytic enzymes present not only at the surface of the lactobacilli but also released during ripening (Sadat-Mekmene et al., 2013).

Peptidomics of fermented milk products provides information about product authenticity, origin, and history (Gagnaire et al., 2009), biological activities of peptides, functional properties, allergenicity, and sensory properties (Lacou et al., 2016; Sánchez-Rivera et al., 2014b). The review of Lopez-Exposito et al. (2012) highlighted that cheeses are a major source of bioactive peptides. Namely, recent peptide identification includes bitter peptides (Karametsi et al., 2014) and bioactive peptides with a potential or proven impact on health from fermented milk and cheeses, for example, phosphopeptides, antimicrobial peptides, and antihypertensive peptides (Adt et al., 2011; Contreras et al., 2008; Gagnaire et al., 2012; Losito et al., 2006; Pritchard et al., 2010; Sagardia et al., 2013; Sánchez-Rivera et al., 2014a). Some of these studies also submitted the peptides to simulate in vitro digestion to examine their stability in the digestive tract. Even if no clear sequence consensus exists to ascertain proven bioactivities in vivo from all the bioactive peptide dataset, many databases have been created to have a survey of all bioactive peptides from milk and other food components, such as BIOPEP (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php), and MilkAMP databases, the latter one dedicated to milk antimicrobial peptides (<http://milkampdb.org/home.php>). The recent review of Sánchez-Rivera et al. (2014b) presents the mechanism of bioactive peptide modification during digestion at cellular and animal levels with a special focus on dairy peptides.

22.5 Probiotics Dairy Microorganisms: How Bacteria Express Their Health Benefits

Bacterial adaptation under technological stresses predisposes these organisms to survive gastrointestinal digestion and to exert beneficial effects in the digestive tract as probiotics. Proteomic tools have allowed major breakthroughs in the understanding of the mechanisms responsible for the adaptation of probiotic bacteria toward stresses encountered within the digestive tract, including acid and bile salts stresses (van de Guchte et al., 2012).

Although stress responses varied among bacterial species, acid adaptation was shown to coincide with overexpression of key proteins involved in protein folding, energetic metabolism, and internal pH homeostasis in the studied probiotic bacteria (Jan et al., 2001; Lee et al., 2008b; Sánchez et al., 2007a,b). A similar approach was used to investigate mechanisms responsible for adaptive response to the presence of bile salts, major stressing agents of the intestinal milieu. This was shown to involve signal sensing and transduction, general stress response as well as remediation of oxidative toxicity in probiotic bacteria (Lee et al., 2008a; Leverrier et al., 2003; Sánchez et al., 2007b).

For acid and bile salts, the two major stresses encountered within the digestive tract, sensing of a sublethal dose of stress triggers an adaptive response, which in turn affords enhanced tolerance toward an otherwise lethal dose of the same stress. As an example, *L. delbrueckii*

subsp. *lactis* overexpressed proteins, when exposed to bile salts, as general stress response chaperones, proteins involved in transcription and translation, in peptidoglycan/exopolysaccharide biosynthesis, in the lipid and nucleotide metabolism, and several glycolytic and pyruvate catabolism enzymes (Burns et al., 2010). Inhibition of protein neo-synthesis by the ribosome binding antibiotic chloramphenicol was furthermore shown to hinder tolerance acquisition (Jan et al., 2001). By contrast, overexpression of key stress proteins may confer constitutive tolerance in probiotic bacteria (Khaskheli et al., 2015).

This molecular approach evidenced both common and distinct stress response pathways in probiotic bacteria, some of the key stress proteins, the so-called general stress proteins, being common to responses to distinct stresses. In accordance, physiological data evidenced cross-protection between distinct stimuli (Leverrier et al., 2004). In particular, adaptation to salt stress (or hyperosmolarity), called osmoadaptation, was shown to induce cross-protection against digestive stresses (Kim et al., 2001), which is particularly relevant, some fermented food products being salted for extended shelf-life. Indeed, Emmental cheese environment was shown to enhance both adaptation proteins and stress tolerance of propionibacteria (Gagnaire et al., 2015). Furthermore, growth of these bacteria in hyperosmotic conditions in cheese whey afforded enhanced tolerance toward digestive and technological stresses (Huang et al., 2016).

One major trend in this area is the in vivo proteomic investigation of probiotics adaptation to the gut. To address this challenge, ingenious experimental devices were developed, such as the gut-implanted dialysis bag containing a pure culture of bifidobacteria (Yuan et al., 2008), allowing the proteomic profile induced within the intestine of rabbits to be monitored.

Another approach combined gnotobiology and proteomics by colonizing the digestive tract of axenic mice with *L. lactis* to investigate the proteome of bacteria grown in the intestine (Roy et al., 2008). The intestinal environment was shown to induce proteins involved in repair and protection of proteins, carbohydrate metabolism adaptation to the intestinal nutritional environment, and physical interaction with the host cells (Yuan et al., 2008). Accordingly, inactivation of genes encoding ClpC (protein repair), PTS (carbohydrate metabolism), or S-layer protein (interaction with the host) leads to reduced persistence of lactobacilli in vivo (see review of Lebeer et al., 2008).

Among the proteins involved in interactions with the host, proteomic investigations in probiotic bacteria revealed adhesins. As an example, an inventory of the *Bifidobacterium animalis* extracellular proteome identified 18 proteins thought to interact with host cells, including adhesion to collagen, mucin, and intestinal cells (Gilad et al., 2011). In the same species, enzymatic shaving of cellular surface proteins with trypsin also revealed proteins involved in adhesion, such as pilus structure proteins and moonlighting proteins (Zhu et al., 2016). In *Bifidobacterium bifidum*, proteomic profiling of cellular proteins spotted two proteins, EF-TU and enolase, with a known moonlighting function in adhesion, as well as six pilin proteins

potentially involved in adhesion (Wei et al., 2016). Interestingly, proteomic analysis of *Bifidobacterium longum* bile response spotted the bile-induced esterase and sortase associated with adhesion and colonization in the intestinal tract (An et al., 2014). In various lactobacilli species, surface layer proteins were first described as involved in adhesion and in inhibition of enterobacteria adhesion to cultured colon cells (Zhang et al., 2013). In *L. rhamnosus*, surface shaving identified piluslike structure and several moonlighting proteins with potential role in adhesion (Espino et al., 2014). Proteomics further revealed that adhesion proteins were induced by iron (Fe^{3+}) in *Lactobacillus jensenii* (Martín et al., 2015) and by raffinose, an emerging prebiotic, in *Lactobacillus acidophilus* (Celebioglu et al., 2016). Pili able to bind to human intestinal epithelial cells were also revealed by surface proteome analysis in *L. lactis* (Meyrand et al., 2013). Interestingly, the surface proteins revealed by this approach may also play a role in modulation of the host immune system (Gilad et al., 2011). In *P. freudenreichii*, the surface proteome was deciphered using a combination of the three methods currently used, enzymatic shaving, surface labeling, and chaotrope extraction (Le Maréchal et al., 2014). This allowed an inventory of the surface proteins to be made, some of which play a key role in immunomodulation. Indeed, surface-extracted proteins induce the production of the immunomodulatory cytokine IL-10 in human peripheral blood mononuclear cells (Le Maréchal et al., 2015). These S-layer-type proteins, which were shown to be expressed at the surface of bacteria within the cheese matrix, led to the development of an experimental cheese exerting protective effects in a mice model of colitis (Plé et al., 2015). This is to our knowledge the first surfaceome study of a beneficial bacterium within a cheese. In *L. plantarum*, combination of proteomics and molecular biology revealed that sortase-dependent surface proteins play a key role in the host immune response modulation (Remus et al., 2013). Surface proteins involved in immune modulation were also identified in bifidobacteria (Gilad et al., 2011), including the lipoprotein BopA, whose expression was shown to be linked with adhesion and with antiinflammatory properties in *B. bifidum* (Guglielmetti et al., 2008).

Altogether, these reports reveal that the use of cutting-edge proteomics tools, together with molecular biology and bioinformatics, will bring crucial new insights at the molecular and cellular levels, contributing to the understanding of the interplay between probiotic bacteria and the host. They open avenues for the pragmatic and experienced development of new functional food supplements and fermented probiotic foods and to select candidate probiotic strain appropriately (Fig. 22.1).

22.6 Future Challenges

One of the main experimental challenges for future investigation will be linked with the complexity of the sample in terms of microbial community, composition of fermented dairy products, kinetics aspects of the modification of these two elements (microbiota and composition). The bottlenecks might reside either in the analytical tools to address such complexity or in the processing of the resulting big data.

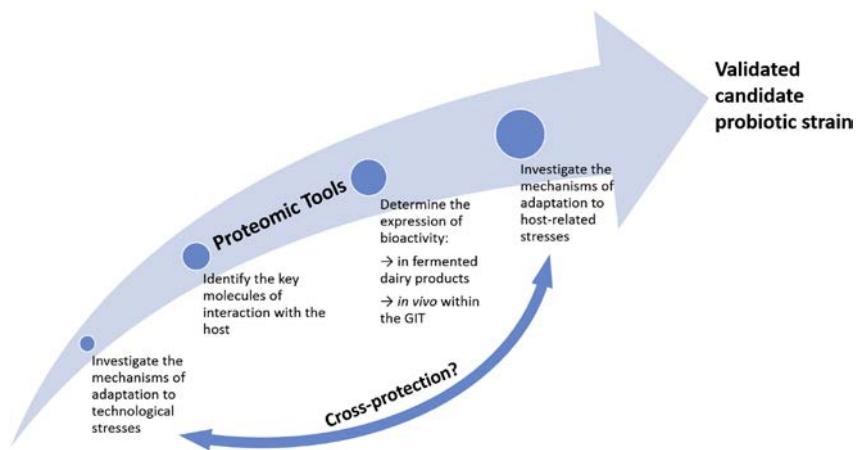


Figure 22.1

Schematic approach to find a candidate probiotic dairy bacterium by using proteomic tools.

Another major issue is the strain-dependency of the probiotic bacteria biological effect. As an example, the panel of proteins decorating the surface of different strains of the same species may determine proinflammatory or antiinflammatory properties that have to be dissected by protein identification. Such a strain-dependency can also give specific bioactive peptide profile that may orientate a fermented food product toward a targeted specific population.

A major opportunity lies in associating proteomics with the other -omics technologies, such as metabolomics, fluxomics, and volatilomics (Le Boucher et al., 2013; Pogačić et al., 2015; Rossell et al., 2011). Multiomic approaches, including genomics and transcriptomics, proteomics and peptidomics, untargeted metabolomics, and a targeted view of lipidomics, glycomics, are essential to complete the view of the bacterial community in the food and to enable the management of the microbiome to bring health benefits (Han et al., 2011). This will give a new era dealing with functional proteomics of complex community including bacterial cross-talk with quorum sensing (Di Cagno et al., 2011) and the metabolic pathway complementation by the interplay between the different members of such a community. Such interplay also includes synergetic or antagonistic relationships between microorganisms within the fermented milk products or present at their surface. This will give new clues to predict the production of bioactive compounds, including peptides, vitamins, aroma compounds, and more.

Another avenue will lead to the development of proteomic tools to decipher probiotic–host interactions at the molecular level and in a strain-specific manner. This will allow prediction of the targets of each probiotic strain at the host level. Taking a long-term perspective, personalization of the diet will be addressed by reverse engineering of fermented milk products designed for targeted populations (Plé et al., 2016).

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Proteomic Analysis of Beer

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23.1 Introduction

Beer is the most ancient and most consumed alcoholic beverage worldwide. It has a huge economical and sociocultural importance. The composition of beer is very complex as it encompasses hundreds of small constituents, among which are volatiles, amino acids, salts, phenolics, hop resins and sugars, as well as macromolecules, including proteins, nucleic acids, and polysaccharides. Some of the components deriving from the raw materials survive unmodified in beer. In contrast, many other components are the result of complex biochemical and chemical transformations induced by malting and brewing. In particular, proteins undergo heavy multiform modifications, such as denaturation, proteolysis, glycation, deamidation, reduction/oxidation, and aggregation.

The protein content of beer is relatively low when compared to protein-rich food matrices, ranging between 0.2 and 2.9 g/L depending on the brewing style. Nonetheless, beer proteins have been investigated in depth for a number of reasons. Proteins and polypeptides have a primary impact on important technological and sensory traits of beer, including color, foam firmness, haze formation, and colloidal stability. Thus, proteins directly affect the appearance, texture, carbonation, mouthfeel, and palatability of beer. Beer contains immunologically active proteins and peptides. Proteins of beer can elicit IgE-mediated allergic reactions, although the prevalence of allergy to beer is relatively low (Bonadonna et al., 1999). On the other hand, among the beer (poly)peptides some are derived from gluten and can trigger an autoimmune reaction in subjects suffering from celiac disease. Beer is an interesting model system to investigate the changes to proteins undergoing harsh technological and high-thermal processing. Due to the simultaneous occurrence of intact proteins, proteolytic oligo-/poly-peptides and process-modified sequences (e.g., glycated), the protein/peptide fraction of beer severely challenges classical analytical methods. For this reason, much of the detailed information about the composition of the protein/peptide fraction of beer has been obtained recently, using modern proteomic and peptidomic approaches based on high-resolution mass spectrometry (MS).

This chapter provides a general overview about the knowledge of the protein/peptide composition of beer and relevant functional, technological, and immunological implications, with special focus on the impressive achievements obtained using state-of-the-art proteomic methodologies.

23.2 The Origin of Proteins and Peptides in Beer

The vast majority of beer proteins originate from malt barley (*Hordeum vulgare*) or from wheat (*Triticum aestivum*) in the case of wheat beers (weissbier) popular in Germany. Proteins derived from other malted or unmalted cereals used for brewing (adjuncts) and from yeast are observed to a lesser extent. Two varieties of barley are commonly used for malting purposes, namely two-row and six-row barley, which are classified according to the number of kernel arrays around the spike shaft. Two-row barley is usually preferred by both large-scale and traditional breweries, especially because it yields malts with greater extracts, lighter color, and lower protein content than six-row type. In contrast, six-row cultivars possess a higher diastatic power (a measure of the potential starch-converting enzymatic activity) and requires shorter steeping time for malting. To balance the effect of a higher protein content, malt from six-row varieties is usually cut with variable percentages of unmalted corn or rice grains.

Barley genetic makeup, climate, breeding, and malting practices have contributed to diversify the range of malts currently used for brewing. Therefore, the “protein fraction” of beer is expected to vary enormously according to the malt used, the brewing style, and the brand. The variety of available malt types combined to the different brewing procedures generates the huge diversification of beers currently on the market. However, at least under a qualitative standpoint, the main protein components are common to practically all the beer brands.

23.2.1 Barley Seed Proteins

The total protein content of barley seeds is around 10%–15%. As for other cereals, the protein fraction of barley is complex, being constituted by heterogeneous families of storage and metabolic gene products. Despite the fact that cataloging cereal proteins according to functional properties appears more appropriate ([Shewry and Halford, 2002](#)), the classification based on Osborne’s procedure of fractionated protein extraction is still largely adopted ([Osborne, 1895](#)).

Albumins and globulins are soluble in water and aqueous saline solutions respectively. They constitute a group of structurally heterogeneous proteins, many of which reside in the aleurone layer or in the embryo of caryopsis and have metabolic functions. In barley, as in other cereals and legumes, some saline soluble globulins are nonprolamin storage proteins, stocked as protein bodies in the grain endosperm.

Hordeins, the most represented class of barley storage proteins accounting for at least half of the total grain proteins, constitute a heterogeneous mixture of polymorphic polypeptides, encoded by multigene families. Hordeins are characterized by a high degree of genetic polymorphism and, in general, *cultivars* have characteristic protein expression patterns. According to the electrophoretic mobility and amino acid composition, hordeins have been classified in subfamilies, named the B-, C-, D-, and γ -hordeins (Shewry and Milfin, 1985). The group of low MW A-hordeins, formerly included within the hordeins, are now classified among the salt-soluble globulins. Sulfur-rich B- (30–45 kDa) and sulfur-poor C- (45–75 kDa) account for 70%–80% and 10%–15% of hordeins, respectively. D- (105 kDa) and γ -hordeins (35–40 kDa) are less represented components, accounting for less than 5% each. Hordeins are synthesized during the mid to late stages of grain filling and are deposited in protein bodies that form a protein matrix within the endosperm cells.

Being the 60%–70% ethanol-soluble barley prolamins, hordeins correspond to gliadins in wheat. C-hordeins, for instance, are homologous to wheat ω -gliadins. However, MW and structural organization of B-hordeins, with a single N-terminal sequence preceding a QQPFQPQ poly-repeated domain, correspond to the low-MW glutenin subunits of wheat (Han et al., 2010). Based on sequence and phylogenetic parentage, B-hordeins have been further subdivided into two major (B1 and B3) and one minor (B2) subclasses (Skerritt and Janes, 1992). Similar to gluten organization, in the presence of water B-hordeins can in part associate via disulfides with high-MW D-hordeins to form a gel-like aggregate (Moonen et al., 1987). Indeed, barley glutelins, extractable only in denaturing and sulphydryl reducing buffers, actually are chains of D- and B-hordeins, associated by means of intermolecular disulfide bridges (Skerritt and Janes, 1992).

Starch granule-associated proteins (SGAP) constitute a group of hydrophobic proteins, in part adsorbed to the (external) surface and in part tightly bound to (internal) starch granules, the latter soluble in heated SDS-containing buffers (Borén et al., 2004). Although quantitatively minor, SGAP contribute primarily to determine the malting properties of barley, as they control size, shape, and hardness of starch granules. Barley seeds also contain a minor fraction of peculiar proteins with unknown molecular function that do not share homology with functionally characterized gene products (Finnie and Svensson, 2009).

23.2.2 Protein Modification During Brewing

The whole brewing process is an amazing practice of applied enzymology, formerly accomplished empirically and now more or less consciously. The general brewing scheme has been sketched in a previous report (Picariello et al., 2013). Beer production starts with malting, which is a controlled germination of the barley grains. During malting, barley storage proteins are partially hydrolyzed by endogenous proteases into more soluble peptides and free amino acids. More than 40 different active proteases have been cataloged in malt (Jones, 2005a).

Cysteine-proteases, especially endoprotease (EP)-A (37 kDa) and EP-B (30 kDa) with different substrate specificity, play a pivotal role in mobilizing barley storage proteins. However, different endoproteases including metallo-, aspartic-, and serine-proteases participate effectively to protein modification (Jones and Budde, 2005). Malt exopeptidases, including aminopeptidases (Spannen and Mikola, 1975) and at least five carboxypeptidases with complementary specificity, contribute to release free amino acids (Mikola, 1983). Storage proteins, especially hordeins, are physiologically deputed to provide the building blocks for protein synthesis in the growing embryo. Malt endoproteases maintain activity even at temperatures higher than 60°C. Owing to protection within the caryopsis compartments, kilning (65–220°C) does not destroy the hydrolytic potential of proteases, which act in later stages, during mashing. Similarly, the amylases are not inactivated by kilning and hydrolyze carbohydrates in the mash at temperatures higher than 60°C.

Thermally induced protein conformational changes and nonhydrolytic enzymatic activities, such as those of disulfide isomerase, indirectly contribute to proteolysis, enabling the access of proteases to substrates. In cereal grains, the free-sulphydryl/disulfide equilibrium controls a panel of events during the dormancy-to-germination transition, including the mobilization of storage proteins (Montrichard et al., 2009). The reducing conditions of barley malting promote the depolymerization of D-/B-hordein aggregates and induce the partial release of monomeric chains, which are rendered available for proteolysis. In contrast to malting, mashing occurs under oxidative conditions that can partially restore covalent aggregation of hordeins. In particular, B-hordeins are believed to form disulfide-linked aggregates in which C-hordein chains are entrapped (Celus et al., 2006). Thus, hordein aggregates became largely insoluble, ending up in spent grain or precipitating from the low-alcohol wort.

Aside from determining the ratio between soluble and total protein amount (“Kolbach” index), the breakdown of the network of barley storage proteins dictates the accessibility to starchy reservoirs that will then provide the fermentable carbohydrates. In effect, the Kolbach index is a reliable predictor of the potential degree of starch conversion. Hordeins are known to affect the diastatic power of malt wherein the total hordein grain content negatively correlates with the malting quality (Smith and Simpson, 1983). However, the total hordein content alone is a poor indicator of malting properties, because the composition of cultivar-specific hordeins is unquestionably relevant to the technological parameters. Overall, the relationships between the content of total or individual protein classes and malting traits are conflicting, as brewing is a highly intricate process, difficult to reproduce in simple model systems (Gupta et al., 2010).

For example, the content of D-hordein appears to display a negative correlation with malting properties (Howard et al., 1996). Similarly, it has been proposed that endosperm proteins, especially B-hordeins in the subaleurone layer, can impede the absorption of water during steeping, thus affecting the enzymatic activity during malting (Molina-Cano et al., 2002). On the other hand, dedicated studies have demonstrated that relationships between protein

content of endosperm and water uptake is complex, while genetic factors determine unique combinations of polymorphic protein patterns, which variably concur to the malting quality (Cozzolino et al., 2014). A number of additional chemical-physical parameters critically affect proteolysis. Indeed, mashing temperature, time and pH, position within the caryopsis, grist particle size, and grist to liquor ratio influence the balance among the activity of proteases, solution diffusion of substrates, and solubility of polypeptides. Furthermore, starch granules are embedded within a rigid network of proteins, also including external and internal SGAP (Weurding et al., 2001), the latter determining the hardness of the starchy endosperm, in turn related to poor or high malting aptitude. Friabilin (ca. 15 kDa) that consists of two puroindolines appears decisive to loosen the aggregation among granules, thereby promoting amylolysis (Darlington et al., 2000). Malt modification is also modulated by a complex set of thermally stable inhibitors of proteases and amylases (Jones, 2005b).

Overall, it is clear that the pattern of beer proteins does not reflect the original content of barley, but it is the result of a complex and variable series of modifications barley proteins undergo. Brewing is a harsh process having a drastic impact on the majority of barley proteins. Due to their structural properties, related to their physiological role, protease inhibitors endure proteolysis and are found to be enriched in beer. Surviving polypeptides also carry evidence of partial structural modifications, including unfolding, glycation, and deamidation. Yeast enzymes are minor protein components of beer released during fermentation or at a stage that succeeds the major proteolytic events. The different strains of ale brewer's yeast (*Saccharomyces cerevisiae*) that are used appear to have limited impact on the beer proteome (Berner et al., 2013) (Table 23.1).

23.3 The Characterization of Beer Proteome

23.3.1 Classical Analytical Methods

Due to limitations of the traditional methods of analysis, the protein composition of beer has remained relatively unexplored until recently. In a series of pioneering works, Hejgaard et al. purified the most abundant protein of beer, formerly referred to as "antigen one" and later identified it as Z4-barley protein (Hejgaard, 1977, 1982). Starting from a large-scale purification involving 100 L beer, Sørensen and Ottesen (1978) separated two main polypeptide fractions by Sephadex G-150 size exclusion chromatography, at estimated MW of 44 and 10 kDa.

The 44 kDa component, Z4-barley protein, is variably glycated. Z4-barley was later found to be composed of two homologue polypeptides that were named Z4 and Z7, as they are encoded by genes on chromosomes 4 and 7 of barley respectively (Hejgaard and Kaersgaard, 1983). Z7-barley was identified initially as a 39 kDa component. Subsequent analysis demonstrated that Z7-barley protein comigrates within the dominant Z4-barley band when separated by electrophoresis owing to close MW and pI (Lundgard and Svensson, 1989; Curioni et al., 1995).

Table 23.1: The “core” proteome of beer: barley-derived proteins recurrently described in beer.

| Protein | NCBI Accession | Uniprot Accession | MW | Theoretical pI |
|---|----------------|-------------------|------|----------------|
| Z4 serpin | gi 1310677 | P06293 | 43.3 | 5.7 |
| Z7 serpin | gi 75282567 | Q43492 | 42.8 | 5.4 |
| ns-LTP 1 | gi 128376 | P07597 | 9.7 | 8.2 |
| ns-LTP 2 | gi 128377 | P20145 | 7.0 | 7.0 |
| D-hordein ^a | gi 1167498 | Q40054 | 75.0 | 8.0 |
| B3-hordein ^a | gi 123459 | P06471 | 30.2 | 7.7 |
| B1-hordein ^a | gi 18929 | P06470 | 31.5 | 6.5 |
| γ1-hordein | gi 123464 | P17990 | 32.7 | 8.1 |
| γ3-hordein | gi 288709 | P80198 | 33.2 | 6.7 |
| Avenin-like protein A1 ^a | gi 156630232 | A7XUQ7 | 16.3 | 8.2 |
| α-Amylase/trypsin inhibitor CMd | gi 585291 | P11643 | 16.1 | 5.2 |
| α-Amylase inhibitor BDAI-1 | gi 123970 | P13691 | 16.4 | 5.4 |
| α-Amylase inhibitor BMAI-1 | gi 2506771 | P16698 | 15.8 | 6.4 |
| Trypsin inhibitor CMe | gi 1405736 | P01086 | 16.1 | 7.5 |
| Trypsin inhibitor BTI-CMe2.1 | gi 2707924 | O49861 | 16.2 | 6.8 |
| Subtilisin-chymotrypsin inhibitor CI-1A | gi 124125 | P16062 | 8.9 | 5.2 |
| Subtilisin-chymotrypsin inhibitor CI-1C | gi 124129 | P01054 | 8.2 | 5.7 |
| α-Amylase/trypsin inhibitor CMb | gi 585290 | P32936 | 16.5 | 5.8 |
| α-Amylase/trypsin inhibitor CMa | gi 585289 | P28041 | 15.5 | 5.9 |
| Trypsin/amylase inhibitor pUP13 | gi 225102 | Missing | 14.7 | 5.3 |
| Oleosin 18 kDa | gi 75282620 | Q43769 | 19.4 | 9.7 |
| Hordoindoline A | gi 7671688 | Q9M4E3 | 13.2 | 8.6 |
| Hordoindoline B1 | gi 75172332 | Q9FSI9 | 13.2 | 8.6 |
| Hordoindoline B2 | gi 374258553 | Q9LEH8 | 13.2 | 8.7 |
| Barwin | gi 114832 | P28814 | 13.7 | 7.8 |

^aMultiple isoforms detected dependent on both the style of beer and source of barley (cultivar).

Z proteins belong to the superfamily of serine protease inhibitors (serpins; [Hejgaard et al., 1985](#)). The Z-type cereal serpins are classified into five subfamilies, with homology degree of 70%–75%. Members within a subfamily share sequence identity higher than 89%. Barley serpins are subdivided into three subfamilies, namely barley seed (BS)Z4, BSZ7, and BSZx. Zx, only sequenced at a gene level ([Rasmussen, 1993](#)), has never been detected as a polypeptide. Hexaploid *T. aestivum* wheat encodes a higher number of serpin proteoforms compared to diploid barley. Five wheat Z-type serpins have been cloned and sequenced and all of them have been detected in Weissbier, brewed with 40%–60% wheat grist ([Picariello et al., 2015](#)).

The dominant 10kDa component of beer was identified as the barley nonspecific lipid transfer protein 1 (LTP1, [Sørensen et al., 1993](#)). Two strictly homologue polypeptides, named LTP1a and LTP1b, were characterized after LTP purification ([Jégo et al., 2000](#)). LTP1b differs from the main “a” isoform by an unusual posttranslational modification, identified as a α-ketol 9-hydroxy-10-oxo-12(Z)-octadecenoic acid esterifying an aspartic acid residue ([Bakan et al., 2006](#)). Further studies allowed the identification of an additional lower MW

component (7.0 kDa) referred to as LTP2. LTPs are no longer considered inhibitors of cysteine-proteases as previously believed (Davy et al., 1999). Malting and kilning processes, under reducing chemical environment, induce heavy glycation of LTPs by Maillard reactions (Perrocheau et al., 2006). Glycated LTP1 has been proposed as a probe to monitor the degree of malt modification by MALDI-TOF MS (Bobalova and Chmelik, 2007). In general, nonglycated LTPs possess exceptional stability to heat, proteases, and detergents (Lindorff-Larsen and Winther, 2001). On the contrary, wort boiling conditions have a drastic impact on the conformational changes of glycated LTPs (van Nierop et al., 2004). The unfolded and glycated LTP chains acquire increased flexibility and amphiphilic properties, because of the simultaneous exposure of covalently bound polar sugars and hydrophobic residues, the latter being internally buried in the native state. These structural transitions are the basis for the foaming and surface active properties of LTPs. It has been proposed that glycated and partly unfolded LTPs contribute to foam formation, while glycated Z4 affects foam stability (Sørensen et al., 1993; Evans et al., 1999). Interestingly, native LTP1 does not display foaming properties (Mills et al., 2009). Through sulfhydryl exchange, wort boiling can produce chimeric aggregates of LTP1 and LTP2 (Jégou et al., 2001) in addition to covalent protein aggregates, such as adducts between barley barwin fragments and Z4 (Iimure et al., 2012a). Wheat LTP1 and LTP2 have been detected in Weissbier, although they appear as lesser abundant components compared to their barley counterparts (Picariello et al., 2015).

In general, the study of the dynamic evolution of growing barley grain has demonstrated that, aside from the storage proteins, the proteome of the mature seed is dominated by the so-called “pathogenesis-related (PR) proteins” (Gogjanović, 2009). PR proteins are part of a natural resistance mechanism, selectively evolved to protect dormant and germinating seeds against insects or pathogenic pests and microorganisms. Extreme vacuolar and apoplastic chemical conditions as well as proteolysis only minimally affect the pattern of PR proteins. Thus, it is not surprising that the harsh brewing processes select and enrich PR proteins, among which Z-barley, LTPs, and small inhibitors of proteases are the most represented. Due to their structural stability, PR proteins are potential food allergens, either in raw or processed foodstuffs. In fact, even after severe thermal treatments, a certain proportion of LTPs retains the native fold and can survive human gastrointestinal digestion after consumption. The whole protein or its immunologically active fragments can come in contact with the mucosal immune system, eliciting IgE-mediated allergic response in predisposed individuals.

The detailed characterization of additional proteins in beer has remained vague and undefined until the recent application of proteomic methods. In particular, major amounts of haze-forming unhydrolyzed hordeins or their large fragments in beer, detected by immunochemical assays with either polyclonal antibodies raised against whole hordein extracts (Asano et al., 1982; Ellis et al., 1990) or monoclonal antibodies against hordein subfamilies (Sheehan and Skerritt, 1997), did not find support by recent investigations.

23.3.2 The Age of Proteomics

Due to the complexity of the matrix, the detailed protein composition of beer, also including the “deep” proteome, has remained out of reach until the development of high-resolution separation methods (Colgrave et al., 2013). The pioneering two-dimensional electrophoresis (2-DE) analyses provided a rough snapshot of the beer protein system (Marshall and Williams, 1987; Pressi et al., 1993). The popularity and socioeconomical relevance of beer contributed to fuel a series of systematic investigations aimed at optimizing the analytical performance of 2-DE (Weiss et al., 1992; Görg et al., 1992) in order to define the pattern of barley proteins and the changes induced by brewing.

Perrocheau et al. (2005) explored a “classical” proteomic approach, based on the 2-DE separation followed by MS-based characterization of trypsinized protein spots, to track the protein transition from barley to malt and finally to beer. This study allowed the characterization of many of the minor protein constituents of beer, including α -amylase/trypsin inhibitors (ATIs), metabolic proteins from yeast (*Saccharomyces* spp.), and a γ 3-hordein. The 2-DE map of beer evidenced a complex pattern of Z4 and LTP1 spots, dispersed over a wide range of pI, due to variable glycation. Combined to this, ns-LTP1 is an alkaline protein (pI 8.2) and generally occurs in the map as a series of poorly focused spots. On the contrary, γ 3-hordein occurred as a single faint spot, probably due to the low number of lysine residues available for coupling to reducing sugars. Additional foam-active proteins, such as barley dimeric amylase inhibitor-1 (BDAI-1) and yeast thioredoxin, as well as haze-active proteins, including chloroform/methanol soluble trypsin inhibitors (CMb and CMe), have been identified using the same proteomic approach (Okada et al., 2008; Iimure et al., 2008, 2009). The proteomic analyses confirmed that intact hordeins occur at low concentrations in beer, because of their scarce solubility in the low-alcohol solutions. These findings have been further supported by successive proteomic investigations (Picariello et al., 2011; Iimure et al., 2009).

Due to an intrinsically limited dynamic range, the 2-DE-based analysis only shows the tip of the iceberg, that is, the subset of the most abundant protein components, while a plethora of low-abundance protein entities escapes detection. The lack of protein and genomic databases has delayed the construction of a comprehensive inventory of proteins potentially occurring in beer. Such an information gap has been progressively bridged firstly by detailed proteomic investigations covering individually the barley seed compartments, including the starchy endosperm, aleurone layer, and embryo (Finnie and Svensson, 2003; Bønsager et al., 2007), and then with the release of the complete barley genome (The International Barley Genome Sequencing Consortium, 2012).

Several strategies have been devised to dig deep in the beer proteome. To better resolve the separation among components, protein extracts from 11 beer samples were separated by 2-DE using two different pI ranges, namely 4–7 and 6–9 (Iimure et al., 2010). Overall, 85 out of 199 detected spots were successfully identified by peptide mass mapping and categorized into 12 gene products. The “core” of beer specific proteins is common to all the analyzed beer

samples, so that these authors proposed the construction of a reference map of proteins in beer to be used in quality control and assessment. In order to identify minor haze-active proteins, [Hao et al. \(2006\)](#) separated proteins extracted from beer foam by SDS-PAGE and sliced thin sections of the resulting gel. Proteins were in-gel trypsinized and peptides analyzed by LC-ESI MS/MS. In this way, they identified several protein chains not described before, also including a series of large-sized fragments of foam-stabilizing D- and B-hordeins in the 17–20 kDa range. Major advances in the analysis of beer proteome have been introduced by gel-free shotgun analysis of beer proteomes. According to this strategy, the whole protein extract of beer is digested by trypsin and resulting peptides are analyzed by HPLC coupled to high-resolution MS and MS/MS. Despite the increased complexity of the system, because each protein generates multiple fragments, the probability of detecting and sequencing at least one peptide as an analytical surrogate of the parent protein is much higher.

Furthermore, LC and MS techniques are much more effective for conveniently sized peptides than for large proteins. The first paper in this sense was by [Weber et al. \(2009\)](#), who identified several B-, D-, and γ -hordeins and traces of undeclared wheat gluten proteins in beer, using nanoflow capillary chromatography coupled to a hybrid Q-TOF mass spectrometer.

Interestingly, these authors identified for the first time avenin-like protein A (ALP-A), a 16.4 kDa protein chain thereafter described as one of the recurrent proteins of beer, regardless of the brand. More than a single ALP-A isoform has been detected in beer ([Picariello et al., 2012](#)). An analysis extended to 60 beer samples from the market, confirmed the findings about the occurrence of hordeins in beer, although their content is highly variable according to the malt typology, the brewing process, and the beer style ([Colgrave et al., 2012](#)). Shotgun proteomic experiments overcome many of the 2-DE limitations related to the detection of proteins at extreme pI. On the other hand, in shotgun experiments the information about the origin of the sequenced peptides is generally lost. Thus, in beer it is difficult to establish if peptides derive from small-sized fragments or, alternatively, from intact proteins. For this reason, we separated the large and the small polypeptides of beer (exclusion limit 6 kDa) by size exclusion chromatography, before shotgun analysis ([Picariello et al., 2012](#)). With the aid of an additional fractionation step that reduced the impact of the protein dynamic range based on methanol solubility of the proteins, we identified 33 gene products occurring as intact chains or large-sized polypeptides in beer, including B1-, B3, D-, and γ -hordeins, though present at low levels.

The “deep” proteome of beer has been explored using the combinatorial peptide ligand library technology (ProteoMiner). According to this method, large amounts of beer (an entire bottle) were treated with a library of resin-bound hexapeptides, which exhibit a large array of chemicophysical properties. Based on their structural properties, proteins have different affinity and compete to bind specific hexapeptides. Most abundant proteins quickly saturate their affinity ligands while the excess is eluted in washing steps. On the contrary, low abundant proteins progressively bind to their specific affinity sites. Accumulating low-abundance components and depleting partly the highest-abundance ones, the ProteoMiner approach

tends to equalize protein amounts and results in a compression of the dynamic range. A preliminary treatment of beer with polyvinylpyrrolidone was required to remove polyphenols from beer. Righetti and coworkers separated the ProteoMiner-eluted proteins by SDS-PAGE and digested proteins in-gel slices prior to high-resolution MS/MS analysis. In this manner, they identified 40 different *Saccharomyces* spp-derived proteins never described before in addition to more than 20 barley proteins (Fasoli et al., 2010). It is noteworthy that no protein from hop (*Humulus lupulus*) or possibly ascribable to it were identified even after ProteoMiner enrichment. In an elegant investigation, the beer proteome was explored comparing several methods of extraction and separation (Konečná et al., 2012). The most effective strategy consisted of protein isolation by Sephadex-G25 trapping/desalting columns followed by OFFGEL fractionation of proteins and 2-DE with Sypro Ruby staining. This method allowed the authors to single out 70 different proteins, 30 of which from *H. vulgare* and many others from yeast. It can be reasonably expected that the recent advances in MS techniques and the widespread adoption of high-resolution benchtop MS instruments, such as those based on the high-throughput Orbitrap technology, will further expand the inventory of the beer proteome and render routine protocols for beer quality assessment by detailed proteomic analysis (Iimure and Sato, 2013; Iimure et al., 2014). To this end, we recently carried out an analysis of traditional Weissbier, brewed with 40%–60% wheat grist adjunct. The presence of relatively high percentage of hexaploid wheat (*T. aestivum*) further increases the complexity of the protein system, severely challenging the performance of the proteomic protocols. While the 2-DE map of a representative wheat beer (Fig. 23.1) did not differ substantially from that of a typical all-barley malt beer, using an Orbitrap Q-Exactive mass spectrometer we characterized 58 intact proteins or large fragments deriving from barley, wheat, and yeast in Weissbier (Picariello et al., 2015). Interestingly, the wheat-derived proteins identified corresponded to the majority of the previously identified barley homologues and included gliadins as well as both high and low MW glutenin subunits.

23.3.3 The Analysis of Beer Peptidome

The occurrence of small polypeptides in beer was documented over 40 years ago when Clapperton (1971) fractionated peptides from wort and beer using anionic exchange chromatography. Thus, it was quickly accepted that the bulk (60%–75%) of the nitrogenous constituents of beer are small MW compounds, including peptides released from barley proteins through proteolysis (Bishop, 1975). Nevertheless, the peptide fraction of beer has remained uncharacterized until a few years ago, most likely due to the complexity of the fraction and the occurrence of many interfering small molecules (e.g., amino acids, sugars). More than 15 years later, beer polypeptides were separated by molecular weight (MW) using size exclusion chromatography (Dale and Young, 1987) and the complexity of the peptide fraction was partly resolved using reversed phase-HPLC (Dale and Young, 1989). The amino acid composition was consistent with a proteolytic release of peptides from malt proteins.

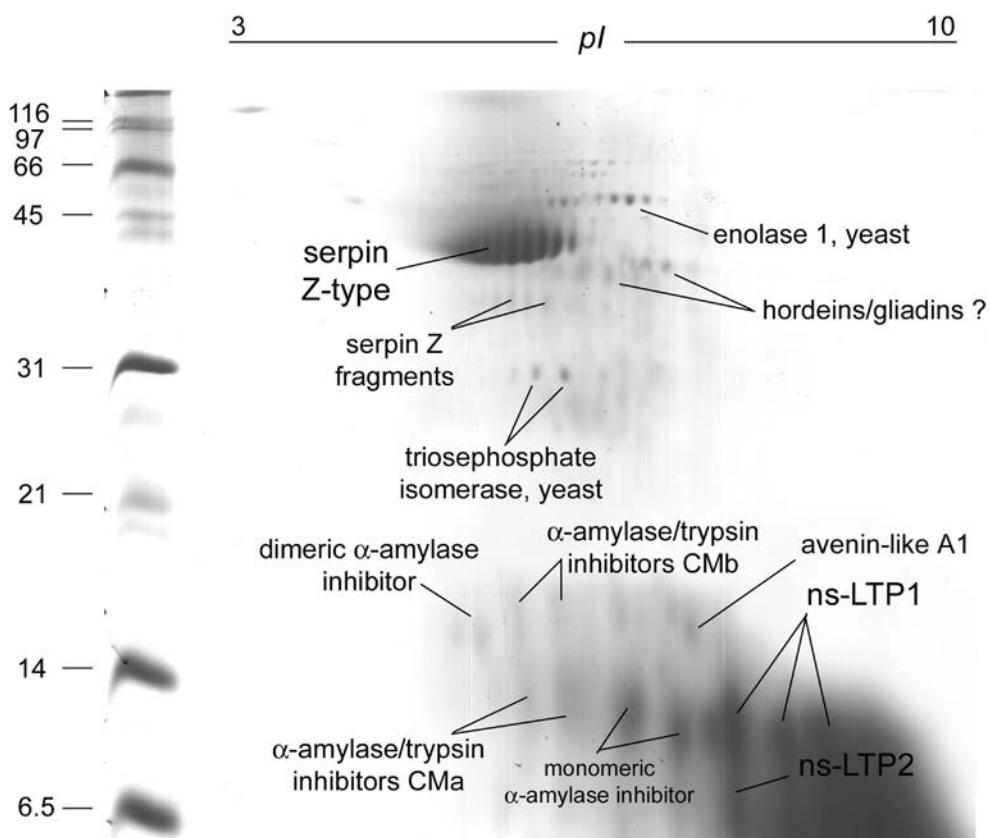


Figure 23.1

Two-dimensional electrophoresis of a representative Weissbier sample. Proteins were identified by peptide mass fingerprinting. *Reprinted with permission from Picariello, G., Mamone, G., Cutignano, A., Fontana, A., Zurlo, L., Addeo, F., Ferranti, P., 2015. Proteomics, peptidomics, and immunogenic potential of wheat beer (Weissbier). J. Agric. Food Chem. 63, 3579; Copyright 2015, American Chemical Society.*

Hordein-derived peptides covering a wide MW range were detected by immunochemical methods ([Sheehan and Skerritt, 1997](#)). Similar findings were also obtained in subsequent investigations, despite these hordeins were thought to be almost completely degraded into free amino acids during malting ([Osman et al., 2003](#)). Our first LC–MS/MS-based characterization of peptides in beer primed the annotation of the peptide components of beer ([Picariello et al., 2011](#)), which is far to be completed as it strongly depends on beer brand.

Along with subsequent investigations ([Picariello et al., 2012](#)), we demonstrated that beer peptides derive from albumins and globulins as well as hordeins. Many among the beer peptides arise from proteins that are also found intact in beer, including LTPs, Z4, ATIs, and ALP-A. This finding confirms that these proteins undergo structural modifications (e.g.,

unfolding) and partial proteolysis, probably during downstream malting. A single-stage MALDI-TOF MS analysis of the low MW (poly)peptides of beer provided an impressive, though not exhaustive, snapshot of the complexity of the fraction, which include hundreds of signals, arising from a multitude of different proteins (Mamone et al., 2011). A C-terminal fragment of Z4-serpin with MH^+ 4034.7 (average mass), generated by a trypsin-like cleavage, dominated the intermediate mass range of barley beer peptides, after cutoff of $<3\text{ kDa}$. The presence of an additional mass, deriving from wheat Z2B-serpin, distinguishes beers brewed with significant amount of wheat (Weissbier), as observed by comparing spectra of at least eight different wheat beer brands to all-barley counterparts. Thus, a simple MALDI-TOF MS approach that monitored the signature trypsin-like fragment of wheat Z2B-serpin (MH^+ 4471.2 as N-terminal pyroglutamic acid) allowed discrimination between all-barley from wheat-containing beers (Fig. 23.2, unpublished data).

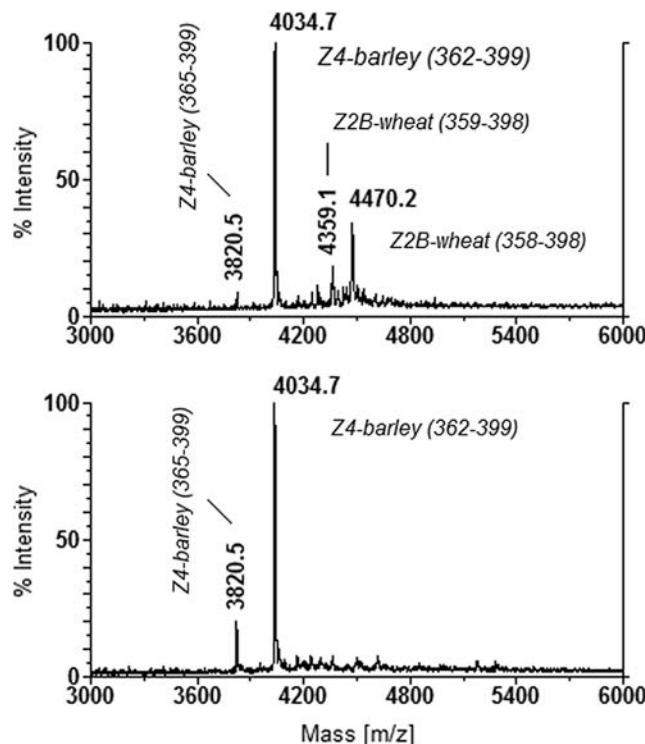


Figure 23.2

MALDI-TOF MS comparison of wheat (upper panel) and all-barley malt beer samples in the 3–6 kDa mass range. Small-sized peptides were removed by cutoff membrane ($<3\text{ kDa}$) after protein precipitation (20% TCA). C-terminal fragments of Z4-barley serpin (all-barley malt beer) and additional ones of wheat Z2B-serpin, the latter distinguishing wheat-containing beers, dominate the spectra. Z2B-wheat peptide (358–398) occurs with an N-terminal pyroglutamic acid (data unpublished).

Among the beer peptides several differ by single or short amino acid sequences, due to the action of barley and/or yeast exopeptidases. Interestingly, many fragments as well as intact small proteins, such as LTPs (Bobalova and Chmelik, 2007; Lašovičková et al., 2010), displayed clear signals of heat-induced glycation. Several factors challenge the potentiality of MS techniques to characterize the beer peptidome, including the heterogeneity of the proteins from which the detected peptides originate, the unpredictable or absent proteolytic specificity, the potential glycation and additional modifications, such as deamidation or oxidation and the shortage of basic amino acids in prolamins. However, the recent introduction of the high-resolution/high-throughput MS coupled to high-resolution LC separation has overcome in part some of these complexities. Thus, Colgrave et al. (2012) further expanded the list of beer peptides, with a focus on the hordein-derived fragments. In this investigation, proteins and small MW peptides were together subjected to trypsin or chymotrypsin digestion before LC–MS/MS bottom-up analysis. Given the low amount of intact prolamins predicted to be present in beer, the hordein peptides potentially also arose from small-/medium-size hordein fragments. Later on, using nanoflow LC coupled to high-resolution MS/MS, hundreds of peptides have been characterized in the sub-10 kDa fraction of beer, confirming that B1-, B3-, D-, and γ -hordeins are particularly susceptible to hydrolysis, most likely at the stage of malting (Colgrave et al., 2014). Peptides from C-hordeins are scarce, probably due to the substantial insolubility of the hydrophobic repeated octapeptide motifs PQQPFPQQ in the mashing solution (Colgrave et al., 2012). However, fragments of C-hordeins released by malting proteolysis have been detected in beer as well, although at trace amount (Picariello et al., 2012). Using LC-Orbitrap MS we characterized hundreds of peptides in wheat beer (Weissbier), which belonged to 44 gene products, including hordeins, wheat gliadins, and high- and low-MW glutenin subunits (Picariello et al., 2015). In this case, the identification of protein fragments was further complicated by abundant yeast-derived polypeptides and by the additional heterogeneity of the parent proteins derived from both barley and hexaploid wheat (*T. aestivum*).

23.4 Technological Role of Beer Polypeptides

Beer proteins affect two key quality parameters, namely foam stability and haze formation, which in turn have primary impact on sensory traits of beer. The majority of the studies that have been carried out examining the protein complement in beer were aimed to identify foam-stabilizing and haze-active components. However, the chemical–physical mechanisms through which proteins influence quality parameters are difficult to extrapolate, also because they depend on a series of additional factors. In fact, foam stability is also influenced by hop iso- α -acids, nonstarch polysaccharides, and metal ions. In the past an important role in foam formation, rather than its stability, has been ascribed to Z4-barley, as it was isolated in relatively large amounts from beer foam. The evaluation of foam stability in beers brewed with mutant Z4-and or Z7-deficient barley has reconsidered the foam-active role of Z proteins, which appears comparable to other beer proteins (Imure et al., 2012b). Native barley

LTPs are not foam-forming, while they are converted in part to foam-active components by brewing-induced conformational transitions and glycation (Sørensen et al., 1993; Mills et al., 2009). In its “foaming structure” LTP1 is susceptible to degradation by yeast proteinase A (Leisegang and Stahl, 2005). We enriched ALP-A from beer foam using silica gel adsorption (Picariello et al., 2011). However, a targeted investigation demonstrated that ALP-A should not be foam active at all (Imure et al., 2015). In their proteomic analysis of beer foam, Hao et al. (2006) detected practically all the main beer proteins, also including large fragments of hordeins, demonstrating that the protein interaction in foam can have a level of complexity higher than previously supposed (Kauffman et al., 1994; Kaverva et al., 2005). Gushing, which is an overfoaming upon the opening of the beer bottle, also seems related to high level of PR proteins, probably due to the use of infected barley (Gorjanović, 2009). The role of hordein polypeptides in foam formation remains contentious, as they might also act as negative effectors (Leiper et al., 2003a). On the other hand, specific studies have conferred a major role in foam stabilization to specific components such as BDAI-1 (Okada et al., 2008). Conversely, yeast thioreodoxin has been described as a foam-negative protein (Pedrajas et al., 1999). Similar to sparkling wine, yeast-derived proteins can also affect the flavor stability of beer (Guido et al., 2004), even though massive yeast autolysis can generate off-flavors in beer.

Haze formation is one of the most serious drawbacks affecting beer quality. Undoubtedly, haze depends on interactions between specific proteins and polyphenols (tannins), but the specific factors and mechanisms ruling haze formation are still unclear (Asano et al., 1982). CMb and CMe trypsin inhibitors appears to be haze-active proteins as they have been isolated from silica adsorbates after beer clarification (Leiper et al., 2003b; Robinson et al., 2007; Imure et al., 2009). However, these proteins can be nucleation and growth factors of colloidal haze rather than actual haze-active proteins. Hordein peptides can also be involved in haze formation, but the coagulation of proteins and the colloidal aggregation with polyphenols strongly depends on thermal processing of brewing steps. Opportune wort boiling and cooling steps can remove great part of colloidal haze, while the acceptance of unclarified and unfiltered beer is increasing.

23.5 Immunological Aspects of Beer Proteins and Peptides

Proteins that survive brewing have structural traits that confer them high stability against proteolysis. Thus, these proteins or their large fragments can also survive gastrointestinal digestion and come in touch with the intestinal mucosa in an immunologically active form. Nevertheless, allergic reactions to beer are rare and have a very low prevalence, in spite of its large worldwide consumption. The clinical traits of the allergic response suggest that reactions to beer are IgE-mediated and triggered in most of cases by barley LTP1 (Curioni et al., 1999), which is a well-established food “pan-allergen.” In fact, individuals allergic to beer are often sensitized and cross-react with LTP from other plant sources (Asero et al., 2001). On the other hand, only a few subjects sensitized to LTP, also including the barley isoform, elicit

an IgE-mediated reaction to beer (Asero et al., 2002). This apparent incongruity can most likely be explained by the structural changes of LTP1 during brewing (i.e., partial unfolding, proteolysis, and glycation), which also affect the immunological potential of the protein. Other malted or unmalted cereals, such as maize or wheat, used as brewing adjuncts can also modify the allergenic potential of the beer, which depends on the brewing process (Quercia et al., 2012). To this purpose, Herzinger et al. (2004) reported a severe anaphylaxis in a subject following the ingestion of wheat beer, with no sensitization to all-barley beer. In this case, the injuring component was a 35 kDa wheat protein.

LTP1 is not the only potential allergen in beer. Figueiredo et al. (1999) associated a case of beer-induced anaphylaxis in a 21 year-old woman to immunoreactive proteins in the 31–56 kDa range, with major response to a 38 kDa component that probably was a fragment of Z4-barley protein. Indeed, Z4-barley is a possible IgE-binding protein and can evoke positive response in skin-prick tests (García-Casado et al., 2001). Similarly, an 18 kDa polypeptide of beer, probably the ALP-A, has been reported as an additional IgE-binding component (Hiemori et al., 2008). Beer is relatively rich in ATIs that also can survive human gastrointestinal digestion (Mamone et al., 2015) and are supposedly involved in the onset of nonceliac gluten sensitivity (Biesiekierski and Iven, 2015), a delayed reaction to gluten with undefined symptoms, whose traits are still vague. ATIs may also increase the gluten-specific T-cell response in celiac disease (Junker et al., 2012).

An even more intricate issue, with several aspects still unresolved, concerns the content of gluten-like epitopes in beer, which trigger celiac disease in genetically predisposed individuals. An intact D-hordein of beer, alongside the additional wheat prolamins of Weissbier, was immunoreactive to gliadin-specific IgA from celiacs' sera (Picariello et al., 2012, 2015). The Western immunoblotting of beer proteins from all barley and wheat beer is shown in Fig. 23.3. We and other authors identified a number of hordein-derived peptides containing the typical amino acid motifs belonging to DQ2 and DQ8 restricted epitopes (Picariello et al., 2012; Colgrave et al., 2012; Real et al., 2014). Some of these motifs, such as B-hordein PQQPY or C-hordein-derived PQQPF, are shared with immunodominant epitopes of wheat gliadins. We demonstrated that these peptides are targets of tissue transglutaminase-2 mediated deamidation and induce the release of proinflammatory chemokines by intestinal gliadin reactive T-lymphocytes from celiacs (Picariello et al., 2012).

Quantification is a main concern related to the presence of these epitopes in beer. The first-generation ELISA determinations relying on the sandwich Mendez R5 monoclonal antibodies were inadequate for detecting extensively hydrolyzed gluten and were replaced by competitive R5 assays. Next generation monoclonal antibodies (i.e., G12/A1) overcome in part the issue of the reactivity to short sequences that are also found in nongluten-containing foods. In fact, G12 is raised against the gliadin 33-mer immunodominant epitope (Comino et al., 2013). However, due to the possible pitfalls of the immunochemical determinations, ELISA results do not correlate with the content of hordein peptides determined by multiple reaction

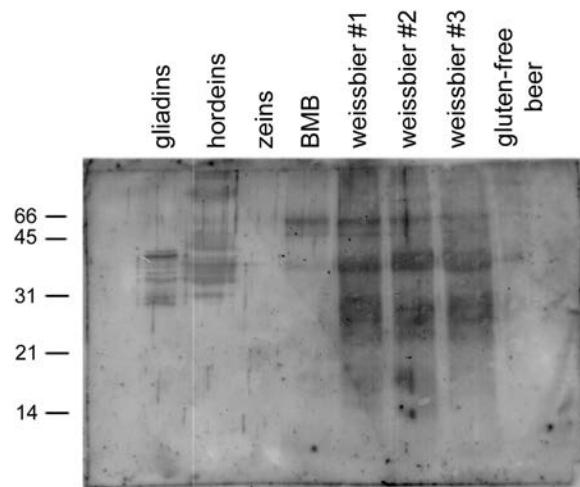


Figure 23.3

Western immunoblotting of all-barley malt (BMB) and three Weissbier samples immunostained with antigliadin IgA from pooled sera of four celiac subjects. Wheat (gliadins) and barley (hordeins) prolamins were used as the positive controls, whereas maize prolamins (zeins) and gluten-free beer were the negative control. *Reprinted with permission from Picariello, G., Mamone, G., Cutignano, A., Fontana, A., Zurlo, L., Addeo, F., Ferranti, P., 2015. Proteomics, peptidomics, and immunogenic potential of wheat beer (Weissbier). J. Agric. Food Chem. 63, 3579; Copyright 2015, American Chemical Society.*

monitoring MS (Tanner et al., 2013a, 2013b). ELISA determinations of beer containing relatively high levels of hordein, especially B- subtypes, gave false-negative responses (Colgrave et al., 2014). According to ELISA determinations a large number of commercial beers display a gluten content much lower than 20 ppm (Dostálek et al., 2006; Guerdrum, 2011), despite the unambiguous detection of gluten epitopes by MS even in apparent gluten-free beer samples (Real et al., 2014). In other cases, ELISA methods provided clearly aberrant figures, mainly due to the lack of an adequate standard (Tanner et al., 2013a). For example, gluten content ranging from 5 to 47 mg/g has been reported for wheat-containing beers. These values are obviously unreliable as they exceed largely the whole protein content of beer. Apart from beer brewed with nonceliacogenic cereals, beer producers have recently introduced barley-based beer brands declared as gluten-free, in which gluten is removed or degraded by bacterial or mold (*Aspergillus niger*) prolyl endopeptidases (van Landschott, 2011; Knorr et al., 2016). The effective removal of these gluten-like epitopes is commonly assessed with ELISA methods that might provide unreliable responses. For these reasons, the development of robust assays to quantify hydrolyzed gluten in foods and beer is an urgent question that may be answered through antibody-independent methods such as MS (Colgrave et al., 2014).

An ultra-low gluten barley (<5 ppm) has been recently obtained by conventional breeding programs and the use of this grain for brewing has been realized with commercial introduction in Germany and has the potential to provide a safe, gluten-free alternative for celiacs (Tanner et al., 2016).

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Proteomics of Grapevines and Wines

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24.1 Introduction

Grapevine (*Vitis vinifera* L.) has largely been studied by genomic and proteomic approaches (Moreno Arribas et al., 2002; Ferreira et al., 2002; Flamini and De Rosso, 2006; Giribaldi and Giuffrida, 2010). It is the most cultivated fruit plant throughout the world for the production of wines and other alcoholic beverages. Grape manufacturing products are generally assessed via measurements of titratable acidity, soluble solid content, and concentration of volatile aroma compounds (Moreno Arribas et al., 2002).

Recent proteomics investigations have focused on two aspects of wine analysis: on the one side, the assessment of residual proteins added as fining agents and in-depth analysis of its proteome content in untreated products; on the other side, the study of the quality of the grapes by the verification of bacterial infections before ripening. Furthermore, the European Community (EC) legislation is focusing attention on the proteinaceous content of grapevine beverages, to verify the presence of allergens or unhealthy chemical agents, used to clarify or adulterate the products.

For many years, wine producers have been adopting fining agents, aimed at reducing or totally eliminating potential sediments. The denaturation and subsequent aggregation products of proteins can flocculate, leading to amorphous sediments, causing turbidity. A haze or deposit in bottled wine indicates that the product is unstable, has a low commercial value, and is therefore unacceptable for sale. The classical fining process involves addition of bovine casein or egg albumin, potentially allergenic proteins, which form complexes with residual grape proteins and induce massive flocculation. The subsequent treatments with bentonite should remove any residual initial fining agents.

Wine proteins play an important role in the quality of wine; they affect taste, clarity, and stability of products. About 30% of residual grape proteins are present in a medium–high abundance level in an unclarified bottle of wine while the remaining 70% are low abundance proteins (D'Amato et al., 2011). Different classes of grape, yeast, and bacteria proteins, such as chitinases, thaumatin-like proteins, endochitinase, and glucosidase, persist through the vinification process and cause hazes and sediments in bottled wines. On the other hand, proteins

contribute to the formation and stability of foam in champagne-based wines (Cilindre et al., 2008). Small molecules, such as peptides, affect wines in terms of sensory properties that can influence the organoleptic characteristics of the product. Moreover, the presence of pathogenesis-related (PR) proteins, produced by the plant during the ripening of the berries, influences the quality of wine too.

In the food industry, proteomics and peptidomics approaches, in synergy with metabolomics and molecular biology, represent novel tools to certify the quality of grapevines and the shelf products, but also in fighting counterfeiting and fraudulent practices.

24.2 Proteomics Methodologies in Food Products

Proteins have been scarcely investigated in food products, even if they play an important role in food allergy and stability, they are a source of biologically active peptides, and they can provide information about product authenticity. The main challenges in the application of proteomics are: (1) the high dynamic range in protein concentration, as well as the high concentration of different plant-specific cellular components (polysaccharides, lipids, and polyphenols) and secondary metabolites that can interfere with protein separation analysis; and (2) incomplete protein databases resulting from the lack of sequenced genomes in many important food species.

Protein sample preparation optimization is critical for establishing reproducible high throughput proteomic analysis. The combinatorial peptide ligand library (CPLL) technology, a modern prefractionation technology, has been developed over the years and permits the detection of the low- to very-low abundance proteins in complex matrices (Boschetti and Righetti, 2013, 2014; Righetti et al., 2014). CPLLS appear to be a unique tool for exploring the “dark side” of any proteome. CPLLS comprise several million hexapeptides (probably made by using 16 different amino acids) which are able to recognize and bind to a complementary amino acid sequence in a bait protein, thus harvesting it from the sample matrix (Boschetti and Righetti, 2013, 2014; Righetti et al., 2014). CPLLS act to normalize the protein concentration and hence minimize the dynamic range by enriching for trace proteins, while concomitantly reducing the relative concentration of the more abundant species.

Other different fractionation sample preparation techniques (in-gel digestion and in-solution digestion) for shotgun proteomics had been tested to quantitatively compare proteins identified in *V. vinifera* samples. The combination of filter-aided sample preparation coupled with gas phase fractionation showed a higher number of identified proteins and a higher reproducibility related to gel-based (SDS-PAGE) methods (George et al., 2015).

The classical methods used for the analysis of grape beverages include dialysis, ultrafiltration, precipitation, 2D electrophoresis, immune detection, and capillary electrophoresis (CE). The combination of high-performance liquid chromatography (HPLC) with

advanced mass spectrometry (MS) technology and protein database interrogation has allowed the in-depth characterization of grape and wine proteome fingerprinting. In particular, the advanced prefractionation methodologies in combination with 1D and 2D electrophoresis have allowed the visualization of several bands or spots, corresponding to low-abundance proteins. The following endoprotease digestions, nHPLC MS/MS, and the use of protein database, such as the Uniprot *Viriplantae* or EST database, such as the *V. vinifera* database, resulted in the discovery of a surprisingly high number of new protein species in foodstuffs and alcoholic and nonalcoholic beverages (Righetti et al., 2012; Fasoli et al., 2011). For the first time, trace proteins/peptides exhibiting negative effects on health (e.g., allergens) have been detected. Trace proteins/peptides displaying positive effects on health (e.g., antimicrobial, antihypertensive, and antioxidant activities) have also been discovered.

Proteomic technology has also the potentiality to discover fraud in commercial food products and provide a measure of the authenticity of commercial foods, as found in supermarkets (D'Amato et al., 2010; Cereda et al., 2010).

24.3 Additives in Wine

The European Union (EU-28) is the world's leader in wine production, with almost half of the global vine-growing area. France, Italy, and Spain are the largest EU wine-producing countries, followed by Germany, Portugal, Romania, Greece, Hungary, and Austria.

The presence of amorphous sediments and turbidity, that affect the customer's selection of wine bottles off the shelf, is avoided by using fining agents. To control lactic bacteria growth in malolactic fermentation of wine, the producers utilize lysozyme addition in wines containing lower levels of sulfur dioxide. The EU has established a maximum lysozyme level in the final product of 500 µg/mL (Commission Regulation No. 2066/2001) because this protein can induce allergic reactions in the consumer. Casein and water-soluble potassium caseinate, major food allergens in bovine milk, are the most popular fining agents. Nevertheless, since traces of casein can still remain after coagulation, because they are nearly insoluble at the pH of wine, most winemakers prefer to perform a second treatment with bentonite (a claylike material of volcanic origin). At acidic pH, bentonite is negatively charged and binds casein via electrostatic interaction, thus allowing its removal by filtration. The treated wines can achieve clarity and improve color, flavor, and physical stability.

However, according to Directive 2007/68/EC, "any substance used in production of a foodstuff and still present in the finished product" must be declared in the label, especially if it originates from allergenic material. As such, the European Community has encouraged the food research area to develop new sophisticated methodologies to detect low abundance allergens and additives. As of July 1, 2012, the fining agents used in wine had to be declared

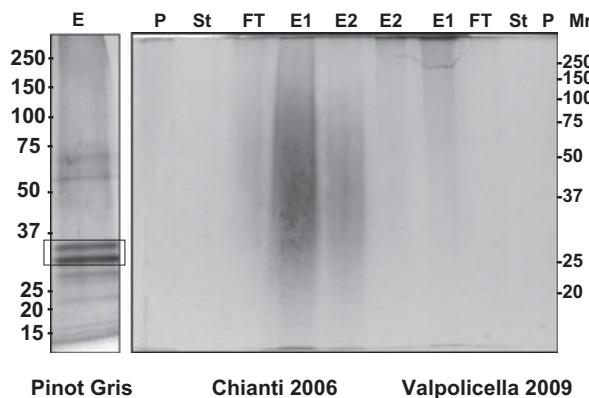


Figure 24.1

1D SDS-PAGE of one white wine (Pinot Gris) and two red wines (Chianti 2006 and Valpolicella 2009) treated by CPLLS at pH 3.3. FT, flow through; E1 and E2, eluates 1 and 2 from the beads; P, pellets, St, untreated starting material; Mr, molecular mass ladder. The highlighted gel region represents the bands identified as the caseins.

on the wine label under Regulation (EU) No. 579/2012, in conjunction to article 120g of Regulation (EU) No. 1234/2007 if exceeding the threshold of 0.25 mg/L allergenic protein.

ELISA methods for the detection of casein in white and red wines and to investigate the risk of allergenic residues in fined wines result in an LOD of 0.1 mg/L. For red wine the LOD was 0.2 mg/L in an indirect sandwich ELISA setup. The LOD of the indirect sandwich ELISA for white wine depends on the calibration standard. It is 0.1 mg/L for the fining agent casein and 0.01 mg/L for casein from a chemical trader. However, the use of different technological procedures during winemaking would lead to no detectable amounts of fining agents (Deckwart et al., 2014).

Further study, using CPLLS in combination with advanced mass spectrometry, enabled the detection of far lower casein levels. The analyses of untreated wine proved that as low as 1 µg/L of added casein can be detected with efficiency by SDS-PAGE, after CPLLS capture (Cereda et al., 2010). The analyses of an Italian white wine (Pinot Gris) and even some red wines (Chianti and Valpolicella) revealed the presence of bovine casein as a fining agent, although it is stated that red wines are in general fined with egg albumin (Fig. 24.1) (D'Amato et al., 2010). The caseins amount was in the range of 75–100 ng per bottle of 750 mL, representing an extremely high level of sensitivity, verified by analysis of calibration standards (D'Amato et al., 2010; Cereda et al., 2010). The identity of the captured proteins was verified by excising gel bands (Fig. 24.1), performing trypsin digestion, nLC MSMS analyses, and protein database interrogation with unrestricted taxonomy (Table 24.1).

The accurate detection of extremely low levels of fining agents in treated white wines has significant implications for winemakers in labeling their products and for EC regulation.

Table 24.1: Proteins identified in white wine after elution from combinatorial peptide ligand libraries.

| Gel Band | Accession Number | Protein Name | Taxonomy | Mascot Score | Mr | No. Peptides |
|----------|------------------|-------------------------|-------------------------------|--------------|--------|--------------|
| 1 | B5B3R8 | Alpha-S1 casein | <i>Bos taurus</i> | 654 | 24,484 | 12 |
| | P02663 | Alpha-S2 casein | <i>B. taurus</i> | 466 | 26,173 | 10 |
| | A3FJ56 | Kappa-casein | <i>B. taurus</i> | 113 | 19,676 | 2 |
| 2 | B5B3R8 | Alpha-S1 casein | <i>B. taurus</i> | 458 | 24,484 | 9 |
| | P02663 | Alpha-S2 casein | <i>B. taurus</i> | 348 | 26,173 | 7 |
| | P02666 | Beta-casein | <i>B. taurus</i> | 325 | 25,148 | 10 |
| | A3FJ56 | Kappa-casein (fragment) | <i>B. taurus</i> | 106 | 19,676 | 2 |
| 3 | B5B3R8 | Alpha-S1 casein | <i>B. taurus</i> | 349 | 24,484 | 8 |
| | O81228 | PR-4-type protein | <i>Vitis vinifera</i> | 215 | 15,674 | 3 |
| | B0FZ26 | Class IV chitinase | <i>Vitis pseudoreticulata</i> | 166 | 28,143 | 2 |
| | P02663 | Beta-casein | <i>B. taurus</i> | 161 | 25,147 | 6 |
| | A3FJ56 | Kappa-casein (fragment) | <i>B. taurus</i> | 115 | 19,676 | 2 |
| | P02663 | Alpha-S2 casein | <i>B. taurus</i> | 113 | 26,173 | 3 |

24.4 Proteomics of Grapevine Berries

The protein content of *V. vinifera* has been studied in depth in order to identify the berry proteins that persist during fermentation and that can form sediment in the wine bottle as well as potential protein allergens and bacterial and yeast proteins that could alter the quality of berry products. The investigation of berry development and ripening is highly related to yield and quality of the final product and as such is of importance to berry and wine producers.

The proteome profiling of Nebbiolo berries from 1 month after flowering up to the ripe stage showed 118 proteins differentially expressed during development, involved in energy metabolism and protein synthesis. The results suggested a general decrease in glycolysis during ripening and an increase in pathogen-related proteins in the 20–35 kDa range (Giribaldi et al., 2007).

Proteomics studies have also allowed the characterization of infections on berries before ripening. The infection of grape berries by *Botrytis cinerea*, a widespread fungus responsible for gray mold disease that degrades both must and wine proteins by exhibiting proteolytic activity, causes reductions in both quality and quantity of grapes and wine (Cilindre et al., 2008). The proteomic analysis of two champagne base wines prepared with either healthy or *Botrytis* Chardonnay grapes showed differentially expressed proteins. The study resulted in the identification of nine proteins distributed into several isoform zones: vacuolar invertase I, class IV chitinase, class IV endochitinase, VVTL1, putative thaumatin like protein, osmolin

like protein, PR4-type protein, and ss-1,3-glucanase. The disappearance of numerous grape proteins was observed in the *Botrytis* grape wine, suggesting that they were probably degraded or even repressed or the result of a differential expression of grape proteins upon fungal infection. On the other hand, two pectinolytic enzymes secreted by *B. cinerea* were found only in the *Botrytis* grape wine (Cilindre et al., 2008).

The discovery of mechanisms involved in growth and mycotoxin biosynthesis, implicated in the induction of grape protein modifications, could improve the control on infection and on secretion of toxins due to the presence of fungus. A proteomic analysis of Amarone wine grapes, infected by two strains of *Penicillium expansum* (Pe1) and *Penicillium crustosum* (Pc4) described the physiological mechanisms underlying the pathogen attack (Lorenzini et al., 2016). The Pe1 strain had a major impact on *V. vinifera* protein expression by inducing pathogenesis-related proteins and other protein species involved in energy metabolism. Increased expression of new *Penicillium* proteins involved in energy metabolism and some protein species related to redox homeostasis has been observed in grapes infected by the Pc4 strain (Lorenzini et al., 2016). Moreover, the newly induced proteins in infected grapes could represent potential markers in withered grapes, thus creating the chance to develop case-sensitive prevention strategies to inhibit fungal growth. The modification of grape protein patterns after *Penicillium* infection could provide useful data on its effects on grape and wine quality and gives information to prevent fungus development (Lorenzini et al., 2016).

Another serious disease of grapevine is the downy mildew, caused by the oomycete *Plasmopara viticola*. The beneficial microorganism *Trichoderma harzianum* T39 (T39) induces plant-mediated resistance and reduction of the severity of downy mildew in susceptible grapevines (Palmieri et al., 2012). Quantitative proteomic analysis of T39-induced resistance in grapevine showed 800 differentially expressed proteins, involved in signal transduction, indicating activation of a complete microbial recognition machinery in response to stress and redox balance, thus suggesting an active defense response to downy mildew (Palmieri et al., 2012).

24.5 Proteomics of Wine and Other Alcoholic Beverages

The proteomic analyses of red wines (Chianti and Valpolicella), by CPLPs at different binding pHs, in combination with high resolution MS and the interrogation of a protein database with unrestricted taxonomy, allowed the discovery of unpredictable species, such as the caseins and bacterial proteins (D'Amato et al., 2010). In particular, the analyses resulted in the identifications of proteins belonging to *V. vinifera* (thaumatin-like proteins), to plant pathogens and fungi (e.g., *Botryotinia fuckeliana*, *Sclerotinia sclerotiorum*, *Aspergillus aculeatus*), to *Saccharomyces cerevisiae* and to *Bos taurus* (alpha-S1 casein, albumin, and beta-lactoglobulin) (Table 24.2) (D'Amato et al., 2010). Surprisingly, after clarification treatments, the CPLP capture enabled the recovery and identification of very small amounts of residual proteins, showing the bacterial infection of grapes used in the production of wines.

In addition, glycoprotein profiling of untreated Chardonnay white wine revealed the presence of a number of potential allergens sharing homology with known plant and fruit allergens including 13 grape and 15 yeast proteins with 44 N-linked glycosylation sites identified (Palmisano et al., 2010).

The proteinaceous content of a nonclarified wine, a home-produced Recioto wine, was examined by proteomic profiling of the residual grape proteins (D'Amato et al., 2011). Capture by CPLLs at different pH values resulted in different intensity profile on 1D SDS-PAGE (Fig. 24.2). The eluates of untreated wine, or after capture at pH 3.8, 7.2, 9.3 and finally at pH 2.2, compared to the control, showed a large spectrum of polypeptide chains, extending from 8 up to 250 kDa (Fig. 24.2, Panel A). Moreover, among all eluates, the pH 2.2 capture generated the most intense gel image, with a total optical density at least three times higher than the SDS profiling of the pH 7.2. The identifications, upon consulting the EST_Vitis vinifera and Uniprot_Viridiplantae databases, enabled the discovery of a total of 106 unique gene products, the largest description so far of any wine proteome (Fig. 24.2, Panel B). In addition to these identifications, the screening of the *S. cerevisiae* protein database enabled the identification of an additional 11 unique gene products belonging to yeast (Table 24.2). The Venn diagrams of Fig. 24.2 summarize the contributions of the various fractions to the total identifications: the precipitate alone gave the highest contribution (52 proteins), followed by the summed contributions of all four CPLL captures (49 proteins).

Table 24.2: Proteins identified in Valpolicella red wine eluted from combinatorial peptide ligand libraries (binding at pH 7.2 and 9.3).

| Accession number | Protein Name | Taxonomy | Mascot Score | pH 7.2 | pH 9.3 |
|------------------|---|---|--------------|--------|--------|
| B3LI46 | Cell wall protein | <i>Saccharomyces cerevisiae</i> (strain RM11-1a) | 403 | x | - |
| A6RS43 | Putative uncharacterized protein | <i>Botryotinia fuckeliana</i> (strain B05.10) | 285 | x | - |
| B3LP15 | Protein YGP1 | <i>Saccharomyces cerevisiae</i> (strain RM11-1a) | 244 | x | x |
| P02662 | Alpha-S1 casein | <i>Bos taurus</i> | 197 | x | x |
| A6S6M5 | Putative uncharacterized protein | <i>Botryotinia fuckeliana</i> (strain B05.10) | 168 | x | x |
| A2QAC9 | Remark: Alternate name for <i>S. cerevisiae</i> Crh1: YGR189 C. | <i>Aspergillus niger</i> (strain CBS 513.88/FGSC A1513) | 163 | x | - |
| Q8NK89 | Alpha-L-arabinofuranosidase B | <i>Aspergillus kawachi</i> | 146 | - | x |
| B0JYQ0 | ALB protein | <i>B. taurus</i> | 145 | x | x |
| P02754 | Beta-lactoglobulin | <i>B. taurus</i> | 139 | x | x |
| A3QRB5 | Thaumatin-like protein | <i>Vitis vinifera PE</i> | 136 | x | - |
| A6SNL1 | Putative uncharacterized protein | <i>Botryotinia fuckeliana</i> (strain B05.10) | 119 | x | - |
| Q00012 | Mannan endo-1,4-beta-mannosidase A | <i>Aspergillus aculeatus</i> | 109 | x | x |

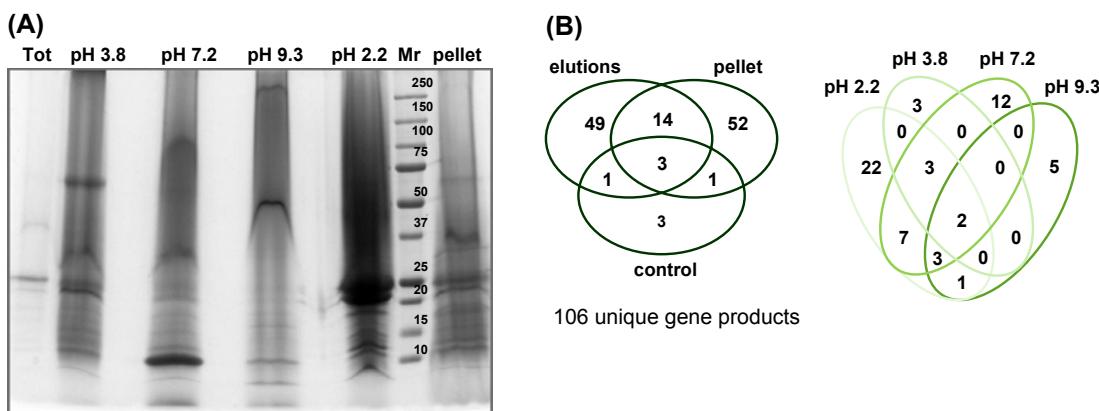


Figure 24.2

Panel A: 1D SDS-PAGE of Recioto untreated red wines. Control and SDS eluates after treatment at pH 3.8, 7.2, 9.3, and 2.2. Panel B: Venn diagrams of identified proteins in the different eluates and control.

These datasets might be exploited to obtain a proteomic signature (proteotyping) of high-quality wines for protection against counterfeited products (D'Amato et al., 2011).

The described proteomics approach was also applied to Champagne bottles (Cilindre et al., 2014) using CPLLS; a wide variety of proteins were identified as belonging to various classes of proteins from grape and yeast. Among the identified proteins were candidates that are thought to act to enhance the foaming properties of Champagne and sparkling wines in part as a result of their glycosylation status. A total of 43 unique gene products were identified from both Champagne wines, among which 15 were common to both samples (Cilindre et al., 2014). Champagne base wines contain a majority of proteins of grape origin (12 from *V. vinifera*), compared to a relatively poor diversity of yeast proteins and fungi, including proteins from PR-4, PR-5, and PR-14 families (i.e., thaumatin-like, VVTL1, and osmotin-like proteins) and *S. cerevisiae*. However, the proteomics approach has not yet enabled differentiation according to the grape varieties since a high number (24) of proteins from other *Viridiplantae* organisms were identified in both Champagne wines (Cilindre et al., 2014).

Finally, a proteomics study of vinegar showed for the first time its proteome content. The trace proteome of white-wine vinegar, after CPLL capture at pH 2.2 and in presence of 0.1% trifluoroacetic acid resulted in a total of 27 unique gene products, of which 10 were specific to the *V. vinifera* database. The most abundant species detected was PN40024 scaffold_22, a protein of the glycosyl hydrolase family (Di Girolamo et al., 2011).

24.6 Conclusions

The combination of CPLL technology with MS and in general the proteomic approach has allowed the identification of very-low abundance proteins in commercial food products.

Proteomics is indeed a robust methodology with potential applications in food manufacturing to monitor contamination or the presence of allergens and to enable transparency in product labeling. The comprehensive data collected using proteomics approaches will serve as the foundation for researchers to detect, characterize, and understand the mechanisms underlying fungal or bacterial infections. Secondly, the induction of secreted proteins and their effect on the quality of wines can be investigated. Lastly, the potential allergenic reactions due to the presence of unknown or known molecules, still not declared by wine makers, may be avoided. Thus, in the food industry, proteomics and/or peptidomics represents a novel, but formidable tool, useful for certifying the authenticity of wines and enabling producers to follow the labeling directives of European Commission.

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SECTION 5

Food Spoilage, Pathogenic Organisms and Allergens

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Proteomics of Food Spoilage Pathogens

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25.1 Introduction

A major issue of the food industry is to assure the safety and high quality of foodstuffs provided to consumers. In this sense, foodborne pathogenic bacteria have to be precisely detected and identified in a rapid and reliable way. Once the nature of the bacterial species present in the food product is determined, measures can be taken to control and minimize the microbiological hazard. Early detection and characterization of foodborne pathogens may significantly improve the ability of a risk management system to avoid the spread of food-borne outbreaks. Likewise, the identification of the sources of outbreaks and contaminations provides information on transmission routes and critical hazard points in the food chain, allowing the industry and government to target efforts to control foodborne pathogens.

The study of food spoilage pathogens traditionally relied on culturing processes coupled to morphological, physiological, and biochemical characterization. Over the last few decades, the implementation of rapid and sensitive biomolecular tools has included miniaturized biochemical systems, antibody-based assays, and DNA-based methods. Nowadays, “omics” technologies, including genomics, transcriptomics, proteomics, and metabolomics, together with important advances in bioinformatics tools, offer extensive information of the biomolecules produced by an organism.

Proteomic tools enable protein identification and quantification but also allow the study of protein expression, function, interaction, and structure. These techniques mainly include two-dimensional (2D) gel electrophoresis and mass spectrometry (MS) that were both introduced to the characterization and identification of microorganisms. With 2D gel electrophoresis, proteins are separated on a gel by their isoelectric point and mass. Bacterial identification has been carried out by comparing the migration pattern with reference gel patterns in an established database. However, 2D gel electrophoresis is time and labor intensive, being inadequate for rapid pathogen identification. Instead, the approach of 2D gel electrophoresis is mainly used for the analysis of protein mixtures, with the aim to resolve and compare the total protein abundance for bacterial pathogens or

studying the response of a bacterial strain to a certain stress or growth condition. In this sense, proteins of interest may be isolated and identified by combining 2D gel electrophoresis with mass spectrometry.

Mass spectrometric-based proteomics was greatly advanced by the development of soft ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI), and electrospray ionization (ESI) that permit the analysis of intact large biomolecules. A variety of targets such as bacteria, whole cells, cell lysates, or isolated proteins are analyzed using these two MS approaches. These ionization methods are most commonly coupled to time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography (LC-ESI-MS) for bacterial identification and characterization. The resulting spectral profiles that reveal the masses of the proteins are compared to databases (experimental or *in silico*), with the aim to identify organism-specific signatures (fingerprints) or proteins (biomarkers). Multistage MS (tandem MS or MS/MS) allows the fragmentation of selected proteins and gives information about the amino acid sequences. With the help of bioinformatics tools, selected proteins may be identified by the spectral profiles of peptides obtained after digestion (bottom-up approach) or by the spectral profiles derived from fragmentation of intact proteins (top-down approach).

In the following sections, the applications of MALDI-TOF and LC-ESI MS to the analysis of food spoilage pathogens are reviewed, with special focus on bacterial identification and typing. Furthermore, the use of proteomics to study the response of foodborne pathogens to a stress or growth condition, such as the adaption to a certain food matrix, is described briefly.

25.2 MALDI-TOF MS Fingerprinting for Bacterial Identification of Food Spoilage Pathogens

Of all the proteomic approaches, MALDI-TOF MS is the most widely applied technique for fast bacterial identification, due to its rapid nature, simplicity, and cost-effectiveness. MALDI is a very soft ionization technique that allows large molecules, such as proteins, to be analyzed without fragmentation of the molecules. The sample is mixed and crystallized with a matrix solution that protects the target molecules when subjected to ablation by a laser beam by absorbing the applied energy. Subsequently, the matrix ions transfer the energy to the sample molecules, resulting in ions of low charge (typically 1+ and sometimes 2+). The ions generated are separated in an electric field with relation to their mass/charge (m/z) ratio by a time-of-flight analyzer (TOF).

With MALDI-TOF MS soluble and low molecular weight bacterial proteins (1500–20,000 Da) are preferentially detected wherein ribosomal proteins are frequently encountered and to a lesser extent structural proteins, such as cold shock proteins and DNA-binding proteins

(Ryzhov and Fenselau, 2001). The use of a strong acid and organic solvent can cause the lysis of the cell wall and liberation of intracellular proteins. Many studies have demonstrated that whole bacterial cells can be analyzed directly without any sample pretreatment, just by picking single colonies and overlaying with the matrix solution, containing formic acid and acetonitrile. Nevertheless, the quality and resolution of spectral profiles is improved when analyzing protein extracts, obtained with a short mixing and centrifugation step employing a strong acid and organic solvent (Böhme et al., 2010). The resulting spectral profiles are highly specific and represent a fingerprint for the corresponding organism (Clark et al., 2013a). However, it has been shown that the spectra are variable when different culture conditions and sample preparation protocols are applied and emphasized the need for a standardized procedure to allow spectral comparison (Wunschel et al., 2005).

To solve the problem of reproducibility, bacterial identification by MALDI-TOF MS has been carried out by determining the masses of biomarker proteins. Searches are then conducted against a protein database by matching the masses against theoretical sequence-derived masses, taking into account possible posttranslational modifications (Fenselau et al., 2007). Although this approach is robust to experimental variations, relatively few studies have described its successful application for bacterial discrimination (Sun et al., 2006; Fenselau et al., 2007). The database searches require high mass accuracy to unequivocally assign the corresponding proteins by their masses alone, which is difficult to achieve with MALDI-TOF MS instruments. In addition, the continuously expanding protein sequence databases have led to an increase in the number of proteins with very similar masses.

The MALDI-TOF MS profiling approach has been established as the most promising technique for the characterization of microorganisms and the number of studies employing this approach has increased significantly in the last decades. Several detailed reviews give an overview over the methodology, sample preparation, and application to bacteria, yeasts, and fungi (Clark et al., 2013a; Welker and Moore, 2011). Although following a standardized protocol is important for spectral reproducibility, it has been shown that comparison and identification of bacterial strains is also possible even if different protocols have been applied, due to a set of conserved biomarker proteins.

Bacterial identification by MALDI-TOF MS profiling is carried out by comparing the spectral profile to a previously created reference library and searching for the best match. In this sense, a library with a great number and variety of spectral entries significantly improves the success for correct species identification. The creation of representative databases and the establishment of adequate search algorithms to find the best match, taking into account possible mass variations and peak intensities, are fundamental for the reliable identification of an unknown bacterial strain. Two commercial systems, the MALDI Biotyper from Bruker Daltonics (Bremen, Germany) and the Vitek MS platform from bioMérieux (Marcy l'Etoile,

France) have been developed over the last years and are nowadays implemented into routine clinical analysis. The Food and Drug Administration (FDA) approved the MALDI Biotyper for the official identification of 40 bacterial species and the Vitek MS platform for 194 species (Deak et al., 2015). Both include comprehensive databases comprised of spectral profiles of bacteria, mycobacteria, and fungi and have been successfully validated with huge numbers of clinical isolates, achieving 92%–93% of correct species identification (Bizzini et al., 2010; van Veen et al., 2010; Martiny et al., 2012). Other databases include the Andromas database from Andromas SAS (Paris, France) and the MicrobeLynx bacterial identification system from Waters Corporation (Manchester, UK). The Andromas database has been implemented into the clinical microbiology laboratory of the Necker-Enfants malades hospital to identify all microorganisms isolated routinely, achieving 93%–99% correct species identification (Bille et al., 2012). Likewise, the MicrobeLynx database has been successfully applied to identify clinical isolates (Rajakaruna et al., 2009).

The aforementioned databases are under continuous improvement with respect to both their content and the data analysis tools and allow the addition of intralaboratory results as new entries. The main drawback is that they are only available commercially and are costly to access. Furthermore, the strains included in the databases are not always sufficient for a correct species identification and the creation of in-house spectral databases may lead to better results as described by Erler et al. (2015) that developed the spectral library VibrioBase for the discrimination of *Vibrio* species (Erler et al., 2015).

MALDI-TOF MS fingerprinting has been studied extensively for the identification of clinically relevant bacterial species and has been successfully applied for routine clinical analysis to identify strains causing human diseases. In the cases of sepsis, the infectious bacterial species may be identified directly in the blood of the patient within minutes (Klein et al., 2012). Since many human diseases are caused by the consumption of contaminated food, MALDI-TOF MS fingerprinting studies and commercial databases include a huge number of bacterial strains that correspond to food spoilage pathogens. Table 25.1 gives an overview of studies that were focused on the analysis and discrimination of foodborne pathogens. In a further study, histamine-producing bacterial species have successfully been differentiated by MALDI-TOF MS fingerprinting (Fernández-No et al., 2010). Mazzeo et al. (2006) analyzed species of the genera *Escherichia*, *Yersinia*, *Proteus*, *Morganella*, *Salmonella*, *Staphylococcus*, *Micrococcus*, *Lactococcus*, *Pseudomonas*, *Leuconostoc*, and *Listeria* that are commonly implicated in food spoilage and made their spectral profiles and peak mass lists freely available on the internet (http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm).

However, identification has been mainly applied to clinical isolates and to a lesser extent to bacterial strains isolated from food. For food control purposes and to perform efficient bacterial species identification of foodborne pathogens in food products, it is important to include spectral information of strains isolated from different food matrices into the spectral

Table 25.1: Overview of intact cell MALDI-TOF MS fingerprinting studies carried out on food spoilage pathogens.

| Species | References |
|--------------------------------|--|
| <i>Acinetobacter baumanii</i> | Sousa et al. (2014) and Alvarez-Buylla et al. (2012) |
| <i>Aeromonas hydrophila</i> | Donohue et al. (2006) and Dieckmann et al. (2010) |
| <i>Bacillus</i> spp. | Branquinho et al. (2014) and Fernández-No et al. (2013) |
| <i>Campylobacter</i> spp. | Mandrell et al. (2005) , Bessède et al. (2011) , Zautner et al. (2013) and Kolínská et al. (2008) |
| <i>Clostridium</i> spp. | Grosse-Herrenthey et al. (2009) and Reil et al. (2011) |
| <i>Cronobacter sakazakii</i> | Zhu et al. (2011) and Stephan et al. (2010) |
| <i>Escherichia coli</i> | Siegrist et al. (2007) , Novais et al. (2014) , Matsumura et al. (2014) , Clark et al. (2013b) , Christner et al. (2014) and Khot and Fisher (2013) |
| <i>Legionella</i> spp. | Gaia et al. (2011) , Moliner et al. (2010) and Pennanec et al. (2010) |
| <i>Listeria</i> spp. | Barbuddhe et al. (2008) and Jadhav et al. (2014) |
| <i>Mycobacterium</i> spp. | Amlerová et al. (2014) and Adams et al. (2015) |
| <i>Salmonella enterica</i> | Kuhns et al. (2012) , Dieckmann and Malorny (2011) , Dieckmann et al. (2008) and Sparbier et al. (2012) |
| <i>Shigella</i> spp. | Khot and Fisher (2013) |
| <i>Staphylococcus aureus</i> | Szabados et al. (2010) , Böhme et al. (2012b) , Du et al. (2002) , Jackson et al. (2005) , Wolters et al. (2010) , Carbonnelle et al. (2007) , Dubois et al. (2010) and Josten et al. (2013) |
| <i>Vibrio</i> spp. | Hazen et al. (2009) , Dieckmann et al. (2010) and Erler et al. (2015) |
| <i>Yersinia enterocolitica</i> | Lasch et al. (2010) , Ayyadurai et al. (2010) and Stephan et al. (2011) |

libraries. Spectral profiles of wild-type strains may differ significantly from those of reference strains due to changes in their phenotypic and proteotypic profiles, as a response to environmental changes and stress conditions they are subjected to in a concrete food matrix. In this sense, [Böhme et al. \(2012a\)](#) constructed a spectral library including reference strains, as well as strains isolated from food of bacterial species related to seafood spoilage. The created spectral library SpectraBank ([Böhme et al., 2012a](#)) is publicly available to other researchers and has been successfully applied to identify bacterial strains isolated from seafood products ([Böhme et al., 2011](#)).

In further studies, bacterial strains isolated from food products could be clearly identified at the species level and distinguished from other, less pathogenic species of the same genus. These included enterotoxin-producing *Staphylococcus aureus* strains isolated from dairy products ([Böhme et al., 2012b](#)), strains of the emerging foodborne pathogen *Cronobacter sakazakii* in infant formulas ([Stephan et al., 2010](#); [Zhu et al., 2011](#)), and *Vibrio parahaemolyticus*, a main causative agent of pandemic outbreaks of seafoodborne gastroenteritis ([Hazen et al., 2009](#)).

In comparison to other identification tools that are commonly applied to bacterial identification in the food sector, such as classical culturing, phenotypic tests, immunological assays, and DNA-sequencing, MALDI-TOF MS fingerprinting is faster and less laborious, achieving

specificity (with 92%–98% of correct species identification) which is a significantly better result than obtained with commonly applied microbial identification tools. At the species and subspecies level, MALDI-TOF MS has been demonstrated to be more discriminative than 16S rRNA sequencing, allowing the differentiation of genetically closely related species (Böhme et al., 2013). However, the fingerprinting approach is limited to isolated strains, thus requiring time-consuming culturing steps prior to analysis. The determination of characteristic biomarkers may achieve the identification of mixed cultures of two distinct species, but bacterial discrimination in complex mixtures or matrices is not yet possible, because the number of overlapping signal ions increases. Nevertheless, the search for specific peak masses by MALDI-TOF MS profiling and the identification of the corresponding proteins is of great interest for bacterial pathogen control, since they may be targeted in further approaches and thus allow the detection and even quantification of the corresponding species in a complex food matrix. In this sense, a number of species-specific and genus-specific peak masses have been described for bacterial pathogens by MALDI-TOF MS analysis and identified by additional proteomics methods (Suarez et al., 2013; Fagerquist, 2007).

Using a different strategy, top-down proteomics allowed the discrimination of closely related species by the resulting different fragmentation patterns, although peak masses of the original biomarkers were nearly identical (Demirev et al., 2005).

25.3 Foodborne Pathogen Detection by LC-ESI-MS

The aforementioned limitations of MALDI-MS can be overcome by other applications of mass spectrometry such as ESI-MS, wherein higher mass resolution can be achieved, allowing the distinction of specific biomarkers with high confidence. In addition, ESI-MS is commonly combined with separation techniques such as liquid chromatography (LC) that facilitates a more accurate and efficient identification of specific biomolecules and the detection of target molecules in complex mixtures and matrices. LC-ESI-MS has been applied to analyze different pathogenic strains and compared to MALDI-TOF MS profiling. As expected, more proteins were detected by LC-ESI-MS with a higher mass accuracy, resulting in the determination of more biomarker proteins and allowing the discrimination down to the strain level (Mott et al., 2010).

The fragmentation of characteristic proteins by top-down proteomics methodologies allowed the identification of the intact proteins and the corresponding bacterial species, either pure or in mixtures (Wynne et al., 2010).

The bottom-up approach is commonly employed in microbiological studies and includes sample preparation procedures involving enzymatic protein digestion and fragmentation of the resulting peptides by tandem mass spectrometry. The observed masses of the proteolytic fragments are compared to the calculated masses of peptides, derived from the amino acid sequences of the

corresponding proteins stored in protein databases. This technique provides a huge amount of data allowing confirmation of the protein sequence(s) and yielding much information about the studied sample. Another advantage over MALDI-MS profiling is the availability of comprehensive public protein databases that include protein sequences that have been experimentally determined or theoretically derived from DNA sequences. The bottom-up proteomics approach has been successfully applied to bacterial classification of pathogens at the species level and strain level (Jabbour et al., 2010; Alves et al., 2016). However, the whole process can be labor intensive and requires statistical tools for reliable identification, thus being currently inefficient for routine bacterial identification purposes (Sauer and Kliem, 2010).

In contrast, LC-ESI-MS/MS is an important tool for the detection and identification of microbial toxins and to discover proteins that are associated with virulence or antibiotic resistance (Martinović et al., 2016; Cifuentes, 2012). Cell surface proteins have been studied to investigate factors that are involved in the surface attachment and biofilm formation by pathogenic bacteria that are problematic in food processing facilities (Tiong et al., 2016).

The search for biomarkers that allow the determination of the pathogenicity of an unknown strain and the detection of bacterial species in mixtures is of crucial interest for pathogen control in food. In this sense, characteristic peptides that permit the discrimination of the corresponding pathogenic bacterial species have been described for *Clostridium perfringens* (Sengupta et al., 2010), *S. aureus* (Calo-Mata et al., 2016), *Yersinia pestis* (Chenau et al., 2014), and the *Bacillus cereus* group (Dworzanski et al., 2006).

To reduce the huge amount of information obtained with bottom-up proteomics when whole bacterial lysates are analyzed, the combination of LC-ESI-MS/MS with previous results from MALDI-TOF MS fingerprinting can be an effective strategy (Fig. 25.1). Protein extracts are obtained from whole bacterial cells in a rapid manner as described for MALDI-TOF MS experiments. The same extracts are analyzed by LC-ESI-MS/MS and specific proteins determined by the MALDI fingerprinting approach are filtered out from the results. In this way, specific peptides have been determined that could be targeted by proteomics tools and allow the selective detection of the corresponding species in food matrices.

25.4 Typing of Food Spoilage Pathogens by Proteomics

The differentiation of subspecies and serotypes is of crucial importance for risk assessment in the food sector, due to the varying pathogenic character. Likewise, comparison of strains isolated from food to clinical isolates and the determination of clonal lineages is fundamental for epidemiological studies of foodborne disease outbreaks. Microbial source tracking (MST) aims at the detection of foodborne pathogens in the food chain and to determine the source of contamination and consequently take corrective actions, as well as prevent foodborne outbreaks.

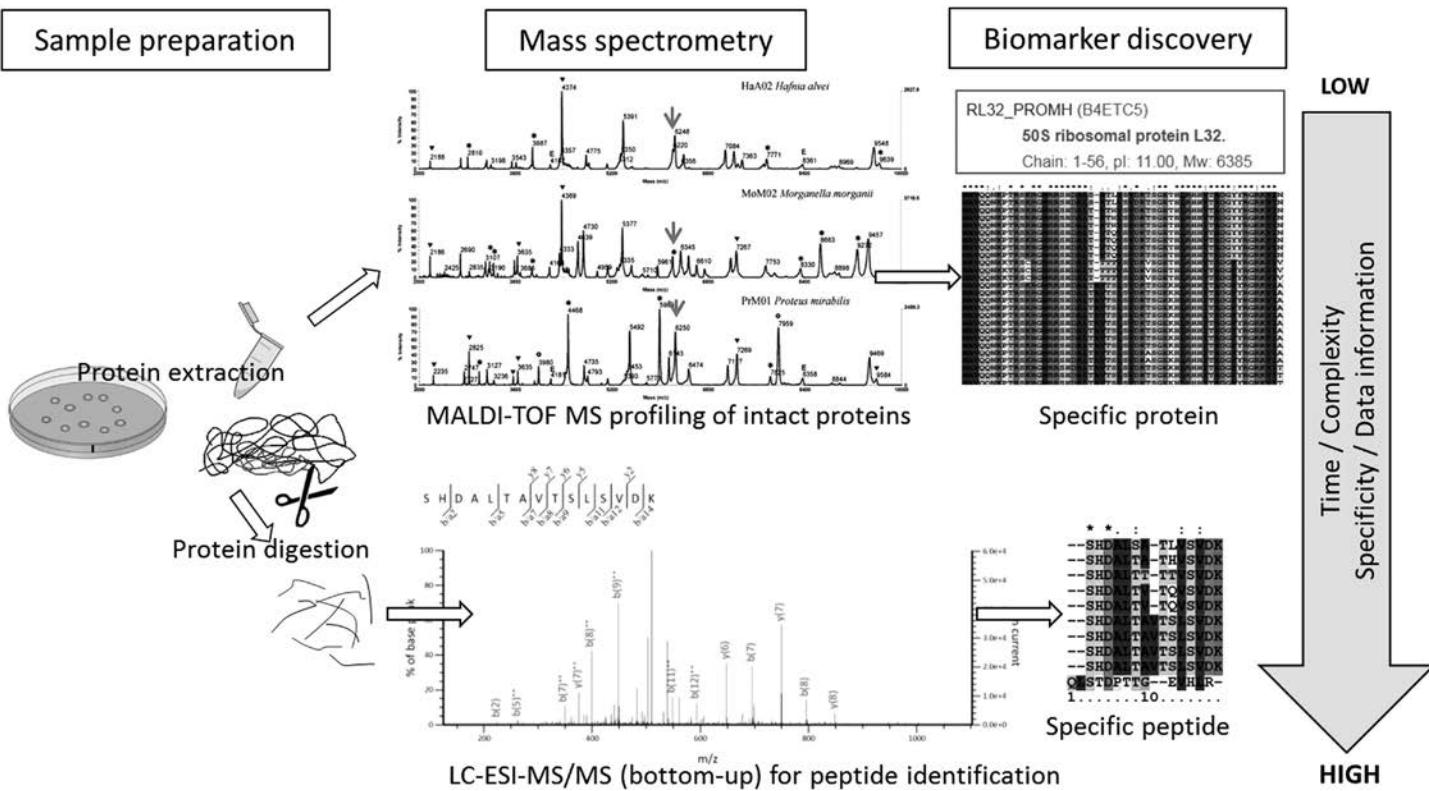


Figure 25.1

Scheme of the combined approach of MALDI-TOF MS and LC-ESI-MS/MS for biomarker discovery.

The protein expression profiles of bacterial strains that represent different physiological states and/or responses to certain conditions are commonly compared by 2D gel electrophoresis of crude extracts derived from intact bacterial cells. Differentially expressed proteins are determined and identified by MS/MS proteomics approaches. More recently, quantitative LC-MS/MS is being applied with the same objective but achieving higher resolution and much more information (Van Oudenhove and Devreese, 2013).

Due to the simplicity and speed, MALDI-TOF MS has been used as a bacterial typing tool, with the aim to detect pathogenicity, antimicrobial resistance, and carry out epidemiological studies (Clark et al., 2013a; Sandrin et al., 2013). Bioinformatics applications classify reference and unknown strains into a dendrogram and group them into different clusters. Although MALDI-TOF MS profiling was demonstrated to have a higher discriminatory potential compared to DNA-based clustering, the obtained grouping originating from spectral differences could rarely be related to pathogenicity, toxin production, or antibiotic resistance (Böhme et al., 2012b). In contrast, the analysis of methicillin-resistant *S. aureus* strains isolated during an outbreak allowed the differentiation from methicillin-sensitive strains, as well as the rapid typing of the outbreak strains and detection of epidemic lineages (Josten et al., 2013). Also successful was the differentiation of *Enterococcus faecium* and *Enterococcus faecalis* strains in relation to their isolation sources (meat or dairy products) (Quintela-Baluja et al., 2013). Similar, spectral variability could be observed at the strain level in relation to the geographical origin and moment of isolation of *V. parahaemolyticus* strains (Hazen et al., 2009). Nevertheless, to perform bacterial typing of serotypes, pathotypes or clonal lineages, in most cases the determination of subtype-specific biomarker peaks is required (Suarez et al., 2013). In this sense, subtyping of *Yersinia enterocolitica* (Stephan et al., 2011), *Y. pestis* (Ayyadurai et al., 2010), *Campylobacter jejuni* (Mandrell et al., 2005), *Escherichia coli* (Novaïs et al., 2014; Matsumura et al., 2014; Christner et al., 2014), *Salmonella enterica* (Dieckmann and Malorny, 2011; Kuhns et al., 2012), and *S. aureus* (Wolters et al., 2010) could be achieved after identifying specific biomarkers.

As mentioned in the previous sections, MALDI-TOF MS profiling has its limitations with respect to biomarker discovery and needs considerable refinement for routine bacterial typing. LC-ESI-MS/MS in contrast has a much higher potential to search for characteristic peptides at all taxonomic levels. Virulence factors and specific peptides have been identified to detect pathogenic and nonpathogenic *Y. pestis* strains in food samples after immune capture of intact cells (Chenau et al., 2014). Likewise, toxin-producing serotypes of *E. coli* have been compared to nonpathogenic strains (Lim et al., 2007) and biomarkers determined for shiga toxin identification (Fagerquist et al., 2014; Jabbour et al., 2014). A unique biomarker has been described that allowed the tracking of the pandemic O3:K6 strain and its clonal derivatives of *V. parahaemolyticus* (Williams et al., 2004). *Listeria monocytogenes* has also been studied by LC-ESI-MS/MS and virulence factors, as well as further biomarkers could be identified,

permitting the differentiation of pathogenic and nonpathogenic strains (Trost et al., 2005). Likewise, virulent and nonvirulent *S. aureus* strains could be distinguished by a reference map of characteristic proteins (Gatlin et al., 2006). A top-down proteomics approach based on the intact protein expression profiles of bacterial isolates has been applied to distinguish serovars of *S. enterica* (McFarland et al., 2014).

There is tremendous potential for proteomics tools to identify and classify food spoilage pathogens and develop control strategies. One of the most promising approaches is the identification of biomarkers to trace sources of contamination to ensure food safety and prevent foodborne disease outbreaks. Another important aim is the study of factors that are related to survival under stress conditions and in important food matrices. In this sense, the response and adaption of different foodborne pathogens to food matrices, such as meat and lettuce, and food characteristic conditions, such as salt content and heat treatment, have been studied by proteomics tools (Kim et al., 2009; Mujahid et al., 2008; Zhang et al., 2014; Williams et al., 2005).

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Biotyping Meets Proteomics: Mass Spectrometry-Based Approaches for Characterization of Microorganisms

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26.1 Introduction

Identification and differentiation of microorganisms by mass spectrometry (MS) has developed to a robust cutting-edge diagnostic technology in microbiological laboratories in recent years (Dallagassa et al., 2014; Welker and Moore, 2011; Giebel et al., 2010; Seng et al., 2009; Freiwald and Sauer, 2009; Demirev and Fenselau, 2008). For this approach, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS based on profiling of mainly taxonomic relevant ribosomal proteins and comparison to a reference mass spectra database has proved to be superior. High speed of analysis, short time-to-result, and a streamlined protocol allow a cost-effective identification within less than 20 min. Application areas comprise clinical and veterinary diagnostics, food safety control, outbreak tracking, environmental microbiology, biotechnology, and biodefense (Wenning et al., 2014; Böhme et al., 2013; Quintela-Baluja et al., 2013; Kuhnert et al., 2012; Hahn et al., 2011; Lasch et al., 2009, 2010; Murray, 2010; Seng et al., 2009; Teramoto et al., 2009; Dieckmann et al., 2008; Siegrist et al., 2007; Maier et al., 2006). The main challenge of MS-based identification is to reliably increase the taxonomic resolution to below-species level. This is due to the substantial genetic overlap and thus high protein similarity. Currently, two approaches are being used to resolve mass peak variations below the species level: the library-based and the proteomics-based approach. Within the library-based approach, sample pretreatment or data reduction strategies have been developed. Proteomics-based approaches include bottom-up and top-down characterization of biomarkers applying publicly available databases. The focus of this chapter is on characterization of microorganisms and the combination of rapid sample pretreatment and subsequent proteomics approaches for microbial species and subspecies differentiation is highlighted. Specifically, we demonstrate the application of tryptic peptides as a recent development in enhancing the discriminatory power for bacterial profiling. Rapid identification at the below-species level is crucial in the diagnosis of food-borne pathogens to determine

appropriate drug therapy, to reliably trace back contamination sources in elucidation of epidemics, and to improve food safety during processing. Furthermore, we shortly comment on the MALDI top-down approach as a method to identify discriminatory protein masses present in biotyping protein profiles (Fig. 26.1).

26.2 Library-Based Approach

In the library-based approach, peak lists extracted from a mass spectral fingerprint of unknown microorganisms are compared to the peak lists of reference spectra deposited in a database, which contains a collection of well-characterized strains. Subtle and reproducible differences detected in mass spectra were applied in most studies reporting successful profiling of bacteria below the species level using MALDI-TOF MS. A general prerequisite for MALDI-TOF MS-based identification is cultivation of microorganisms on solid or liquid media and subsequent direct smear of inactivated whole cells onto the MALDI target or short chemical extraction with formic acid and acetonitrile and spotting of supernatants onto the MALDI target. In both cases (direct smear or extract), the sample is covered with a MALDI matrix, for example, α -cyano-4-hydroxycinnamic acid. Mass spectra are acquired in positive ion mode from random locations on the target spot and comprise a mass range of 2–20 kDa (Ghyselinck et al., 2011; Ilina et al., 2010), of a broader (Teramoto et al., 2009; Hettick et al., 2006; Jackson et al., 2005) or narrower mass range (Rajakaruna et al., 2009; Keys et al., 2004). Particularly, high mass ions are promising for differentiation on below-species level due to rarity of these ions and to absence of background signals. Single mass peaks of spectra in library-based approaches are not given proof of identity; however, most of the peaks are attributed to basic, abundant, and conserved proteins, in particular ribosomal proteins (Sauer and Kliem, 2010; Fenselau and Demirev, 2001) and to a minor degree to cell wall-associated proteins (Evanson et al., 2001). Approximately 30% of total proteins in a cell being in the exponential growth phase are ribosomal proteins. Success in below-species identification using library-based approaches requires robust software, reliable algorithms, as well as databases in order to precisely compare acquired spectra to database entries and to calculate the similarity. In addition, mass spectral quality (resolution, accuracy, reproducible acquisition of spectra) is crucial and standardized experimental conditions including culture conditions need to be strictly followed to ensure reproducibility of the MALDI mass spectra and to detect specific protein biomarker masses for microorganisms below the species level.

Regarding categorization on below-species level, serovars of *Salmonella enterica* subsp. *enterica* have been categorized by comparison of their MALDI-TOF mass spectra (Leuschner et al., 2003). Karger et al. (2011) employed a library-based approach to categorize STEC serovars and Stephan et al. (2011) categorized *Yersinia enterocolitica* as pathogenic or nonpathogenic strains. Strains of *Yersinia pestis* could be categorized according to their biotypes (Ayyadurai et al., 2010) and clinical strains of *Moraxella catarrhalis* have been categorized at the subpopulation level (Schaller et al., 2006). In a

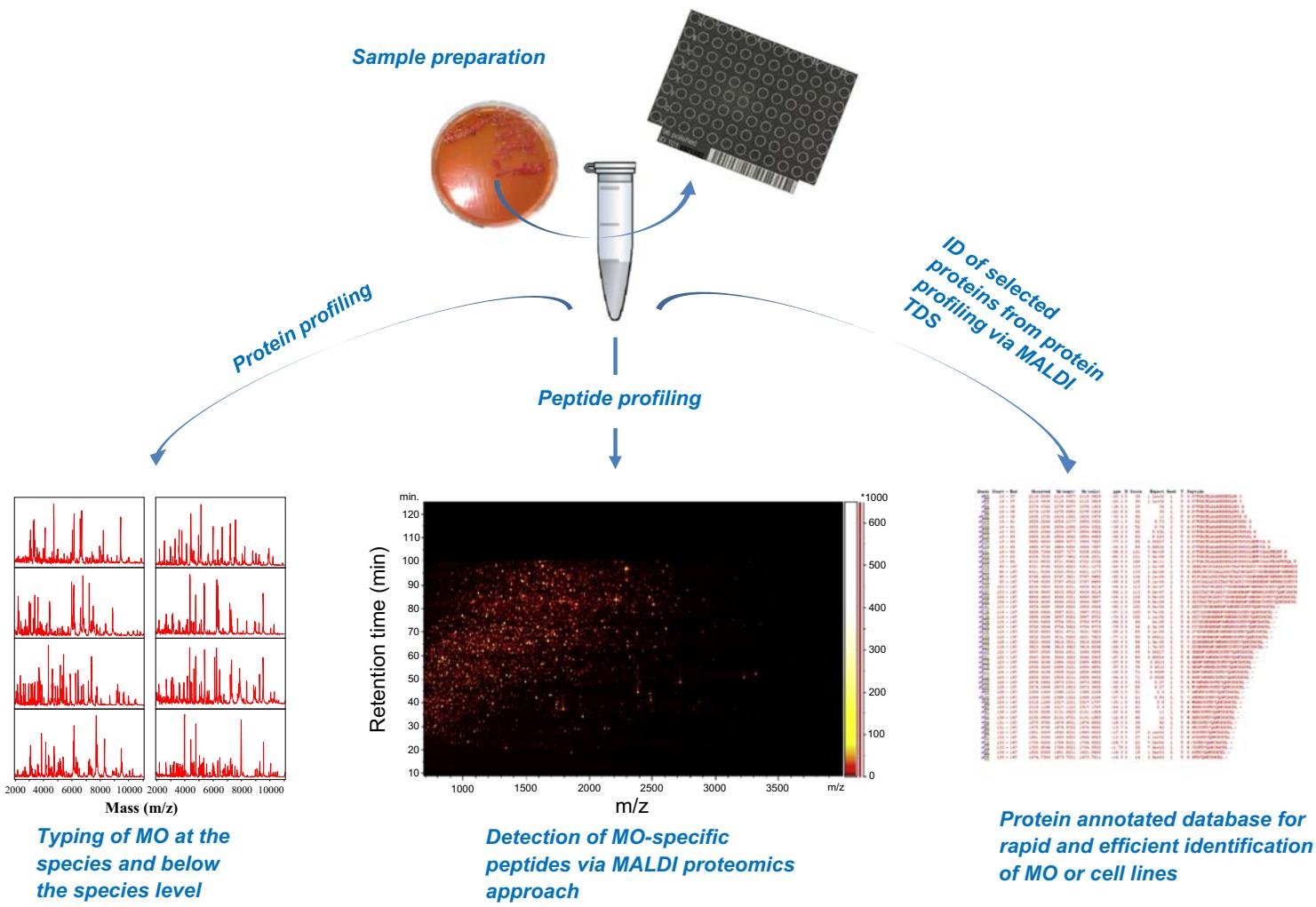


Figure 26.1

Pushing the limits of MALDI-TOF MS based on classical sample preparation (extract) toward typing of microorganisms (MO) below the species level, detection of MO-specific peptides in bottom-up approaches, and identification of proteins via top-down sequencing.

study by Siegrist et al. (2007) strains of the fecal indicator *Escherichia coli* were categorized according to their environmental origin and furthermore, five biomarkers were identified that were conserved among strains isolated from avian species. *Listeria monocytogenes* was categorized at the level of clonal lineage showing characteristic peaks and MALDI MS-derived lineage agreed with those from pulsed-field gel electrophoresis (PFGE) (Barbuddhe et al., 2008). Categorization of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains using specific markers for the methicillin resistance status were reported by Edwards-Jones et al. (2000) and by Shah et al. (2011) using artificial neural networks. Josten et al. (2013) categorized strains according to major clonal complexes of MRSA and Dubois et al. (2010) demonstrated by MALDI-TOF MS different clonal lineages of *Staphylococcus* which were of environmental or human origin.

For differentiation between single bacterial strains, MALDI-TOF mass spectra were used to identify biomarker mass peaks of respective strains. This approach was applied to *Helicobacter pylori* (Nilsson, 1999), *E. coli* (Lynn et al., 1999), *Campylobacter* (Mandrell et al., 2005), *Mycobacterium* (Hettick et al., 2006), and MRSA (Majcherczyk et al., 2006). Williamson et al. (2008) differentiated strains of *Streptococcus pneumoniae* using unique mass peaks. Masses in the range of 5000–11,000 Da matched ribosomal proteins of *S. pneumoniae* and spectrum clustering revealed the relationship between an outbreak of *S. pneumoniae* conjunctivitis and corresponding isolates. Similarly, *Streptococcus pyogenes* strains were differentiated by Moura et al. (2008) into invasive and noninvasive isolates using specific biomarkers. Differentiation of *Enterococcus faecium* and *Enterococcus faecalis* at the strain level was described by Albesharat et al. (2011). Intact mycobacteria were differentiated at strain level by linear discriminant analysis (Hettick et al., 2006). Pierce et al. (2007) demonstrated differentiation of *Coxiella burnetii* strains using partial least squares discriminant analysis (PLS-DA) of MALDI-TOF mass peaks.

For identification of single, unknown strains—compared to categorization or differentiation—the complete mass spectrum is used and compared to a library of reference spectra of known strains. Distinct algorithms were employed in correlations calculated and often small spectral differences between strains that have been given more weight (weighted pattern matching) increased the sensitivity of these small differences and contributed to successful identification, for example, in studies using *E. coli* (Arnold and Reilly, 1998) or *Micrococcaceae* (Carbognolle et al., 2007). Arnold and Reilly (1998) demonstrated that strains exhibited both common peaks and strain-specific peaks in the range of 3.5–10 kDa. Applying an algorithm calculating both cross-correlation and auto-correlation values for each of 13 intervals, 25 strains were distinguished. Bright et al. (2002) applied a pattern recognition algorithm to the mass spectra and each spectrum was translated into a point vector in an *n*-dimensional space. Data from 35 strains from 20 species, mainly enterobacteria were included in a reference

library and correct identification on the strain level was achieved for 79% of the samples. The algorithm was successful even in the distinction of species for which biochemical typing failed, for example, for *E. coli* O122 and *Citrobacter freundii*. A hierarchical cluster algorithm combined to ANOVA was employed in a study by [Hsieh et al. \(2008\)](#) to extract biomarkers from several isolates of six human pathogens.

In principle, two kinds of algorithms exist: one employs analysis of peak intensities and the second uses presence and absence of mass peaks. It is worth mentioning that mass signals exhibit an analytical error due to slight variability of acceleration voltage, to the status of matrix crystals, and to peak recognition by the software. With respect to linear MALDI-TOF MS, an analytical error of approximately 500 ppm (representing a 5 Da deviation for a signal at $m/z = 10,000$) is generally accepted. Two main automated software systems including validated reference databases are commercially available [BioTyper, Bruker Daltonics ([Sauer et al., 2008](#)) and SARAMIS, bioMérieux ([Kallow et al., 2000](#))] which also allow analysis of mass spectra on below-species level as shown, for example, by [Grosse-Herrenthey et al. \(2008\)](#) using the BioTyper to identify clostridia at the strain level or by [Stephan et al. \(2011\)](#) using SARAMIS for characterization of *Y. enterocolitica* strains according to their biotype. These databases are constantly being improved by inclusion of new microorganisms relevant to clinical diagnostics, veterinary medicine, food safety, and environmental microbiology. To obtain more mass peaks serving as putative biomarkers and to increase sensitivity, in several studies samples have been treated by enzymes, detergent, sonication, corona plasma discharge, or heat ([Horneffer et al., 2004](#); [Ryzhov et al., 2000](#); [Nilsson, 1999](#); [Krishnamurthy et al., 1996](#)). Furthermore, in some studies mass spectra that contained less peaks have been applied for discrimination of strains as shown, for example, for *M. catarrhalis* strains ([Schaller et al., 2006](#)), *S. aureus* ([Shah et al., 2011](#)), or *Francisella tularensis* ([Seibold et al., 2007](#)). In the latter study, a method applying surface enhanced laser desorption/ionization (SELDI) was used.

Identification of a single or multiple species in bacterial mixtures using MALDI-TOF MS remains challenging. In a study by [Jarman et al. \(2000\)](#) nine bacterial species were used to generate 50 mixed cultures and MALDI-TOF MS in combination with automated data extraction and novel analysis algorithms were employed. In all but one of the samples, the species were correctly identified. Using liquid chromatography (LC)-MS/MS, [Lo et al. \(2006\)](#) could identify up to eight pathogens present in bacterial mixtures.

In contrast to the most common routine application of MALDI-TOF MS using whole cells from solid growth media, [Jadhav et al. \(2014\)](#) developed a rapid workflow for detection of *L. monocytogenes* directly from selective enrichment broth allowing the detection of 1 CFU/mL initial broth culture within 30h.

A further challenge in MALDI-TOF MS addresses identification of microorganisms without prior in vitro cultivation directly from food samples. Recently, a workflow was established for reliable identification of *Monilinia* species using crude protein extracts that were prepared from fungal material obtained directly from infected fruits and application of in-house generated reference spectra. Furthermore, specific low-mass peaks characteristic of *M. polystroma* and *M. fructicola* were observed and could be directly subjected to MALDI-TOF/TOF MS analysis without prior separation by chromatography (Freimoser et al., 2016). Chenau et al. (2014) demonstrated the direct detection of *Y. pestis* using a combined approach consisting of immunocapture of intact bacterial cells from food and environmental samples and subsequent LC-MS/MS.

Compared to MALDI-TOF MS applications, ESI-MS is employed less frequently to differentiate microorganisms at the below-species level. LC-ESI-MS was applied, for example, to identify putative biomarkers for nonpathogenic *E. coli*, O157 EHEC, non-O157 EHEC, *Shigella flexneri*, and *Shigella sonnei* (Everley et al., 2008). Ho and Hsu (2002) investigated the effects of sample preparation methods on the detection of bacterial proteins by LC-ESI-MS and showed that pH and polarity of the solvent used for extraction influences the number of proteins.

26.3 Proteomics-Based Approaches

26.3.1 Bottom-Up Approach

A rapid increase in the number of complete genomes of microorganisms in public databases has boosted research utilizing proteomics-based approaches toward identification of single mass peaks in order to profile microorganisms at the below-species level.

Both MALDI-TOF MS and MALDI-TOF/TOF MS have been described for identification of intact protein biomarkers. This approach involves the comparison of their masses against the masses derived from in silico-generated protein databases arising from translation of genomic databases. Intact protein identification was used to identify strain-specific protein biomarkers, for example, for *E. coli* O157:H7 (Ochoa and Harrington, 2005), *Campylobacter* (Mandrell et al., 2005), and *Salmonella* (Dieckmann et al., 2008). Two fundamental approaches are employed for protein identification in proteomics: the bottom-up and the top-down approach.

In bottom-up approaches, proteins extracted from microbial cultures are digested enzymatically at specific sites and resulting peptides are identified by MS/MS (postsource decay, laser-induced dissociation, or collision-induced dissociation). Digestion is performed using proteolytic enzymes such as trypsin (Aebersold and Mann, 2003; Yao et al., 2002).

The first bottom-up approach developed was achieved by 2D-polyacrylamide gel electrophoresis (2D-PAGE) prior to in-gel digestion of excised spots. Fagerquist et al. (2005)

applied HPLC and 1D SDS-PAGE to proteins from *Campylobacter* before identifying strain-specific biomarker proteins. 2D SDS-PAGE has been used by Schaller et al. (2006) prior to biomarker identification for *M. catarrhalis* strains. We further optimized and accelerated the bottom-up identification workflow to discriminate subspecies of *S. enterica* (Drissner et al., 2016; Gekenidis et al., 2014) and species of the *Bacillus cereus* group (Pfrunder et al., 2016). For the first time, whole cell protein extracts generated via an established extraction procedure (MALDI biotyping according to the Bruker protocol) were applied directly to high-intensity focused ultrasound (HIFU)-assisted trypsin digestion prior to liquid chromatography and identification of specific peptides and proteins by means of MALDI-TOF/TOF MS or ESI-MS/MS respectively.

In the proof of concept study by Gekenidis et al. *S. enterica* subspecies *arizonae*, *S. enterica* subspecies *enterica* and *S. enterica* subspecies *houtenae* were used to demonstrate ultrafast generation of tryptic peptides from whole-cell extracts within minutes applying HIFU. Resulting peptides did not need any further processing before identification by nano-LC-MALDI-TOF/TOF MS. MS/MS data acquired were searched on the UniProtKB/SwissProt database and the unique peptides identified could be used to eventually discriminate between the closely related subspecies.

Recently, in a study by Pfrunder et al. the same workflow for generation of tryptic peptides as described earlier was applied to *B. cereus* group species which are difficult to distinguish also by MALDI-TOF MS due to their close relatedness. Therefore a novel approach was developed to identify specific diagnostic peptides. This approach comprised the analysis of proteomes by LC-ESI MS/MS, selection of candidate diagnostic peptides, and verification of diagnostic peptides by confirming that the diagnostic peptide sequences were uniquely present in one particular *Bacillus* species and absent in all the other *Bacillus* species listed in a *Bacillus*-specific database by string comparison.

26.3.2 Top-Down Approach

In contrast, top-down proteomics approaches in profiling bacteria comprise measurement of the exact mass of intact proteins and fragmentation by MS/MS yielding partial amino acid sequences and/or peptide fragments. Fragmentation can be achieved by collision-induced dissociation, laser-induced dissociation, electron capture dissociation, or electron transfer dissociation. Resulting MS/MS spectra are compared to a database in order to identify the protein and ultimately the source strain. Software applications compare the masses of MS/MS fragment ions to a database of in silico fragment ions (a-, b-, y-fragment ions) derived from numerous protein sequences which exhibit the same mass as that of the biomarker and an algorithm calculates the identification probability.

MALDI-TOF/TOF MS has been used for identification of intact spores that were treated with 10% formic acid on-target to facilitate extraction of small acid-soluble proteins (Demirev et al., 2005). In another study, proteins were extracted with water–acetonitrile–TFA from cell lysates prior to the identification of biomarkers of *E. coli* O157:H7 via MALDI-TOF/TOF MS (Fagerquist et al., 2010). Furthermore, shiga toxins of *E. coli* O157:H7 have been identified by this approach (Fagerquist and Sultan, 2011). Twine et al. (2008) identified flagellin-specific marker ions in a top-down MS analysis exhibiting the potential to differentiate strains of *Clostridium botulinum*. Future applications of MALDI-TOF/TOF MS in top-down approaches will need further developments to make fragmentation of large proteins more efficient (McLuckey, 2010). In comparison to library-based approaches, proteomics-based approaches are advantageous due to higher specificity and independence of producing mass spectral profiles with reproducible relative intensities of mass peaks.

As documented in the previous section, intact protein profiling of cells directly performed on a MALDI target plate has emerged as a fast and efficient routine tool for microbial identification and diagnosis, that is, fast and efficient identification is applicable from a culture plate to a MALDI plate (from “plate to plate”).

This direct identification is mainly possible due to the fact that the solubility of proteins smaller than 20 kDa is greatly enhanced with solvents like formic acid and ion generation is excellent with proteins like ribosomal proteins or histones, both protein categories comprising a high amount of arginine, lysines, and histidine. This elegant, but straightforward laboratory diagnostic approach has focused mainly on MALDI MS-based identification of microbes, using either intact cells or acidic cell extracts. However, the method was recently documented for mammalian cells (Portevin et al., 2015; Povey et al., 2014; Müller et al., 2013; Schwamb et al., 2013; Feltens et al., 2010). Currently, microorganisms are best identified down to the species level. With MALDI MS, subspecies identification can be ambiguous, dependent on the degree of protein sequence homology within the subspecies of interest. Thus, a relevant question to be addressed for the future is whether more information can be extracted from a single biotyping spectrum, for example, by identifying various protein mass peaks. This would open the way for identifying specific protein biomarkers for all kind of cell types, allowing the generation of highly specific protein annotated databases with internally calibrated mass spectra, that is, calibrated with the exact identified biomarker’s mass.

In the past, a number of attempts have been undertaken to obtain information on the individual mass peaks of intact cell protein profiling experiments (see earlier). Many of these experimental trials applied classical proteomic sample preparations, that is, using extraction buffers containing several detergents and other ingredients and 2D electrophoresis as the dominantly applied protein separation technique.

In this respect, our strategy clearly focuses on using as a starting point simply the acid-extracted sample directly from the classical MALDI biotyping workflow. From our initial experiments we have learned that this easily accessible protein extract with, for example, formic acid (20%–40%) offers great benefit to track protein identification. With additional marginal sample conditioning we have been successful in applying both, in a first attempt bottom-up analytics [more details in the previous section and in [Gekenidis et al. \(2014\)](#)] and very recently, the top-down proteomics approach (Brunisholz et al., unpublished results).

As discussed in the previous section, a bottom-up approach, interfacing accelerated tryptic digestion of the acid/organic solvent extracts (classical biotyping sample preparation) with nano-LC, enables identification of microorganism-specific peptides by MALDI-TOF/TOF MS and thus increases the discrimination power to the level of subspecies ([Fig. 26.1](#); [Gekenidis et al., 2014](#)).

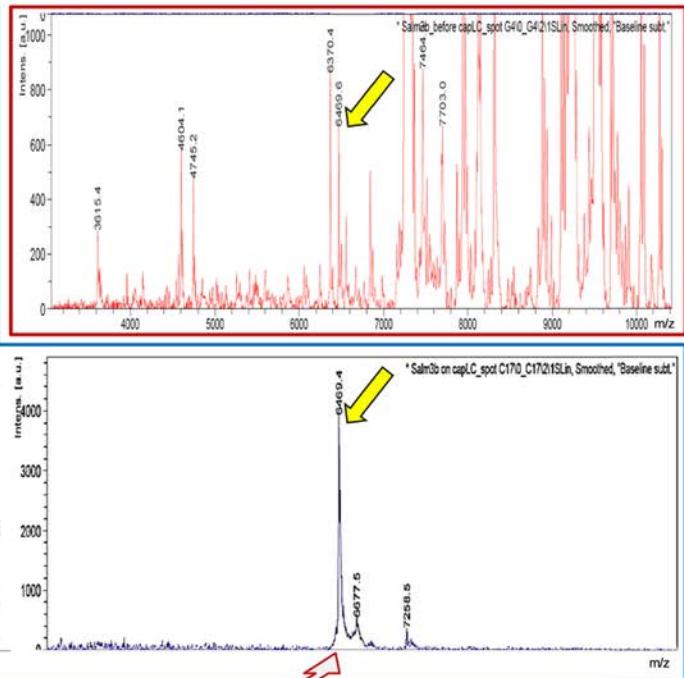
For the top-down approach, it became evident that intact protein separation will play a key role for successful peak identification via a MALDI in-source decay approach (MALDI-TDS, top-down protein sequencing). In classical proteomics approaches, with detergent and other ingredients used in the extraction buffer, laborious cleaning procedures are required before accurate protein separation can be performed. We have been testing in preliminary experiments 1D chromatography, using reversed-phase columns such as C8 and C18 columns. These two ligand specifications are clearly preferred since the targeted proteins to be identified are between 2 and 20 kDa (classical MALDI biotyping mass range). In the following section we will comment on the so-far used protocol to obtain pure or partially pure protein species out of the formic acid (in some cases additionally supplemented with acetonitrile) extract. Depending on the amount of formic acid used, the extract (e.g., 50 μ L) is diluted with about 50 μ L of ddH₂O and evaporated in a speed-vacuum device (leaving some 20 μ L). This partially liberated formic acid sample can then be directly injected onto a reversed-phase column (e.g., 150 mm length \times 0.5 mm inner diameter) on an Agilent1100 capLC apparatus equipped with a diode array photometer to track, for example, proteins at 220 and 280 nm. Additionally, UV-VIS spectra can be recorded during the complete course of separation. After a successful analytical run (injection of approx. 2 μ L), about 8 μ L of extract is sufficient to perform a preparative run employing the spotting unit of the Agilent 1100 HPLC setup. Thereby, a 384 anchorchip plate from Bruker serves as a fractionation unit which then after matrix application is immediately ready for the MALDI recordings ([Fig. 26.2](#)). Using superDHB as matrix allows furthermore to screen selected spots for their identity via the MALDI in-source decay technique ([Suckau and Resemann, 2009](#)).

Protein separation: capHPLC



Spotting onto MALDI target

MALDI protein profiling



Intact protein MALDI-TOF MS of spot C17

Figure 26.2

In-depth strategy for protein separation by capHPLC and spotting onto a 384 spots MALDI target and subsequent MALDI-TOF MS analysis of all spots. Example based on a classical sample preparation (extract, see Fig. 26.1) of *S. enterica* subsp. *enterica* is shown.

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Proteomics Analyses Applied to the Human Foodborne Bacterial Pathogen *Campylobacter spp.*

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27.1 Introduction—An Overview of *Campylobacter*

The number of *Campylobacter* species discovered reached 14 in 2004 and has doubled in 2016 with 28 species and 10 subspecies validated. Among them, *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter upsaliensis*, *Campylobacter hypointestinalis*, and *Campylobacter lari* were isolated from human infections with a higher prevalence of *C. jejuni* (EFSA and ECDC, 2016). *Campylobacter concisus*, *Campylobacter rectus*, *Campylobacter gracilis*, *Campylobacter curvus*, *Campylobacter showae*, and *Campylobacter sputorum* have been associated with the human oral cavity. However, no clear evidence of these buccal species could be observed for their pathogenic role except for *C. concisus*. The number of campylobacteriosis reported cases have been increasing worldwide during the last decade. With 236,851 cases in Europe, this zoonotic agent is the leading cause of bacterial foodborne diseases (EFSA and ECDC, 2016). In USA, an increase of 11% of campylobacteriosis cases was reported in 2014 as compared to 2008 (EFSA and ECDC, 2016).

Campylobacter is an obligate microphilic microorganism. It is often defined as fragile and fastidious for growth requirements. The foodborne pathogenic species are thermophilic and require low level of oxygen (5% O₂) and high level of carbon dioxide (10% CO₂) for their growth (Macé et al., 2015). The two prevalent pathogenic species of *Campylobacter* (*C. jejuni* and *C. coli*) are mainly transmitted by foodstuff originated from farm animals. Poultry, pork, and to a lesser extent beef are the main asymptomatic reservoirs for this human pathogen (Epps et al., 2013). In 2010, a European epidemiological survey revealed that 71.2% of broiler batches and 75.8% of broiler carcasses were contaminated by *Campylobacter* (EFSA and ECDC, 2010). This observation was reinforced by more exhaustive studies indicating a contamination rate over 87% of the broiler carcasses in France and United Kingdom (Hue et al., 2010; Powell et al., 2012). *Campylobacter* constitutes therefore a puzzle as to how it can survive from farm to retail outlets under atmospheric O₂ and CO₂ concentrations.

The genomic plasticity is probably at the origin of the high diversity of strains among the two main pathogenic species of *Campylobacter*. Next-generation sequencing (NGS) analyses revealed that the pathogenicity of *Campylobacter* might result in the acquisition of virulence factors through horizontal transfer during its evolution (Iraola et al., 2014). The genome of *Campylobacter* is composed of highly conserved parts probably due to the presence of essential genes and numerous hypervariable regions. These hypervariable regions include genes mainly involved in bacterial surface components such as lipooligosaccharides (LOS), flagellar system, and surface polysaccharide (Gundogdu et al., 2007). These surface components are important in phenotypes associated with pathogenic mechanisms and adaptation to the environment (Nachamkin, 2002; Konkel et al., 2004; Yuki, 2007; Mortensen et al., 2009). This is illustrated by a wide range of responses of *Campylobacter* spp. to environments influencing their virulence and adaptation abilities. However, for this pathogen we have only a cursory outline of the molecular and cellular mechanisms underlying colonization of poultry digestive tract, survival in food products, adaptation to abiotic and biotic food environments, and virulence process as compared to other foodborne pathogens such as *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus*.

Considering the biological features of *Campylobacter*, the main stresses this pathogen has to cope with are those encountered throughout the food chain and the human digestive tract. The capacity to counteract environmental stresses is crucial for the survival of this pathogen. This capacity depends on the genetic equipment which provides the basis for the adaptive response but also on the protein expression which converts the coding sequence into functionality. Contribution of proteomics approaches to unravel the biological adaptation of *Campylobacter* is reviewed in the following sections.

27.2 The Adaptive Tolerance Response (ATR) of *Campylobacter*

27.2.1 ATR to Acidic Stressful Environments

Acidic conditions refer to environments with a pH below 7. Decreasing pH in food products is one of the commonly used preservation options to prevent growth and survival of pathogens. In addition, the acidic environment in the stomach constitutes a biological barrier for bacterial pathogens to infect humans. A few hundreds of cells of *C. jejuni* were found to be enough to induce a gastrointestinal disorder in humans (Black et al., 1988). This low infective dose indicates that *C. jejuni* is able to survive the acidic gastric conditions during digestion. However, *C. jejuni* is more susceptible to acid exposure than other foodborne pathogens (Birk et al., 2010). To better understand the ATR of *Campylobacter* toward acidic environments at the protein level, two-dimensional electrophoresis (2-DE) profiles were compared (Birk et al., 2012) using HCl or organic acids. HCl was used to mimic the acidic pH in the gastric compartment while acetic acid was selected to simulate the exposure to food containing organic acids for food preservation. Among three *C. jejuni* strains exposed to acidic stresses for

proteomics studies (Birk et al., 2012), the abundance of few proteins were found to be affected. The low number of proteins affected, as compared to other bacterial species exposed to acidic stresses, was related to the limitation of genes present in *Campylobacter* to promote a wide response. De novo synthesized proteins detected in response to acidic stress include proteins involved in the reactive oxygen species (ROS) detoxification such as SodB, AhpC, and TrxB, the iron homeostasis with P19 and Dps, and the molybdenum cofactor biosynthesis protein MogA (Birk et al., 2012). The additional analyses of transcripts indicated that acidic conditions induced an upregulation of genes encoding proteins involved in ROS scavenging and iron homeostasis. The importance of iron uptake regulation and oxygen derivative detoxification were also observed using proteomics in *C. jejuni* exposed to both acidic and oxidative stresses (Varsaki et al., 2015). Transcriptomic analyses performed on *C. jejuni* in acidic conditions confirmed the low number of genes involved in the response to acidic conditions (Reid et al., 2008). Only 26 genes were found to be upregulated in these conditions. Nevertheless, the oxidative stress response and iron homeostasis were not the main pathways highlighted at the transcriptomic level. A correlation between transcriptomic and proteomic analyses is not always expected due to posttranslational modifications. Interestingly, the cross analysis of transcriptomic data with a library of mutants exposed to acidic conditions indicated the contribution of genes encoding proteins related to the surface or membrane of *C. jejuni* (Reid et al., 2008). Among them, genes encoding LOS biosynthesis, the motility, and outer membrane receptor were upregulated under acidic conditions. The corresponding proteins were probably not detected in proteomics studies as these analyses focused mainly on soluble protein content.

27.2.2 ATR to Oxidative Stressful Environments

27.2.2.1 Response of *C. jejuni* to Oxidative Stress

As strict microaerophilic bacteria, *Campylobacter* spp. has to survive atmospheric O₂ concentrations throughout food processing and preservation to be able to reach the human digestive tract. Using 2-DE to compare the relative abundance of proteins from cells exposed to prooxidant agents or oxygen-enriched conditions, the differentially expressed proteins reported were involved in the following cellular processes: (1) response to oxidative stress, (2) intracellular redox status, (3) iron homeostasis, (4) virulence, (5) cell shaping, and (6) motility (Gareniaux et al., 2008; Sulaeman et al., 2012).

27.2.2.2 Proteins Involved in Oxygen Derivative Detoxification, Intracellular Redox Status, and Ion Homeostasis

The oxidative stress response to paraquat (a prooxidant agent) indicated an overexpression of Tpx (Gareniaux et al., 2008). This enzyme seems to play an alternative role of H₂O₂ breakdown in aerotolerance conditions (Atack et al., 2008). The redox status of the cells exposed to paraquat was reinforced by the overexpression of the flavodoxin FldA (Gareniaux et al., 2008),

the electron acceptor of the pyruvate oxidoreductase complex (Por) which ensures the production of NADPH. Por activity, like the activity of 2-oxoglutarate acceptor oxidoreductase (Oor), was shown to be sensitive to atmospheric oxygen concentrations in *Helicobacter pylori*, a close relative to *Campylobacter* (Hughes et al., 1998). These two enzymes are composed of oxygen labile clusters (4Fe-4S) which are rapidly inactivated by ROS generated during oxygen reduction under aerobic respiration (Hofreuter, 2014). Hence, H₂O₂ easily oxidizes ferrous ions (Fe²⁺) to ferric ions (Fe³⁺) and produces highly toxic hydroxyl radicals according to the Fenton reaction. It was recently demonstrated that the oxygen lability of Por and Oor in *C. jejuni* could be limited by the protection effect of hemerythrins HerA and HerB (Kendall et al., 2014). As oxidative stress generates ROS that could affect the (4Fe-4S) clusters of enzymes, iron regulation is of vital importance for *C. jejuni* exposed to oxidative stress. In the proteomic study on *C. jejuni* cells exposed to paraquat (Garenaux et al., 2008), overexpression of the ferritin Cft in *C. jejuni* contributed to this evidence. *C. jejuni* mutants of the *cft* gene were previously found to be more susceptible to oxidative stress (Wai et al., 1996). Indeed, the gene encoding Cft is controlled by peroxide stress regulators (Fur and PerR) in *C. jejuni* (Holmes et al., 2005). In the absence of global regulators in *C. jejuni* such as SoxRS, OxyR, Fur and PerR regulons were found to play a central role in the response to oxidative stress. Noticeably, the expression of *katA* and *ahpC* involved in the breakdown of H₂O₂ has been shown to be repressed by both regulators (van Vliet et al., 1999; van Vliet et al., 2002). More recently, the two-component system (TCS) Cj0355c, namely CosR, was identified as a potential regulator of the oxidative response in *C. jejuni* (Garenaux et al., 2008; Hwang et al., 2011). However, CosR was also found to be involved to the response of *C. jejuni* to antibiotics by negatively regulating the CmeABC efflux pump and to biofilm formation by controlling the maturation phase transition of the biofilm (Hwang et al., 2012; Turonova et al., 2015). Consequently, CosR is likely a key pleiotropic regulator governing adaptive responses in *C. jejuni*.

27.2.2.3 Proteins Involved in the Virulence Mechanism

Oxidative stress also promotes the expression of virulence factors in *C. jejuni*. For instance, a higher abundance of CadF (*Campylobacter* adhesion to fibronectin) in response to oxidative stress was found to be controlled by oxygen and to contribute to adhesion to inert surfaces (Sulaeman et al., 2012; Koolman et al., 2016). This outer membrane protein (OMP) was firstly described as a protein binding to fibronectin and necessary for host cell adhesion and invasion (Konkel et al., 1997; Monteville et al., 2003). Its higher abundance in the outer membrane in response to oxidative stress was revealed using subproteomics analyses of the enriched protein fraction of the outer membrane (Sulaeman et al., 2012). Interestingly, 2-DE-based approach and immunoblotting revealed the presence of various isoforms of CadF (Cordwell et al., 2008; Scott et al., 2010; Sulaeman et al., 2012). Truncated forms of CadF vary in their immunogenic status but not in their fibronectin adhesion ability (Cordwell et al., 2008). Function of CadF could therefore differ according to *C. jejuni* adaptation to the surrounding environment.

27.2.2.4 Cell Shaping and Motility

Campylobacter are nonspore-forming rods harboring a spiral shape in optimal growth conditions. The motility of *C. jejuni* is ensured by one or two amphitrichous flagella. Flagella are responsible for the propulsive torque and the rotary movement of the bacterial cells. *C. jejuni* swims using straight self-propelling motion with few directional changes. Thanks to its spiral shape, the rotary movements provide a corkscrew-like locomotion which is probably at the origin of the ability of the cells to move through viscous liquids such as the mucus. The flagellum apparatus results in a protein complex divided into a hook basal body anchored in the membrane and the extracellular filament. Although two genes (*flaA* and *flaB*) encoding the flagellin monomeric units are present in *C. jejuni* genome, the filament is mainly composed of FlaA. Using proteomics to compare the relative abundance of soluble or membrane proteins between cells grown in microaerobiosis and cells exposed to oxidative conditions, the overexpression of flagellin encoded by *flaA* gene was observed in stressed cells (Garenaux et al., 2008; Sulaeman et al., 2012). This is probably correlated to bacterial move as the motility of *C. jejuni* was increased in these conditions (Sulaeman et al., 2012). In addition, the comparison of 2-DE profiles of *C. jejuni* exposed to oxidative stress revealed the overexpression of the protein encoding a homologous gene to *mreB* (Garenaux et al., 2008). MreB is an actinlike protein that intervenes in the cell morphogenesis in conjunction with the membrane protein MreC (Graumann, 2007). Mutations on *mreB* promote the transition shape of rods to coccoid forms in many bacteria (Jones et al., 2001; Figge et al., 2004; Slovak et al., 2005). As *C. jejuni* could harbor both shapes, MreB is thought to be important for its rod shape maintenance (Ikeda and Karlyshev, 2012). Its overexpression in stressed cells suggests that the rod-shaped status tends to be conserved. The transition to coccoid shape of *C. jejuni* is often associated with the response to stress conditions including oxidative stress conditions (Ikeda and Karlyshev, 2012). Firstly described as a degenerated shape of the cells and associated with nonviable or dormant cells (Moran and Upton, 1986; Boucher et al., 1994), it was shown recently that coccoid shapes of the aerotolerant strain *C. jejuni* Bf could multiply under aerobic conditions (Rodrigues et al., 2015).

27.2.3 ATR to Temperature Stressful Environments

As an opportunist inhabitant of human and poultry intestines, *C. jejuni* is well adapted to temperatures of warm blood animals. The growth of thermophilic *C. jejuni* is inhibited below 30°C and above 47°C although metabolic processes are still active in cells maintained at 7°C (van de Giessen et al., 1996). To identify heat shock proteins (HSPs) in *C. jejuni*, Konkel et al. (1998) compared the 2-DE profiles of *C. jejuni* cultivated at 37°C or 46°C for 10 min using [³⁵S]methionine pulse labeling to identify only de novo synthesized proteins after the thermal shock. Among the 24 HSPs identified, DnaJ, a cochaperone of DnaK with GprE, was reported to contribute to cell viability under high temperature (Sell et al., 1990). In addition, Konkel et al. (1998) showed that a mutant

defected in *DnaJ* was not able to colonize chicken. As the body temperature of chicken is 42°C, this result suggested that HSPs are required at 42°C for specific functions.

Comparative proteomics analyses between *C. jejuni* grown at 37°C and 42°C revealed that the immunogenic protein Peb4 encoded by *cj0596* in NCTC 11168 was induced at human body temperature (Rathbun et al., 2009; Zhang et al., 2009). Mutants defective in *peb4* were associated to a decrease of host cell invasion (Asakura et al., 2007; Rathbun et al., 2009). By comparing proteomics profiles of the mutants and their respective parental strains, proteins involved in transport, motility, and amino acid trafficking were differently expressed suggesting that Peb4 is an outer membrane protein involved in protein export (Asakura et al., 2007; Rathbun et al., 2009). This function was confirmed later on with the analysis of the crystal structure of Peb4 showing a holdase-type chaperone function involved in the protein export and adherence to host cells (Kale et al., 2011).

27.2.4 ATR to Iron Starvation

Free iron bioavailability is critical for life processes. In response to iron starvation, proteomics studies highlighted iron uptake systems in *C. jejuni*. Holmes et al. (2005) observed an overexpression of protein ChuA after exposure to iron limitation conditions concomitantly to higher transcript levels of *chuA* and the putative *chuB*, *chuC*, and *chuD* suggesting the presence a hemin uptake system in *C. jejuni*. One year later, the membrane system ChuABCD was fully described for hemin acquisition in *C. jejuni* by Ridley et al. (2006). In 2014, all proteins of this system were confirmed to be overexpressed under iron starvation using iTRAQ-labeling proteins extracted from *C. jejuni* and proteomics analyses (Clark et al., 2014). A second iron acquisition system was identified with the overexpression of CfrA (Holmes et al., 2005), an enterobactin transporter essential for *C. jejuni* growth under iron starvation. Because *C. jejuni* is not equipped to synthesize siderophores, this transporter which could uptake a wide variety of siderophores plays an important role in *C. jejuni* survival (Naikare et al., 2013). The periplasmic protein P19 was also among the overexpressed proteins in response to iron starvation in accordance with their respective transcript levels (Holmes et al., 2005; Clark et al., 2014). This protein might be an intermediate of a third iron acquisition system in *C. jejuni* (Chan et al., 2010). Two other putative siderophore transport proteins were also overexpressed in *C. jejuni* exposed to iron starvation (Holmes et al., 2005; Clark et al., 2014). The first one CfbpA is a periplasmic protein that might belong to the protein complex composed of CfbpABC based on sequence similarity constituting a fourth iron acquisition system in *C. jejuni* (van Vliet et al., 2002). The second protein Cj1663 could be part of an inner membrane ABC transporter working with P19 (Miller et al., 2009) or a protein involved in phosphonate degradation in *C. jejuni* (Hartley et al., 2009). Proteins of iron pumping (exbBD-TonB) and ferritin enterochelin uptake systems (Ceu) were not detected by Holmes et al. (2005) while they were among the overexpressed proteins in the study conducted by Clark et al. (2014) probably due to the

limitations of the gel-based technology used by Holmes et al. (2005). Apart from proteins involved in iron acquisition systems, iron limitation also resulted in higher abundance of proteins implicated in the ROS detoxification including AhpC, KatA, Trx, and Tpx (Holmes et al., 2005; Clark et al., 2014). As reduction/oxidation status of iron is sensitive to ROS generated during aerobic respiration (H_2O_2 and O_2^-) via Haber-Weiss and Fenton reactions, it is not surprising that these proteins are overexpressed during ion starvation response of *C. jejuni*.

27.2.5 ATR to Bile

Bile is secreted by the hepatocytes and concentrated in the gall bladder before being introduced in the intestinal lumen. Its main function consists on emulsifying fatty acids from the gastric compartment to facilitate their absorption. It is mainly composed of bile salts, inorganic salts, phospholipids, cholesterol and bilirubin and has been shown to have antimicrobial activity. Bile constitutes therefore a barrier to infection of human intestines. *C. jejuni* is able to tolerate 0.3% of bovine bile and could survive up to 5% bile. Using ox-bile at 2.5%, Fox et al. (2007) investigated the adaptive response of *C. jejuni* to bile at the protein level. Proteomic study using 2-DE revealed modifications of the abundance proteins involved in chemotaxis and motility, carbon utilization, protein translation, protein folding, and surface structures. Bile acids were suggested to act as an environmental cue for *C. jejuni* to enhance its host cell invasion via the promotion of virulence factors including Cia and flagellar proteins (Malik-Kale et al., 2008; Clark et al., 2014).

27.2.6 ATR to Food Processes

To minimize food processing and additive input in food products, alternative processing technologies are being investigated for food preservations. Ultrahigh hydrostatic pressure (HHP) is one of the promising techniques for preserving food from bacterial contamination without affecting vitamin content, natural flavor and texture. A bactericide treatment using HHP is obtained at 400 MPa for *Campylobacter* while up to the double is required to reach the same effect for other foodborne pathogens (Solomon and Hoover, 2004; Bièche et al., 2009). At high pressure, a reduction in volume occurs for reactions including conformational changes or phase transition while reactions followed by an increase of volume will be inhibited. As microorganisms contain complex biological systems and reactions, it is not possible to predict the effect of HHP on any particular bacterial population. To better understand the inactivation process of HHP on *C. jejuni*, proteome changes were investigated after a sublethal treatment at 300 MPa for 10 min followed by a recovery phase. The 2-DE dynamics study highlighted the transient overexpression of two proteins: FabH and DnaK. FabH is a key enzyme in membrane biosynthesis (Lai and Cronan, 2003).

It catalyzes the first reaction of fatty acid biosynthesis from acetyl CoA to produce acetyl-ACP. DnaK is one of the main enzymes involved in the network of protein folding (Calloni et al., 2012). As HHP affects predominantly the ternary and quaternary structures of proteins and not the hydrogen or covalent bonds, the protein refolding after HHP shock is important to ensure protein functionality. The transient upregulation of FabH and DnaK, confirmed at the transcriptional level, suggests that *C. jejuni* requires membrane regeneration and protein renaturation after HHP shock. Apart from these proteins, in *C. jejuni* the HHP shock is responsible for the overexpression of proteins also involved in ROS scavenging suggesting that HHP might induce an oxidative burst in *C. jejuni* responsible for its inactivation. The oxidative stress might explain the high sensitivity of *C. jejuni* to this treatment as compared to other bacteria.

27.3 Sample Preparation for Proteomics Analyses

27.3.1 Sample Preparation for 2-DE Analyses

At first, proteomics studies were essentially dedicated to analyze mainly soluble protein content in bacteria due to limitations of the gel-based technologies (2-DE) (Gorg et al., 2004). These limitations were inherent to the separation of proteins using isoelectric focusing (IEF) for the first dimension. The success of protein separation using 2-DE mainly depends on protein solubilization and purification. To be compatible with IEF, the solubilization step could only use specific nonionic detergents like CHAPS and chaotropic reagents like urea and thiourea (Rabilloud, 2009). Then, residual salts have to be removed by protein precipitation or sample dialysis. Preservation of proteins from degradation is also a prerequisite to ensure robust and reproducible data. To avoid protein degradation, low temperature during sample preparation, addition of a cocktail of protease inhibitors and sample aliquoting are recommended practices. Hence, many of the proteomics studies performed on *Campylobacter* used extraction and purification methods optimized for conventional gel-based technologies. As a consequence, protein samples were mainly enriched in hydrophilic proteins which explained the preponderance of soluble proteins among the identified proteins (e.g., Andersen et al., 2005; Holmes et al., 2005; Garenaux et al., 2008; van Alphen et al., 2008; Yun et al., 2008; Kaakoush et al., 2011; Bleumink-Pluym et al., 2013). For instance, gel-based proteomics analyses of *C. jejuni* cells under iron starvation identified only parts of membrane iron uptake systems among overexpressed proteins while most parts of iron uptake systems were identified using transcriptomics analyses or gel-free approaches (Holmes et al., 2005; Clark et al., 2014). Nonetheless, some membrane proteins with hydrophobic regions were identified among the soluble protein content due to their transient passage in the cytoplasmic compartment and before folding maturation. For instance, the OMP CadF was identified in the soluble protein fraction separated using 2-DE method (Garenaux et al., 2008) and in the outer membrane

protein fraction of *C. jejuni* using proteomics analysis focusing on membrane proteins (Scott et al., 2010; Sulaeman et al., 2012).

27.3.2 Sample Preparation for Gel-Free Analyses

With the cutting-edge, sophisticated, and powerful mass spectrometry technologies, gel-free high-throughput analyses on whole cell lysates have been increasing. Although these shotgun proteomics approaches still require high-quality samples ensuring the purity of proteins, they are less stringent than IEF for protein extraction. These approaches could be applied to identify protein composition of a sample or to quantify the relative abundance of each protein between two or more samples.

Concerning *Campylobacter*, gel-based technique was compared to gel-free technique in order to unravel the exoproteome of *C. concisus* using shotgun analysis (LTQ-FT/MS with a preseparation step on SDS-PAGE). As a matter of fact, proteins identified using LTQ-FT-MS predominantly belong to complex fraction while 2-DE gels were more efficient to detect lower abundant proteins indicating a complementarity of the two approaches (Kaakoush et al., 2010). In another example, protein composition of outer membrane vesicles (OMVs) of *C. jejuni* was investigated using LC-MS/MS (Elmi et al., 2012; Jang et al., 2014). A preseparation step of OMVs with SDS-PAGE was selected by Elmi et al. (2012) while Jang et al. (2014) submitted the solubilized and digested whole OMV lysate directly to MS analyses. Using similar strains, these authors found only 64 proteins in common among the 151 and 134 proteins identified, respectively. Apart from the separation and identification technologies, comparison of data obtained from two different labs might be difficult due to culture condition variations and bacterial genome plasticity of *C. jejuni*.

Relative protein quantification in gel-free methods could be achieved using chemical (isotopes or isobaric tags) or metabolic labeling. Metabolic labeling offers the advantage to be used in situ while the chemical labels are applied after protein extraction or protein digestion which could result in some technical biases. In *C. jejuni*, proteins chemically labeled with iTRAQ and separated by LC-MS/MS were used to quantify protein abundance changes after chicken colonization or to investigate protein changes following prophage insertion, chicken colonization, iron starvation, or bile salt stress (Clark et al., 2014; Asakura et al., 2016). While Asakura et al. (2016) extracted proteins based on the methodology developed for 2-DE, Clark et al. (2014) choose to extract proteins using bead-beating and solubilize them with SDS and urea. 2-DE extraction protocols might result in an under representation of hydrophobic proteins. To improve protein resolution using shotgun approach, a preseparation of proteins on SDS-PAGE might be valuable. To do so, the gel lanes of SDS-PAGE are excised into horizontal slices and submitted to LC-MS/MS analyses for protein identification.

27.4 Subproteomic Approaches for *Campylobacter* Analysis

27.4.1 Membrane Proteome

Membrane proteins from *C. jejuni* were investigated using proteomics approaches (Prokhorova et al., 2006; Cordwell et al., 2008; Sulaeman et al., 2012). Using both gel-based and gel-free approaches, Cordwell et al. (2008) identified 453 proteins representing 27.4% of the *C. jejuni* theoretical proteome. Although gel-free methods were able to detect more proteins (432 vs. 77), some of them were only detected using 2-DE indicating the complementarity of the approaches. To maximize extraction of membrane-associated proteins, specific detergents such as tributylphosphine (TBP) and amidosulfobetaine-14 (ASB-14) for a better solubilization of hydrophobic proteins were used (Cordwell et al., 2004; Sulaeman et al., 2012). Many of the proteins detected were in accordance with computational predictions for membrane-associated proteins based on the presence of a signal peptide or with the prediction of membrane-spanning regions. The discrepancy between predicted membrane proteins and identified membrane proteins could be attributed to technical biases and/or bacterial biology. Hence, the extraction of membrane proteins results from a membrane protein enriched fraction with potential contamination of proteins from the cytoplasm. On the other hand, the computational predictions are limited to the presence of membrane signature such as protein conformation, hydrophobic regions, and membrane-addressing signals. Consequently, proteins only associated with membranes without a strong membrane anchorage could not be predicted. The surface proteome investigation led to identify two novel putative periplasmic proteins displaying immunogenic functions in mice (Prokhorova et al., 2006). Nonetheless, these two proteins were not evenly distributed among *C. jejuni* strains to be considered as potential vaccine candidates. By comparing membrane proteome of a *C. jejuni* strain isolated from a patient with only gastrointestinal symptoms (JHH1) and a *C. jejuni* strain originated from a patient with the late onset complication of GBS (ATCC700297), novel surface antigens, OMPs, and proteins involved in motility, chemotaxis, and signal transduction were detected (Cordwell et al., 2008). In *C. jejuni* strain associated with GBS, modifications of LOS by sialylation have been reported to elicit proinflammatory and humoral responses as well as host cell invasion (Godschalk et al., 2007; Louwen et al., 2008; Mortensen et al., 2009; Huizinga et al., 2013). In addition, proteins extracted from the inner membrane and the outer membrane could be explored separately. This could be achieved using sucrose density gradient ultracentrifugation, spheroplasting with lysozyme or membrane specific detergents. In *C. jejuni*, higher efficiency and accuracy of proteins extraction from the outer and the inner membranes were obtained using lauryl-sarkonisate (Sulaeman et al., 2012). Comparing 2-DE profiles before and after exposure of cells to oxygen-enriched conditions, proteins differently expressed are mentioned earlier and discussed in the ATR response to oxidative stress (Sulaeman et al., 2012). In *C. jejuni*, the predominance of a few proteins in the outer membrane of *C. jejuni*, such as MOMP and FlaA, might prevent the detection of lower abundant proteins in both qualitative and quantitative studies. The abundance of MOMP in the *C. jejuni* was evaluated at 45% of total visible

proteins on 2-DE (Cordwell et al., 2008). More recently, using SDS-PAGE for protein preparation following by LC-MS/MS approaches of sarkosinate-insoluble enriched protein fraction, Watson et al. (2014) confirmed the presence of proteins previously detected in the outer membrane proteins. Combining these analyses to genomic comparison, the authors were able to determine the distribution of OMPs and assess their variability across *Campylobacter* species.

27.4.2 Exoproteome

The exoproteome refers to the extracellular proteins released by one organism. The term secretome is often confused with the exoproteome. Hence, in gram-negative, a protein could be secreted (or translocated) in the periplasm without being released in the surrounding medium and the secretome would include all molecules (not only proteins) released by the organism. The exoproteome was rarely explored in *Campylobacter*. Only the exoproteome of *C. concisus* was characterized at 37°C under microaerobic conditions (Kaakoush et al., 2010). Exoproteins contained in the supernatant were retrieved after ultrafiltration and precipitation with trichloroacetic acid (TCA). Proteins were separated by SDS-PAGE or 2-DE and identified using LC-MS/MS or LTQ-FT-MS, respectively. The main concern in such an approach is to distinguish between proteins released by the cells from those coming from cellular lysis content or those coming from vesicles. To partly circumvent these biases, proteins only predicted with a peptide signal or as nonclassical exoproteins could be selected using computational prediction applications. Among the 201 detected proteins by Kaakoush et al. (2010) in *C. concisus*, 86 were predicted to be released outside the cells. Apart from the unknown or putative proteins, these proteins were classified as components related to cell physiology, to host cell interaction during virulence or colonization and to cell protection against environmental stresses.

27.4.3 Vesicle Proteome

Outer membrane vesicles (OMVs) are specific to gram-negative bacteria. These exosomes with a size ranging from 40 to 100 nm derived from cellular membrane. They could be considered as messengers for cell communication, horizontal transfer of DNA, cell survival by securing the surrounding environment and bacterial virulence activation. They are composed of a circular lipid bilayer with membranar proteins and entrapped soluble molecules including nutrients, proteins and nucleic acids. Their content depends on bacterial species, strains and cultures conditions. *C. jejuni*, like its close relative *H. pylori*, is well described for its ability to generate 50 nm outer membrane vesicles. From proteomics analyses of OMVs of *C. jejuni*, three proteases (HtrA, Cj0511, and Cj1365c) were identified and shown to promote bacterial invasion by mediating cleavage of epithelial cell E-cadherin and occludin (Elmi et al., 2012, 2016). The high variation in protein composition of OMVs was confirmed later on by Jang et al. (2014), who reported only 64 proteins over 134 identified proteins in common with Elmi et al. (2012) study.

27.5 Posttranslational Modifications (PTMs)

27.5.1 Glycoproteome

The PTM of proteins by the addition of carbohydrates is crucial in bacteria to control many cellular processes. Without protein glycosylation, bacteria would have a reduced range of interactions with the environment and host cells (Szymanski and Wren, 2005). Glycosylation contributes to structural and functional roles of membrane and secreted proteins including stability, signal transduction and host immune responses (Alemka et al., 2013). In bacteria, two systems of protein glycosylation are described: the *O*-linked protein glycosylation system and *N*-linked protein glycosylation system (*pgl*). Although *O*-glycosylation is widespread among all classes of bacteria, *N*-glycans were detected so far only in epsilon-proteobacteria and few delta-proteobacteria (Nothaft and Szymanski, 2013). Consequently, *Campylobacter* became a model of choice to study *N*-glycosylation in bacteria.

In *C. jejuni*, the surface protein JplA was shown to contribute to the adhesion to epithelial cells (Jin et al., 2001). In order to unravel its immunogenic role, the potential contribution of its glycosylation status was analyzed (Scott et al., 2009). Proteomics studies revealed the prevalence of a diglycosylated JplA exposed at the surface of *C. jejuni*. The results indicated that a diglycosylated JplA is present across strains except for NCTC 11168 which harbors only a monoglycosylated JplA. However, the level of glycosylations of JplA could not be correlated to immunogenic properties of the protein. Nonetheless, glycosylations of JplA might contribute to the pathogenic process as mutants of *C. jejuni* defective in *pgl* system resulted in a decrease of cell invasion and mouse colonization (Szymanski et al., 2003). To identify *N*-linked glycoproteins at the proteome level, glycan structures could be detected using specific LC-MS (hydrophilic interaction liquid chromatography, HILIC) strategies or 2-DE gels of glycoproteins separated on lectin affinity columns. In the latter technique, the glycosylation sites are determined by LC-MS/MS from glycopeptides digested with trypsin and proteinase K and purified on graphite microcolumns. This approach was used to characterize *C. jejuni* glycoproteome which resulted in 38 identified glycoproteins predicted to be located in the periplasm (Young et al., 2002). Combining both HILIC and 2-DE approaches, Scott et al. (2011) detected 81 individual *N*-glycosylation sites among glycopeptides from *C. jejuni* which corresponded to 53 glycoproteins. The data revealed that most of the glycoproteins are associated with the membrane with the majority involved in membrane transport systems. The biosynthesis pathway of *N*-glycoconjugates is now deciphered in *C. jejuni* (Nothaft and Szymanski, 2013). These *N*-glycoconjugates are composed of seven saccharide residues derived from bacillosamine and assembled to an undecaprenyl phosphate lipid carrier. PlgB is responsible for the transfer of the heptasaccharide from the lipid carrier to asparagine residues within the specific glycosylation sequon (D/E)XNZ(S/T) where X and Z cannot be a proline. By comparing the glycoproteome of *C. jejuni* strains, additional 30 *N*-glycosylation sites were recently detected across *C. jejuni* strains (Scott et al., 2014). In

C. jejuni, the encoding genes of enzymes required for *N*-glycan synthesis are all located in a gene cluster.

O-glycoproteins are less numerous in *C. jejuni* and only FlaA, FlaB (encoding flagellins), and MOMP (encoding the major outer membrane porin) were described so far to be decorated by *O*-glycans (Mahdavi et al., 2014). These proteins were previously reported to contribute to the human pathogenesis process. Using LC-MS/MS to analyze glycosylation of flagellin in *C. jejuni*, Ulasi et al. (2015) revealed a flagellin highly glycosylated on both serine and threonine residues. The *O*-glycans, attached to FlaA and MOMP via oligosaccharyltransferase, are mainly composed of derivatives of pseudaminic acid, acetamidino pseudaminic acid, legionaminic acid, and fucose (Thibault et al., 2001; Logan et al., 2009; Merino and Tomas, 2014). However, further analyses are required to determine consensus *O*-glycosylation sequons, *O*-glycan composition, level of glycosylation variations and their impact on *C. jejuni*.

Apart from a better understanding the biology of *Campylobacter* and its interaction with environment and host cells, the progress of knowledge on *N*-glycans composition, location and biosynthesis in *C. jejuni* open new perspectives in terms of glycoengineering to develop promising alternative vaccines against *C. jejuni* in chicken (Nothaft et al., 2016).

27.5.2 Phosphoproteome

A large number of phosphorylated sites were identified among bacterial proteins. Protein phosphorylation is considered as one of the main signal transductions and network regulations in bacteria (Dworkin, 2015). The addition of phosphoryl residues turned the protein into an activated form responsible for the transfer of the signal. Various phosphorylation systems were discovered among bacterial including two-component systems (TCS), phosphoenolpyruvate-phosphotransferase system (PTS), eukaryotic-like serine/threonine kinases (eSTK), bacterial protein-tyrosine kinases (BY kinases), and the protein kinase McsB (Mijakovic et al., 2016). Serine, threonine, tyrosine, histidine, arginine, lysine, aspartate or cysteine is the potential amino acid involved in phosphorylation systems. Depending on the system, some modification sites on the proteins are predictable. Since signal transduction and regulations in bacteria occurred in response to environmental changes, protein phosphorylations are specific and reversible. As a consequence and apart from technical limitations, the phosphoproteome can be challenging to analyse. Using LC-MS/MS of tryptic digested phosphoproteins preseparated on SDS-PAGE, 36 phosphoproteins were identified in *C. jejuni* with a majority of the proteins involved in protein metabolism, iron homeostasis and oxidative stress response (Voisin et al., 2007). Surprisingly, phosphorylations related to the 15 predicted TCS in *C. jejuni* were not detected. Although some technical challenges remain for the bacterial phosphoproteome analysis, more investigations on phosphoproteomes of *C. jejuni* are required before determining and modeling the phosphorylation network of this foodborne pathogen.

27.6 Conclusions

In microorganisms, proteins are essential to the life processes and can harbor a vast range of functions. Proteins in bacteria are present in all compartments and are involved in bacterial structure, enzymatic reactions, molecule trafficking (including storage, transport and communication), and protein complex machineries (e.g. respiration, translation, DNA replication). To detect and analyze proteins at the cell-wide level, holistic approaches and non-a priori methodologies have been developed. Although proteomics studies have been intensively applied to analyze bacteria for over 3 decades, the first studies using proteomics for *Campylobacter* appeared in the early 2000s. This can be explained by the low interest for *Campylobacter* before. As a matter of fact, studies on *Campylobacter* have rapidly benefited from the improvements of proteomics tools which extended the field of investigations. A deeper understanding of *Campylobacter* has arisen from the advent of quantitative proteomics developments. Although some responses to environmental changes remain elusive, proteomics analyses have shed light on the effect of stress conditions to *Campylobacter*. Genomic comparisons of *Campylobacter* are limited due to a high proportion of genes with unknown functions and a low number of complete genomes available. For *Campylobacter* analyses, proteomics analyses often constituted a starting point for the description of biological functions. Because of high genome plasticity, transcriptomic and genomic analyses would be helpful to determine the distribution of genetic events across *Campylobacter* species and strains. The development of gel-free, protein-labeling, and posttranslational analyses during the last decade has brought great benefit to the investigation of *C. jejuni* allowing examination of the functions or regulations systems of *C. jejuni* using novel proteomics tools. Future proteomics analyses on *Campylobacter* should include scientific questions addressing the potential regulation systems involving acetylation, methylation, and dimethylation and protein interactions to characterize and determine the function of protein complexes.

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Proteomic Approaches for Allergen Analysis in Crop Plants

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28.1 Introduction

Crop plants are among the most important sources of foods in the human diet; however, they could potentially trigger allergic reactions. Food allergies are serious health concern whose frequency is increasing globally. Food allergies are defined as adverse health effects caused by a specific immune response when exposed to a given food (Boyce et al., 2010). Foods trigger allergic responses in individuals after ingestion or inhalation of causative proteins contained in various products. In most cases, the substances that cause allergic reactions are proteins with immunoglobulin E (IgE)-binding activities, termed allergens or allergenic molecules. When the organism is first exposed to causative foods, food allergen-specific IgE antibodies are produced and bind to the surface of mast cells in blood. On second exposure to the causative foods, the allergen binds to specific IgE antibodies and causes cross-linking of the high affinity IgE receptor, Fc ϵ RI, on the mast cell surface. The mast cells evoke the degranulation of granules that store inflammatory chemical mediators, such as histamines, proteases, and cytokines. The released chemical mediators then induce the clinical symptoms of allergic reactions (Holowka and Baird, 1996).

Several allergens have been already identified, cDNA clones encoding allergens have been isolated from causative agents, and their amino acid sequences have been determined (Radauer et al., 2008). Most food allergens are proteins with molecular weights of 10,000–70,000 Da and are relatively stable to heat, acids, and proteolysis (Sampson, 1999; van Ree, 2002). Structural features, for example, compact three-dimensional structures, disulfide bonds, and posttranslational glycosylation, contribute to the resistance of allergens to denaturation by food processing and digestive proteolytic attack (Breiteneder and Mills, 2005; Huby et al., 2000; van Ree, 2002). Since IgE antibodies recognize approximately 5–8 amino acid residues on allergen surfaces, the IgE-binding sequence of allergens, known as IgE-binding epitopes or B-cell epitopes, are critical for the reaction against food allergens (Kleter and

Peijnenburg, 2002; Laver et al., 1990). There are two types of IgE-binding epitopes: linear (continuous) and conformational (discontinuous) (Kleter and Peijnenburg, 2002; Zhao and Chait, 1994). Food allergens have been classified into some protein families according to their own structural and functional features (Breiteneder and Radauer, 2004). Each allergen is assigned a name and an abbreviation of the scientific name of its source, which consists of three to four letters indicating the genus and one to two letters identifying the species, and an Arabic number assigned to identify the order (IUIS/WHO Allergen Nomenclature Subcommittee, 1994). All allergens named using this system of nomenclature are registered in the Allergen Nomenclature database (<http://www.allergen.org/>). Moreover, several constantly updated allergen databases are available (Mari et al., 2009). One of the databases, AllergenOnline, contains a tool for identifying the primary potential risks of allergy for GMOs and novel foods (Goodman et al., 2016).

A single food can contain multiple allergens. Furthermore, individuals allergic to the same kind of food may show reactions to different allergens and develop varied symptoms. The primary therapy for food allergies is to avoid the causative foods and/or allergens based on an accurate diagnosis (Ebisawa, 2009; Sicherer and Sampson, 2010). Therefore, an accurate diagnosis of food allergies is necessary for preventing severe allergic reactions and avoiding unnecessary dietary restrictions. In vivo assays, such as the skin prick test and provocation test, are accepted methods to diagnose food allergies. However, these tests may involve a risk to the patient during diagnostic testing, possibly resulting in the onset of severe symptoms. The most common in vitro allergy diagnostics are based on the detection of a specific IgE that reacts with extracts containing allergenic and nonallergenic molecules. However, this test can cause false-positive results, leading to the inappropriate removal of food items from the diet, which can have long-term consequences (Rancé et al., 2002). Recently, new diagnostic strategies, namely component-resolved diagnostics (CRD) or molecular-based allergy (MA) diagnostics, have been developed (Canonica et al., 2013; Valenta et al., 1999). CRD can be applied to the sera of patients with allergies to detect single allergen-specific antibodies against a single allergenic molecule as a causative allergen present in the materials and may be more sensitive than tests employing conventional total food extracts. The diagnostic results supported by such methods, combined with conventional tests that employ total food extracts, are more sensitive and effective for the diagnoses of allergies to wheat and peanuts than the conventional test alone (Ebisawa et al., 2015; Matsuo et al., 2008). Moreover, to further improve diagnostic accuracy, an allergen component-based microarray has been developed (Bublin et al., 2011; Constantin et al., 2009).

Crop plants with suitable agronomic traits, for example, high yield, good taste, texture, and the ability to adapt to environmental conditions, have historically been selected using phenotypic methods. However, more comprehensive analytic methodologies, such as transcriptomics, metabolomics, and proteomics, are increasingly being used to clarify the characteristics of crop plants and enable the selection of suitable varieties depending on

the purpose of cultivation. Proteomics is an analytical method based on the combination of protein separation mainly by electrophoresis or chromatography and identification by mass spectrometry (MS) and subsequent bioinformatics. Recent reports have indicated that proteomic techniques are also useful for identifying novel allergens (Kao et al., 2005), determining the qualitative and quantitative variability of allergens among crops (Hjernø et al., 2006) and detecting posttranslational modifications of various allergen isoforms (Dos Santos-Pinto et al., 2014).

In addition, novel crop plants that can adapt to poor cultivation conditions or that are nutrient-rich are being developed using transgenic techniques. The Codex Alimentarius Commission stated that safety assessments of genetically modified (GM) foods need to include an investigation of allergenic tendencies that may result from gene insertion (ftp://ftp.fao.org/esn/food/guide_plants_en.pdf). Therefore, proteomic techniques may be also useful tools for detecting the unexpected effects of GM crop plants, that is, to detect allergens that have not been detected previously in crop plants. In this review, we focused on the proteomic approach as a powerful tool for the analysis of allergens in crop plants.

28.2 Allergen Identification in Crop Plants

Currently, exhaustive analytic methodologies, such as genomics, transcriptomics, metabolomics, and proteomics, are being used increasingly to clarify the characteristics of crops to enable the selection of suitable plant varieties depending on the purpose. Allergenomics, allergen-targeted proteomic analysis, facilitates the comprehensive detection of IgE-binding proteins (Fasoli et al., 2009; Picariello et al., 2011; Yagami et al., 2004). In most cases, proteins are extracted and separated by gel electrophoresis, detected by immunoblotting with sera from patients with allergies, and then identified by MS technologies.

The combination of protein separation by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D-PAGE) and immunoblot analysis with sera from patients with allergies, referred to as 1D immunoblot, is a traditional and routine method for the detection of IgE-binding proteins suspected as putative allergens or for known allergens. Using this method, food allergens such as in cereals (Satoh et al., 2011; Urisu et al., 1991), pseudo-cereals (Satoh et al., 2008; Tanaka et al., 2002), beans (Mittag et al., 2005), nuts (Clarke et al., 1998; Fernandez et al., 1995; Parra et al., 1993), vegetables (Puimalainen et al., 2015), and fruits (Hemmer et al., 2010; Pastorello et al., 1996) have been identified. The specification of a causative allergen to a particular allergic symptom is attained by 1D immunoblot with sera from patients with allergies who present with the specific allergic symptom (Amano et al., 1998). The differences of allergenomes among several cultivars have been also detected by 1D immunoblot (Weiss et al., 1997). Moreover, it enables the analysis of cross-reactivity between allergens derived from other kinds of organisms (Hemmer et al., 2010; Pastorello et al., 1996; Scheurer et al., 2000).

Two-dimensional (2D)-PAGE is a more effective method for analyzing proteomes by separating proteins according to the differences in both molecular weight (MW) by SDS-PAGE and isoelectric point (pI) by isoelectric focusing (IEF), although this method has some limitations when analyzing hydrophobic and alkaline proteins. The separated proteins in the gel are visible by Coomassie Brilliant Blue or silver staining. The stained gel images are used for identification and comparative analysis of expressed proteins among different cultivars and varieties (Alm et al., 2007; Koo et al., 2011; Kottapalli et al., 2008), developmental stages (Guo et al., 2012), growth conditions (Huang et al., 2012), and storage conditions (Giraldo et al., 2012). The 2D immunoblot with sera from patients with allergies is the most commonly employed method for the comprehensive detection of IgE-binding proteins containing known and putative allergens (Akagawa et al., 2007; Beyer et al., 2002). For example, 2D immunoblot with sera from patients with allergies revealed the differences of allergens obtained from virus-infected and noninfected tomato fruits (Welter et al., 2013). The 2D immunoblot can also detect qualitative and quantitative differences of allergens among different cultivars and varieties (Walczk et al., 2013; Weiss et al., 1997). Several reports utilizing allergenomic techniques demonstrate that the extent of allergenicity among cultivars is dependent on varied allergenic isoforms (Fremont et al., 2002; Reuter et al., 2005) and different types of glycosylated allergens (Alm et al., 2007; Napoli et al., 2008) (Table 28.1).

The conventional method to identify the amino acid sequence of proteins is N-terminal amino acid sequencing by Edman degradation (Beyer et al., 2002; Boldt et al., 2005). With recent technical advances in MS methodologies, even small amount of IgE-binding proteins can be rapidly identified using methods including matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). MALDI-time-of-flight (TOF) MS analysis is a convenient tool for identifying proteins in combination with 1D-PAGE or 2D-PAGE separations and peptide mass fingerprinting (PMF) or tandem mass spectrum (MS/MS) ion searches followed by confirmation using sequence database searching (Akagawa et al., 2007; Boldt et al., 2005; Natarajan et al., 2005; Picariello et al., 2015; Sotkovsky et al., 2008). However, the use of liquid chromatography (LC)-MS/MS for protein identification is growing because of its high throughput, automation, and sensitivity (Bässler et al., 2009; Kottapalli et al., 2008; Larre et al., 2011; Puimalainen et al., 2015; Rogniaux et al., 2015; Serra et al., 2013; Sotkovsky et al., 2008; White et al., 2013). Moreover, de novo sequencing by the MS/MS spectrum followed by a homology search identifies proteins without sequence databases and is suitable for identifying proteins in organisms with incomplete genomic and protein databases (Saha et al., 2015).

28.3 Determining the Qualitative and Quantitative Variability of Allergens Among Crop Plants

Immunoassays, such as immunoblotting and enzyme-linked immunosorbent assays (ELISA), have been used as standard methods for identifying and quantifying food allergens. These assays can detect qualitative and quantitative differences of individual allergens derived from

Table 28.1: Allergenomic identification in crop plants.

| Crop Plants | Detected Allergen(s) | Method for Protein Identification | References |
|-------------|---|---|--|
| Wheat | Serpin α-amylase inhibitor γ-gliadin Low molecular weight (LMW) gliutenin | MALDI-TOF/TOF MS | Akagawa et al. (2007) |
| Maize | Bet v 1.01 C | MALDI-TOF/TOF MS | Huang et al. (2012) |
| Soybean | Gly m Bd 28K | MALDI-TOF/MS LC-MS | Natarajan et al. (2005) |
| | β-conglycinin Gly m Bd 30K Glycinin | LC-MS/MS | Seo and Cho (2016) |
| Peanut | Trypsin inhibitor Ara h 3 Ara h 4 Ara h 1 Ara h 2 Ara h 3 iso-Ara h 3 Ara h 1 Ara h 3 Ara h 1 Ara h 2 Ara h 3 Ara h 6 Ara h 8 Ara h 9 Ara h 10 Ara h 11 Ara h 1 Ara h 3 | nano-ESI-LC-MS/MS N-terminal sequencing ESI-MS/MS nano-ESI-LC-MS/MS LC-MS | Kottapalli et al. (2008) Guo et al. (2008) Walczyk et al. (2013) Johnson et al. (2016) Korte et al. (2016) |
| Almond | Pru du 6 | LC-MS | Korte et al. (2016) |
| Cashew | Ana o 1 | LC-MS | Korte et al., (2016) |
| Hazelnut | Cor a 9 | LC-MS | Korte et al. (2016) |
| Pistachio | Pis v 2 Pis v 3 Pis v 5 | LC-MS | Korte et al. (2016) |
| Walnut | Jug r 1 Jug r 2 Jug r 4 2S albumin Lipid transfer protein (LTP) | LC-MS LC-MS/MS | Korte et al. (2016) Downs et al. (2016) |
| Sesame | 7S globulin 11S globulin Ses i 2 Ses i 3 | MALDI-MS N-terminal sequencing | Beyer et al. (2002) |

Continued

Table 28.1: Allergenomic identification in crop plants.—cont'd

| Crop Plants | Detected Allergen(s) | Method for Protein Identification | References |
|------------------|--|---|------------------------|
| Olive | Ole e 1 | MALDI-TOF-MS/MS | Napoli et al. (2008) |
| Tomato | Lyc e 1 | nano-LC-ESI-MS/MS | Welter et al. (2013) |
| | Lyc e 2 | | |
| | Polygalacturonase | | |
| | Peroxidase | | |
| | Glucanase | | |
| | Pectin methylesterase 1 | LC-MS/MS | Ghiani et al. (2016) |
| | Pectin methyltransferase 2.1 | | |
| | Nonspecific lipid transfer protein (nsLTP) | | |
| | Polygalacturonase 2S | | |
| Cherry | Pru av 1 | MALDI-TOF-MS nano-ESI-MS/MS N-terminal sequencing | Reuter et al. (2005) |
| | Pru av 2 | LC-MS/MS | Ippoushi et al. (2016) |
| Nectarine fruits | Pru du 1.05 | MALDI-TOF/MS | Giraldo et al. (2012) |
| | Pru du 1.06A | | |
| | Pru du 2.01A | | |
| | Pru du 2.01B | | |
| Strawberry | Fra a 1 | MALDI-TOF/TOF MS | Alm et al. (2007) |

some crop plants. However, they are time and labor intensive with allergen-specific antibodies required for each allergen.

For proteomic differential display, gel-based and gel-free methods are used. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) is a gel-based detection method for allergens and is typically used for allergenomic analysis with high-resolution and visible images of protein spots in the gel. 2D-DIGE can detect qualitative and quantitative differences of multiple proteins among two samples in a single gel (Ünlü et al., 1997) and has been used to reveal natural variations of proteomes containing allergens among several crop cultivars (Teshima et al., 2010; Walczyk et al., 2013) as well as proteomes containing allergens at different developmental stages (Guo et al., 2012; Pedreschi et al., 2009).

Gel-free approaches to determine relative and absolute protein levels in a complex biological matrix are available as more effective methods for comprehensive allergen surveys. Compared to gel-based methods, gel-free approaches offer benefits in terms of sensitivity, quantitative power, and throughput (Faeste et al., 2011; Houston et al., 2011). Selective reaction monitoring (SRM; or multiple reaction monitoring, MRM)-based

proteomics represent a targeted approach for quantifying specific protein-derived peptides. Peptide quantification methods by MS include both chemical labeling and label-free methods (Stevenson et al., 2009). Although chemical labeling methods are time-consuming and cost more than label-free methods, the results using labeling methods have a higher accuracy. Peptide labeling methods include cleavable isotope-coded affinity tags (ICAT) (Ong et al., 2002), isobaric tags for relative and absolute quantification (iTRAQ) (Flodrova et al., 2015; Nogueira et al., 2013), and absolute protein quantitation (AQUA) peptides (Ippoushi et al., 2016; Sayers et al., 2016; Stevenson et al., 2015). However, the label-free method has the advantage of being low cost and simple to use (Houston et al., 2011; Stevenson et al., 2009).

28.4 Proteomics for the Assessment of Allergenicity in GM Crop Plants

To use GM crop plants as foods, it is necessary to confirm their safety because potential allergenicity remains a major concern in foods derived from GM crop plants; this is because the production of these plants by transgenic techniques may result in the expression of new proteins (Taylor and Hefle, 2001). Approaches for the assessment of possible allergenicity of newly expressed proteins in GM crop plants include the following: protein source, amino acid sequence homology to known allergens, resistance to pepsin digestion, immunoreactivity with sera from patients with allergies, expression level of the introduced gene, functional characteristics, and prediction using animal models. However, there is no definitive test that can predict the allergenicity of GM crop plants. Therefore, to assess potential allergenicity of newly expressed proteins in GM crop plants, an integrated case-by-case approach should be considered based on a combination of the previously mentioned factors (Goodman et al., 2008).

Proteomic techniques are useful tools for detecting proteins containing known and putative allergens, newly expressed proteins, and unintended off-target effects in GM crop plants. Several reports using allergenomic analysis indicate that there are no obvious differences between allergen expression levels in GM crop plants and their comparators, including cereals (Emami et al., 2010; Gayen et al., 2016; Lupi et al., 2013; Satoh et al., 2011; Scossa et al., 2008), beans (Batista et al., 2007; Goodman et al., 2013; Kim et al., 2006; Rouquie et al., 2010; Sten et al., 2004), and vegetables (Coll et al., 2011; Fonseca et al., 2012). To prove the allergenicity of GM compared to native crop plants, the range of allergenicity that native crop plants can have under various conditions is required. Hence, further information about variations in allergen expression levels among native crops affected by genetic background and environmental factors may provide useful baseline data to facilitate meaningful comparisons of novel crops regarding allergenicity. Several reports show that the unintended effects of gene introduction on the expression of significant proteins were lower than the intended (Gong et al., 2012; Nakamura et al., 2010) or environmental (Nakamura et al., 2014) effects on the expression of these proteins (Table 28.2).

Table 28.2: Allergenomic detection in representative GM crops.

| Crop Plants | Transgene(s) | Characteristics of Transformants | Control | References |
|-------------|---|---|-------------------------------|------------------------|
| Soybean | A gene encoding a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein from <i>Agrobacterium tumefaciens</i> strain CP4 (<i>cp4-epsps</i>) | Glyphosate resistance | Non-GM | Batista et al. (2007) |
| | A gene encoding a modified acetohydroxy acid synthase protein from <i>Arabidopsis thaliana</i> | Imidazolinone herbicides resistance | Non-GM and near isogenic line | Goodman et al. (2013) |
| | A gene encoding a modified phosphinothricin acetyltransferase protein from <i>Streptomyces viridochromogenes</i> | Glufosinate ammonium herbicides resistance | Non-GM and near isogenic line | Goodman et al. (2013) |
| | Genes encoding a modified EPSPS protein from <i>Zea mays</i> and a modified <i>p</i> -hydroxyphenylpyruvate dioxygenase protein from <i>Pseudomonas fluorescens</i> | Glyphosate and isoxaflutole herbicides resistance | Non-GM and near isogenic line | Goodman et al. (2013) |
| | <i>cp4-epsps</i> | Glyphosate resistance | Wild type | Kim et al. (2006) |
| | <i>cp4-epsps</i> | Glyphosate resistance | Near-isogenic line | Rouquie et al. (2010) |
| | <i>cp4-epsps</i> | Glyphosate resistance | Wild type | Sten et al. (2004) |
| Rice | A gene which provides resistance to herbicide phosphinothricin from <i>Streptomyces hygroscopicus</i> (<i>bar</i>) | Herbicide resistance | Non-GM | Gong et al. (2012) |
| | Genes encoding a Cry 1 Ac protoxin from <i>Bacillus thuringiensis</i> (<i>cry 1 Ac</i>) and a modified trypsin inhibitor from <i>Vigna unguiculata</i> (<i>sck</i>) | Insect resistance | Wild type | Gong et al. (2012) |
| | A gene encoding an RNA-binding protein from <i>Mesembryanthemum crystallinum</i> | Salt stress resistance | Non-GM | Nakamura et al. (2014) |
| | A gene encoding disease resistance to <i>Xanthomonas oryzae</i> pv <i>oryzae</i> (<i>Xa21</i>) | Disease resistance | Wild type | Gayen et al. (2016) |
| | A gene encoding a mutant anthranilate synthase α -subunit from <i>Oryza sativa</i> (<i>OASA1</i>) | High-level tryptophan accumulation | Wild type | Satoh et al. (2011) |

| | | | | |
|--------|---|--|---------------------------------|---|
| Wheat | A gene encoding a low-molecular-weight glutenin subunits from <i>Triticum aestivum</i> (<i>lmw-gs</i>) | Manipulation of gluten components | Wild type | Lupi et al. (2013) |
| | A gene encoding a waxy protein from <i>Triticum durum</i> (<i>Wx-B1</i>) <i>lmw-gs</i> | Manipulation of amylose contents | Wild type | Lupi et al. (2013) |
| Maize | A gene encoding a Cry 1 Ab protoxin from <i>Bacillus thuringiensis</i> (<i>cry1Ab</i>) <i>cry1Ab</i> | Manipulation of gluten components Insect resistance | Wild type Near isogenic line | Scossa et al. (2008) Coll et al. (2011) |
| Potato | A gene encoding dehydration responsive element-binding protein (DREB1A) | Insect resistance Dehydration resistance | Non-GM Non-GM | Fonseca et al. (2012) Nakamura et al. (2010) |

28.5 Conclusions

In this review, we described the application of proteomic techniques, which are useful to clarify agriculturally beneficial characteristics of crop plants and to identify and detect their allergens. The combined use of genomic, proteomic, and allergenomic databases and proteomic techniques and their enhancements could provide valuable tools to improve accurate and efficient allergen identification.

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Detection of Microbial Toxins by -Omics Methods: A Growing Role of Proteomics

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29.1 Introduction—Bacterial Foodborne Pathogens and Their Toxins

The World Health Organization (WHO) initiative, aimed at mapping and globally estimating the burden of foodborne diseases, officially acknowledged diarrheal diseases as leading causes of foodborne disease burden worldwide (WHO, 2015). This issue has great relevance for public health in particular in the context of the ever-growing globalization processes that lack proper monitoring and risk management approaches. The first level of foodborne disease control should be performed in the food production and processing chain. Since foodborne bacteria and their toxins are ubiquitous and cannot be totally destroyed due to their vast survival and adaptation potential, several control points across the raw material assessment, whole food production, processing, and distribution chain have to be implemented. Moreover, in recent years the increasing consumption of ready-to-eat (RTE) food and the popularity of semidry products has meant that food preparation steps, such as cooking or washing may be omitted, increasing the possibility of disease outbreak. Similarly, the globalization of the food chain requires a regularly updated public surveillance system and control strategies for risk assessment and management. Reduction of foodborne diseases in such a demanding and constantly changing environment will therefore rely on the development and implementation of rapid, sensitive, and accurate methods for determination of foodborne bacterial pathogens. Such methods will be useful for the assessment of bacterial adaptation and survival mechanisms within the food production chain and postprocessing. Besides modern contributory factors, common causative agents of foodborne outbreaks are often thermostable foodborne bacterial toxins that remain biologically active even after bacterial destruction (Rešetar et al., 2015). Indeed, according to the European Centre for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA), major contributing factors besides water contamination are bacterial food intoxication and toxic-infectious food poisoning. Altogether, almost 50% of all reported foodborne outbreaks in the EU (Fig. 29.1) are associated with foodborne bacterial causative agents (EFSA and ECDC, 2015).

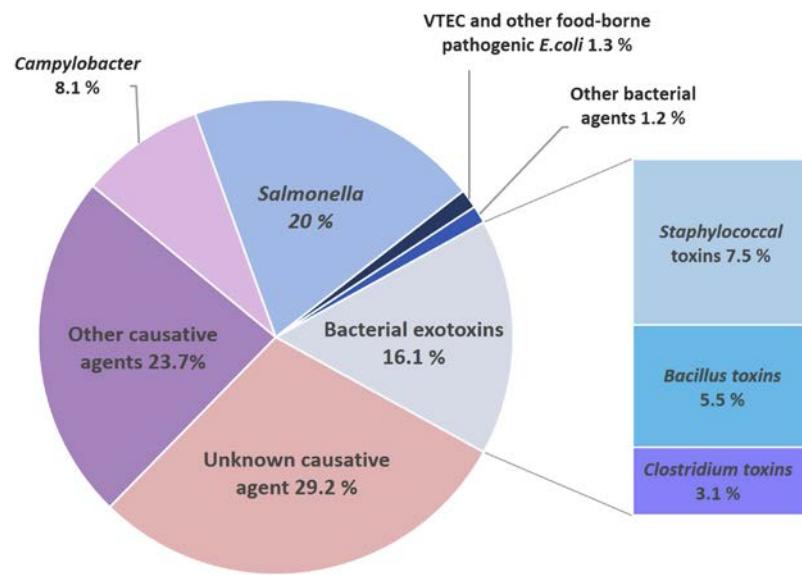


Figure 29.1

Distribution of foodborne outbreaks per causative agent in EU during 2014 ([WHO, 2015](#)).

Foodborne pathologies are a consequence of different invasion mechanisms and present a plethora of disease symptoms that add complexity to this topic. For example, intracellular parasite *Listeria monocytogenes* exerts its action on the host through physical intrusion into the organism's tissue ([Renier et al., 2011](#)). In addition, the majority of bacterial foodborne pathogens act through toxins, upon either auto- or external lysis in the host's body. Furthermore, vegetative foodborne bacteria secrete toxins during growth on food intended for consumption or, less common, in the host's gut. Foodborne bacterial toxins have a large molecular weight ranging from less than 1000 Da to more than 100,000 Da. These substances fall into two main subgroups, namely endotoxins and exotoxins ([Rešetar et al., 2015](#)).

Bacterial endotoxins are chromosomally encoded structural components of the outer membrane of gram-negative bacteria. Endotoxins are highly acylated saccharolipids, for example, the lipopolysaccharides (LPS) have been extensively investigated in the model organisms *Salmonella typhimurium* and *Escherichia coli*. These two bacteria provided important insights into LPS-mediated bacterial immune evasion, host cell adhesion, and invasion mechanisms. Important insights have been obtained on the protective characteristics against the harsh host gut environmental conditions as well as resistance to antibiotics and detergents ([Zhang et al., 2013](#)). A number of gram-negative foodborne bacterial pathogens, including *Campylobacter*, *Yersinia*, *Shigella*, *Listeria*, *Brucella*, *Vibrio*, *Francisella*, and *Leptospira*, are known to induce food poisoning upon consumption of bacteria-contaminated food ([EFSA and ECDC, 2015](#)). Among them, campylobacteriosis has been recognized by WHO as the most frequent

diarrheal illness cause and nontyphoidal *Salmonella enterica* as a diarrheal illness causative agent with the highest mortality rate (WHO, 2015).

Biofilm producing bacterial pathogens such as *S. enterica*, *L. monocytogenes*, *E. coli*, *Staphylococcus aureus*, and *Bacillus cereus* have been well studied (Tan et al., 2014) due to their specific biological characteristics. Moreover, *Vibrio cholerae*, *Shigella flexneri*, and *S. enterica* cannot be cultured, complicating their detection (Senoh et al., 2012). The development of fast, sensitive, and accurate methods is needed in the coming years for this group of bacterial foodborne pathogens.

The second most frequent causative agent of foodborne outbreaks in the EU are bacterial exotoxins (Fig. 29.1) secreted both by gram-negative and gram-positive bacteria. These are most frequently the endospore-forming bacteria *S. aureus*, *B. cereus*, and *Clostridium perfringens*. Structurally, bacterial exotoxins are proteins and polypeptides, usually heat-stable and tolerant to gastrointestinal proteases. These characteristics make them resistant to thermal processing and extreme pH values. After secretion into the environment, it is almost impossible to remove them from raw, dehydrated, or even processed food (Postollec et al., 2012). Indeed, some of bacterial exotoxins are among the world's deadliest reported toxins. Among these agents are botulinum toxins produced by *Clostridium botulinum* group I (proteolytic) and II (nonproteolytic) (Carter and Peck, 2015). Additionally, staphylococcal enterotoxins comprise more than 20 different chromosomally encoded single-chain exotoxins produced by *S. aureus*. However, according to the recent reports, only *S. aureus* serotypes A and B are responsible for the majority of staphylococcal food poisoning cases. These foodborne outbreaks are usually associated with consumption of (1) mixed food, for example, pig and broiler meat contaminated with preformed exotoxins or (2) postprocess contaminated food containing exotoxin-producing strains (EFSA and ECDC, 2015; Castro et al., 2016). Further development of rapid, sensitive, and reliable methods for determination of foodborne bacterial pathogens and identification and quantification of their toxins are of critical importance. These methods are urgently required for monitoring the adaptation and survival mechanisms of foodborne bacterial pathogen within the food production chain and postprocessing steps as well.

Diverse bacterial toxins responsible for foodborne outbreaks can exert their effects in the same way and cause similar immunological responses and clinical symptoms. Some of the observed symptoms include mild diarrhea and emesis, or even severe and lethal neurological disorders. Moreover, accurate clinical diagnosis is often very difficult due to a variable incubation period before symptoms arise, in addition to the complexities from variable consumer food habits in different countries. Direct correlation of the causative agent can be made in up to 70% of all reported foodborne outbreaks, although identification of the contributory factors remain low (EFSA and ECDC, 2015).

Currently, conventional methods for the detection of bacterial foodborne pathogens and their toxins are still used despite numerous limitations, including a lack of sensitivity/specificity or

human errors. These methods are laborious, time-consuming, and also rely on culture-based bacterial enrichment and isolation approaches, subjective cellular morphology observations, and combined growth on selective agar plates with serological, immunological, chemotaxonomic, and biochemical observations (Sintchenko et al., 2007). These conventional methods for the detection of bacterial food pathogens and toxins are not suitable to tackle issues of increased incidence and complexity of bacterial foodborne diseases. The only possible way to address this complex issue is to develop sensitive analytical techniques and protocols for simultaneous multiplex detection of foodborne bacteria and their toxins directly from food matrices or human clinical specimens (Rešetar et al., 2015; Law et al., 2014). Sensitive multiplexing analytical platforms may offer various advantages over traditional methods, comprising both a technological superiority and lower overall costs.

29.2 High-Throughput Technologies for Detection of Bacterial Foodborne Pathogens and Their Toxins

Advances in instrumentation and bioinformatics led to the development of novel high-throughput, culture-independent, and less laborious methodological approaches. Most of them, known as “omics” methods, are suitable for multiple detection and improved characterization of foodborne bacteria and toxins. With the ever-growing introduction of such methods some novel problems may occur. They are most frequently higher probability of interferences from food matrices as well as native microflora present at high levels in raw food. To address this, different sample preparation strategies and enrichment of bacterial food pathogens and toxins present in low quantities in food have been employed (Wang and Salazar, 2016). Approaches that include differential separation and centrifugation steps, dialysis tubing, or advanced approaches that rely on filtration with immunomagnetic separation have been established (Fusi et al., 2011). Recently, detection of bacterial contamination in food by immunological assays based on affinity recognition and that are biosensor-based (Wang and Salazar, 2016) have been established as well as -omics-based technologies for food analyses (foodomics technologies; Gallo and Ferranti, 2016). The immunoassays rely on recognition of the target of interest by an antibody. However, commonly used ELISA double antibody sandwich and automated enzyme-linked fluorescent immunoassay (ELFA) assays may produce false-negative and false-positive signals due to cross-reactions, for example, binding of a polyclonal antibody to different epitopes. Recent improvements in antibody production may provide an enhanced detection of bacterial toxins (Lewis et al., 2014). Two devices for high-throughput screening based on lateral flow assay (LFA) and antibody-antigen recognition for simultaneous detection of 10 different antibodies against *Yersinia pestis* (Hong et al., 2010) and for 10 most common foodborne pathogens (Zhao et al., 2016) have been successfully tested. Moreover, commercially developed protein-affinity microarray kits have been developed for assessment and semi-quantification of *Clostridium* and *Staphylococcus* bacterial exotoxins (Zhao et al., 2014). Recognition of target molecules on bacterial foodborne pathogen cells without

preenrichment steps is increasing in popularity by use of optical, electrochemical, and mass-based biosensors. These technologies may target lipoproteins on the cell surface of gram-negative bacteria, for example, *C. jejuni* and *V. cholerae* (Wilson and Bernstein, 2016). Such analytical devices are a promising basis for standardized detection of bacterial exotoxins. Still, commercialization of such biosensors is hampered by multiple factors (Law et al., 2014) and assessment of exotoxin presence by use of immunochemical methods does not reveal anything about its active/inactive form (Rešetar et al., 2015).

Implementation of genomic (Stasiewicz et al., 2015), transcriptomic (Valdés et al., 2013), metabolomics, and proteomic (Martinović et al., 2016) technologies is already transforming the food safety field and has a tremendous impact on foodborne disease management and development of sanitizing agents and procedures. Nevertheless, fast -omics-based technologies may not be that efficient if bacteria or toxins are present in food at low concentrations or if inadequate sample preparation methods are employed. These issues are the most important ones for faster transfer into public health sector. The “soft” ionization-based mass spectrometry (MS) techniques, namely matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), have evolved rapidly and been approved by European authorities and a few years later by US Food and Drug Administration (FDA). MALDI-TOF MS has been introduced into clinical laboratories as a routine method for the detection of gram-negative bacteria. Indeed, intact cell MALDI-TOF MS (ICMS) has become the gold standard method for bacterial typing in clinical and microbiological laboratories (Schumann and Maier, 2014). Isolated intact bacterial food contaminants, without any prior knowledge about genome or protein sequences, are used to generate ICMS protein spectra often called ICMS fingerprint patterns. The majority of peaks within ICMS represent proteins of ribosomal origin in the mass range of 2–20 kDa (Ryzhov and Fenselau, 2001). ICMS protein spectra are directly compared to a collection of previously collected food pathogen protein reference sequences deposited in a reference database forming the basis for microbial identification. Therefore, the method relies on pattern recognition rather than on individual protein analysis. Up to now, a number of ICMS reference databases have been developed. Some laborious prerequisites for ICMS-based identification of bacteria are the isolation and enrichment of bacterial contaminants and cell culturing either on solid (to eliminate introduction of more than one bacterial species on MALDI target) or liquid medium (Freiwald and Sauer, 2009), but they do not obscure its advantages in comparison to classical procedures. MALDI-TOF MS-based detection of bacteria is highly dependent on the amount of well-characterized mass spectra accessible in the reference sequence database and distinction on the subspecies level might still be problematic when sample preparation procedures are not strictly conducted. Moreover, this method can be further developed for the identification of some gram-positive, demanding anaerobic, or slow-growing bacteria as well (Biswas and Rolain, 2013; Schulthess et al., 2014). In addition, identification problems for *S. enterica* ssp. *enterica* serovar *typhi* and other less clinically relevant groups such as *S. enterica* ssp. *enterica* serotypes (Kuhns et al., 2012) or *C. jejuni* subgroups (Zautner et al., 2013) warrant attention. The ICMS technique is very useful in

assessing the presence/absence of food poisoning bacteria in complex matrices, but cannot give information on the presence/absence of toxin-coding genes or bacterial toxin levels in food. An interesting approach relies on a combination of genomic multilocus sequence typing (MLST) and the proteomic ICMS-spectra method into one coherent and complementary below-species level differentiation technique, termed the S-based phyloproteomics (MSPP) typing method (Zautner et al., 2015).

In summary, the role of mass spectrometry and proteomics in the detection of bacterial food contaminants is constantly increasing. In particular, these methods may be used in combination with genomic technologies that provide information on total genetic variety of foodborne bacterial pathogens and consequent detection of small or untypical outbreaks of foodborne illnesses. Genotyping methods such as multiplex PCR (mPCR) and real-time quantitative polymerase chain reaction (RT-qPCR) are powerful methods for multiplexed detection and quantification of foodborne bacteria at a species level. Hu et al. (2014) developed a multiplexed qPCR method for detection of serogroup-specific and virulence encoded genes of Shiga toxin used for the detection of foodborne pathogen *E. coli*. As bacterial foodborne pathogens often produce bacterial toxins, for example, *S. aureus* and *E. coli* O157, PCR-based assays frequently target toxin-encoded genes. Interference with PCR chemistry by food components and inability to distinguish viable from nonviable cells remain major limitations of these methods. Selective enrichment or filtration steps and alternative mRNA-based PCR methods have therefore been developed, as is the case for the detection of *L. monocytogenes* (Auvolat and Besse, 2016). Furthermore, a digital PCR (dPCR) approach was used to recount chromosome and plasmid copies in three strains of *B. anthracis* (Straub et al., 2013) where the reaction volume was set to several picoliters to nanoliters. Other nucleic acid amplification techniques for bacterial foodborne pathogen detection are DNA microarray assays, loop-mediated isothermal amplification (LAMP) method, and real-time nucleic acid-based sequence amplification (NASBA). The last one is capable of distinguishing viable from nonviable bacterial cells (Law et al., 2014). In addition, several attempts to combine molecular techniques with analytical instrumentation have been introduced, such as a combination of mPCR and capillary gel electrophoresis with laser-induced fluorescence detection (PCR-CGE-LIF). This method has proved useful for the distinction of closely related serotypes and might therefore be suitable for detection of unknown bacterial foodborne pathogens (García-Cañas et al., 2004). A centrifugal microfluidic device which enables multiplex foodborne pathogen identification by LAMP and colorimetric detection was developed by Oh et al. (2016) and three kinds of foodborne pathogens, namely *E. coli* O157:H7, *S. typhimurium*, and *Vibrio parahaemolyticus*, were detected in a simple and high-throughput manner.

When analyzing the superior properties of “omics” methods for the identification of bacteria and bacterial toxins in food, bacterial genome sequencing proved to be the most mature technological platform for routine surveillance of microbiological risk assessment. Indeed, it enables identification of microbes without any prior knowledge of pathogens and without a

need of preparative labor-intensive cloning steps. It is also useful for detection of noncultivable (anaerobic) organisms present alone, or in combination with other pathogens, directly from food samples in an unbiased and robust high-throughput fashion. The next- and third-generation sequencing (NGS) instruments can be used for the discovery of novel pathogens or identification of variants present at low frequencies. Additionally, NGS can be used for deeper bacterial characterization or analysis of genome reorganization of known bacterial foodborne pathogens, whole genome sequencing (WGS), pathotyping, and drug/antibiotic resistance typing (Franz et al., 2016). The NGS technologies can also be used for complete bacterial communities profiling in food or even for profiling of human intestinal microbes. However, relative quantification remains a limitation for serious integration of metagenomics data with food safety aspects (Gallo and Ferranti, 2016).

Therefore, the band pattern-based pulsed-field gel electrophoresis (PFGE) combined with laborious locus-sequencing methods (MLVA and MLST) remains the “gold standard” DNA fingerprinting method for bacterial isolates of diverse origins. WGS is, nevertheless, quickly expanding as it can be easily used for differential analyses of closely related *Salmonella* serovars (Zheng et al., 2011; Allard et al., 2012; Ranieri et al., 2013; Bakker et al., 2011) and most accurate total foodborne bacterial fingerprinting without time-consuming sample preparation steps (Franz et al., 2016). Since 2013, the US CDC implemented WGS for routine surveillance of all human *L. monocytogenes* isolates and it can be expected that WGS will replace other time-consuming and less precise methods for foodborne outbreak investigation in the near future. It will probably remain only an “SOS” method for unsolved cases where issues such as low-intensity and extended time-period foodborne outbreaks will appear, as it was recently the case with two *L. monocytogenes* outbreaks in Denmark caused by smoked fish consumption (Lassen et al., 2016). WGS will contribute to further development of qPCR or DNA microarrays by providing necessary information about targeting genes, as in the case of *E. coli* O104:H4 strains (Delannoy et al., 2012; Ho et al., 2011; Pritchard et al., 2012; Mothershed and Whitney, 2006). Moreover, it might be applied for mutation rates investigation and identification of genes responsible for occurrence of multidrug-resistant foodborne bacteria, as in analyses of different isolates of methicillin-resistant *S. aureus* (Köser et al., 2012). A comprehensive collection and organization of WGS data matched with geographical locations will certainly speed up comparative phylogenomic analyses and reveal possible pathogenesis biomarkers as well as sources of contamination and causative agents in a higher percentage.

29.3 Gene Expression Analysis—Transcriptomics, Proteomics

Foodomic methodologies comprise a set of high-throughput technologies for analysis of bacterial genome at the DNA, RNA, protein, and metabolic levels. These strategies show great promise in food control, prediction of biofilm generation, and surveillance of foodborne outbreaks (Valdés et al., 2013). Foodomic techniques proved effective in monitoring of foodborne bacteria adaptation and survival parameters in relation to environmental conditions

changes as well as for assessment of strain variability of pathogenic species (Lianou and Koutsoumanis, 2013). Although PCR-based and MALDI-based typing methods are recognized as standard techniques for the detection of foodborne bacteria, final conclusion may be achieved only in combination with viability and gene-expression data and identification of bacterial toxins. Both PCR-based and MALDI-based methods do not provide the data on bacterial behavior and growth on food or on resistance to inactivation and food preservation treatments during production. Accordingly, transcriptomic-based and proteomic-based techniques, such as gene-expression microarrays and mass spectrometry combined with liquid chromatography or electrophoretic separation, are under constant development and optimization to tackle these issues. Until now, the majority of gene-expression studies were conducted on artificially contaminated food matrices or directly on monobacterial cultures or mixtures. For that reason, several different *in vitro* and *in situ* approaches have been implemented (Greppi and Rantsiou, 2016). In particular, gene-expression microarrays have been used for bacterial transcriptomes analyses with great success (Fusi et al., 2011; Valdés et al., 2013). More recently, and along with the development of DNA sequencing approaches and the availability of complete genome sequences of major foodborne bacteria, RNA sequencing is a method of choice for studying of foodborne bacterial pathogens physiological state under a specific set of environmental conditions at the molecular level (Valdés et al., 2013; Tessema et al., 2012; Shen et al., 2016; Schultze et al., 2015). Application of reverse transcription qPCR has also been extensively used in transcriptomic studies for quantifying the expression of specific genes and the discovery of potential stress resistance or cell viability biomarkers (Mataragas et al., 2015). Transcriptomics is ideally coupled to proteomics as proteins, such as bacterial toxins, provide the final evidence on processes occurring in foodborne pathogens. For that purpose, antibody-based analytical devices may be used for bacterial toxin identification. Shlyapnikov et al. (2012) developed such a device and enabled identification of up to five bacterial exotoxins, namely cholera toxin (CT) produced by *V. cholerae*, heat-labile toxin of *E. coli* (LT), and three *S. aureus* toxins, namely enterotoxins A (SEA) and B (SEB) and toxic shock syndrome toxin (TSST). Further innovations in antibody-based methods, such as introduction of nanomaterials and new fluorescent probes for proteins or microfluidic devices, continuously enable additional progress in this approach for the detection of bacterial toxins (Zhu et al., 2014). Newly developed protein–protein microarrays are also an excellent tool to study host–pathogen interfaces (Manzano-Román et al., 2013). However, mass spectrometry (MS) is rapidly gaining popularity in the field of foodomics as a dominant identification technique, especially in the studies of foodborne bacteria and their toxins. This is mainly due to the superior performance in descriptive and quantitative applications and significantly longer half-life of proteins in comparison with mRNA. Protocols relying on different gel-based (one-dimensional and two-dimensional gel electrophoresis) or gel-free (mostly liquid chromatography, but also capillary electrophoresis) separation instruments and enrichment techniques that are coupled to MS proved very efficient for the identification of new peptide/

protein markers that are unique for specific bacterial species, strains, or genera (Martinović et al., 2016). Mott et al. (2010) performed a direct comparison of two most commonly used MS platforms, MALDI-TOF MS and LC quadrupole TOF MS equipped with electrospray ionization (ESI) source for the identification of potential intact protein markers from whole cell acetonitrile lysates of *E. coli*, *Shigella sonnei*, and *S. flexneri*. This study summarizes the advantages and limitations of the evaluated techniques, as well as their great advantage over traditional methods. MS analysis of intact bacterial cellular proteins has been recognized as a powerful tool for bacterial subspecies differentiation. Using a tandem MS (MS/MS) confirmation approach in combination with top-down LC, differences in serovar specific nonsynonymous single-nucleotide polymorphism (SNP) expression protein profiles can be observed even in closely related *Salmonella* serovars, *S. enterica* serovar *Typhimurium* strain LT2 and *S. Heidelberg* strain A39 (McFarland et al., 2014). A similar approach enabled identification of specific peptides from *S. aureus* by fast LC-ESI-orbitrap-MS/MS detection protocol of pathogenic bacteria that were present in some food products (Alam et al., 2012). A MALDI-TOF/TOF MS protocol has been successfully used for the identification of *E. coli* Shiga toxin 2 (Stx2) subtypes in a top-down approach (Fagerquist et al., 2014). In contrast to tandem MS, peptide mass fingerprinting (PMF) approach is often applied for the identification of isolated bacterial proteins after protease treatment. Tsilia et al. (2012) conducted PMF for the detection of enterotoxins CytK1 and NHE produced by pathogenic strains of the *B. cereus* group by using trypsin digests after 1D gel electrophoretic separation of bacterial proteins. Even though several different proteases may be used in MS-based bottom-up experiments in proteomics, trypsin remains the most commonly used enzyme (Giansanti et al., 2016). Protein identification from generated PMF spectra has to be compared to the theoretical spectra of masses based on protease digestion patterns or to theoretical peptide fragmentation spectra. Therefore, identification is highly dependent on the number of well-characterized bacterial protein biomarker sequences available in proteome databases.

Interestingly, MALDI MS technology enables easy and fast acquisition of multiple samples and identification of target proteins with relatively easy operational steps. However, this technique has been increasingly replaced by LC-MS and LC-MS/MS technologies due to the increased proteome coverage and superior automation characteristics. LC-MS and LC-MS/MS are also widely used as an alternative to immunoassays for the detection of bacterial pathogens and their toxins due to low solvent consumption, almost completely automated process, drastically lower costs of analysis, possibility of multiplex bacteria and toxin detection, and further characterization of toxin isoforms. Initial high investments in high-resolution accurate mass (HRAM) mass spectrometry instrumentation and still rather complex instrumentation operation and LC column selectivity optimization steps seem to be the major bottleneck that hampers broader use of this technique. However, once established, LC assays together with adequate reference standards and spectral libraries/databases can be easily implemented as a routine method into public health laboratories.

Comparative proteomics experiments are a valuable source of information in food safety research used for measurement and comparison of complete proteomes or relevant sets of proteins isolated from bacterial monocultures or multicultures after exposure to two different conditions. In particular, comparative proteomics enable identification of specific proteins expressed under well-controlled environmental conditions along with their respective quantities. Results of such a comparative study provided new insights on nisin effects on *L. monocytogenes* cells and its survival strategies in a bacteriocin-containing environment (Miyamoto et al., 2015). In addition, Calo-Mata et al. (2016) compared sequences of two *S. aureus* proteins with publicly available protein databases and LC-ESI-MS/MS data upon in-solution generated peptides of several gram-positive and gram-negative bacteria. They proposed a set of *S. aureus* differentiation proteins, namely cysteine synthase, cysteine proteases staphapain A and staphapain B, and 1-pyrroline-5-carboxylate dehydrogenase, while specific *S. aureus* cysteine protease peptides AQKPVDNITQIIGGTPVVK, TESIPTGNNVTQLK, MTTYNEVDNLTK, YTINVSSFLSK, and KTGSPDYLLHFLEQKV were recognized as potential biomarkers for fast detection of bacterial pathogen without the need for a laborious culture enrichment step. Results of proteomic studies are, however, highly dependent on protein isolation, enrichment protocols, and sample preparation steps for subsequent MS. Challenges arise from the wide dynamic range of bacterial proteins, spanning several orders of magnitude, whose complexity is drastically increased when multiculture or several species are analyzed along with possible food protein contaminations. Moreover, highly abundant proteins in the food matrix may mask specific bacterial proteins and/or peptides with low abundance and it can hamper detection and identification of foodborne bacteria or their toxins. Optimization of sample preparation steps in proteomics and MS research is, therefore, crucial. For this sake, several different extraction methods have been applied in *L. monocytogenes* surface protein profiles studies to avoid equipment-incompatible sample preparation and the bias that can be caused by cytosolic, nontargeted proteins before LC-MS/MS, protein identification, and quantification (Tieng et al., 2015). Several proteomic-based studies have been oriented toward detection and relative or absolute quantification of bacterial exotoxins such as *S. aureus* enterotoxins and *B. cereus* cereulide. For this targeted approach, affinity LC has been implemented as well as highly specific and reproducible multiple reaction monitoring (MRM) by use of a triple quadrupole MS system. Synthetic toxins, as well as stable protein/peptide isotope-labeled standard analog to exotoxin of interest (PSAQ strategy), are used as identification and quantification references, for example, internal standards in such MRM-based methodologies. Zuberovic Muratovic et al. (2014) validated absolute quantitative MRM-based method for cereulide toxin from *B. cereus* in pasta and rice samples by using a synthetic cereulide peptide standard along with ¹³C₆-cereulide. The potential of MRM is in monitoring and quantification of multiple toxins of interest during one sample run, as in the study carried out by Andjelkovic et al. (2016), where online solid phase extraction (SPE), coupled to LC-MRM-MS, and based on a bottom-up proteomic approach enabled detection and quantification of *S. aureus* enterotoxin

A and B. In addition, a promising new data-independent MS^E LC-MS/MS method based on label-free approach has been developed for absolute quantification of *Clostridium difficile* TcdA and TcdB toxins (Moura et al., 2013). Label-free approaches are more feasible when low sample volumes are available. Since no labeled compounds are needed, a proteome-wide quantitative analysis can be accomplished. Similarly, quantification based on metabolic ¹⁵N-labelling or SILAC (Stable Isotope Labeling by Amino acids in Cell culture) may be used for simultaneous processing of sample and reference. In this manner, technical variation during sample preparation can be monitored. SILAC quantification strategies allow comparison of multiplex conditions in a single experiment as well. By use of this method, Ravikumar et al. (2014), successfully quantified 1666 proteins in five phases of *B. subtilis* cellular growth. However, metabolic labeling approaches demand cell culturing alike in a study of Shiga and Shiga-like toxins (Type 1 and Type 2) where minimal medium supplemented with ¹⁵NH₄Cl was used (Silva et al., 2015).

29.4 Proteomics of Food Pathogen Fungi and Mycotoxins

Mycotoxin-producing fungi are among eukaryotic food pathogens with the highest toxicity and, together with bacteria, they account for the majority of cases of food spoilage (Rešetar et al., 2015). Food pathogen fungi belong mainly to *Penicillium*, *Fusarium*, and *Aspergillus* genera, and they are well described in plants and animal feed infections. This is the major exposure route to humans where ingestion of food-containing mycotoxins secreted by these fungal pathogens, most frequently as spoiled eggs or dairy products, may cause severe fungi foodborne diseases (Martinović et al., 2016). Most research has been therefore conducted on analysis of mycotoxins by use of high-throughput metabolomics methods based on LC-MS. Indeed, mycotoxins are present in various food matrices at very low concentrations. This fact requires analytical approaches with both high sensitivity and performance, but also well-defined sample preparation protocols (Martinović et al., 2016). “Dilute and shoot” is the simplest sample preparation technique used for mycotoxin detection, constituting a direct injection of a diluted sample into the LC-MS/MS system (Nathanail et al., 2015; Hickert et al., 2015). However, since this method relies on dilution of the matrix/sample, interferences may occur in the column leading to loss of sensitivity, carryover, and/or ion suppression. The most widely used sample preparation technique for mycotoxin analysis is the SPE, consisting of a chromatographic step used to extract or adsorb one or more components from a liquid phase (sample) onto a stationary phase (matrix). Many mycotoxins in food such as ochratoxin A (OTA), aflatoxins, and various *Alternaria* toxins are analyzed using SPE coupled with LC-MS/MS (Campone et al., 2015; Tölgysi and Stroka, 2014). A miniaturized SPE technique, solid phase microextraction (SPME) that requires only few milligrams of sorbent, can be used for separation and consequent determination of OTA and ochratoxin B (OTB) in food matrices (Saito et al., 2012). Although SPME drastically reduces the amounts of organic solvents necessary for SPE, both approaches often result in targeted substance absorption loss, while the interaction

between the sample and the solid phase can be affected by pH and/or solvent type (Yan and Wang, 2013). Therefore, new sample preparation methods have been developed, namely dispersive liquid–liquid microextraction (DLLME) and QuEChERS (quick, easy, cheap, effective, rugged, and safe), both known for their applicability in a multimycotoxin analysis (Martinović et al., 2016). DLLME, an upgraded version of liquid–liquid extraction (LLE) that uses smaller amounts of solvents and is less time-consuming (Razaee et al., 2006), is used for the separation of many mycotoxins, such as estrogenic mycotoxins, aflatoxin B1, aflatoxin B2, OTA, and the *Fusarium* mycotoxin, zearalenone (Emídio et al., 2015; Wang et al., 2015). QuEChERS, a sample preparation method entailing the extraction of the sample by a solvent, partitioning of water with salts and cleanup using SPE, has been successfully used for simultaneous detection of multiple mycotoxins (Dzuman et al., 2015; Myresiotis et al., 2015; Zhu et al., 2015).

Mycotoxins are still most frequently detected by immunochemical or chromatographic methods. One of standard immunochemical techniques is surface plasmon resonance (SPR) that is based on specific antibodies directed against the target substance (Choi et al., 2011; Atar et al., 2015). Aptamers and single-stranded DNA or RNA oligonucleotides can also be imprinted onto the surface of the SPR chip that can be used for mycotoxin detection (Nguyen et al., 2015; Zhu et al., 2014). Besides SPR, indirect competitive enzyme-linked immunoassay (IC ELISA) may be employed as a rapid and accurate immunochemical screening method (Fu et al., 2015; Zhang et al., 2015). Many other immunochemical methods are available for mycotoxin detection as well, for example, immunoaffinity columns (Abd-Elghany and Sallam, 2015) and fluorescence polarization immunoassays (Beloglazova and Eremin, 2015). The preferred chromatographic method for mycotoxin detection in food is LC-MS, preferably using an MS with an extremely high resolving power and a mass accuracy of less than 5 ppm (Senyuva et al., 2015). Isotope dilution (ID) LC-MS seems to be the method of choice for mycotoxin quantification, relying on internal signal ratios instead of on signal intensities (Ahn et al., 2016; Seo et al., 2015). Developments in mycotoxin analysis have been extensively reviewed by Berthiller et al. (2016).

Recently, proteomic methods have been introduced in fungal research, in particular for the portrayal of genus characteristics and assessment of virulence (Yang et al., 2012; Malavazi et al., 2014). The fungal protein secretome has been investigated by use of proteomic methods, mostly gel-based techniques, that are still widely used (Bianco and Perrotta, 2015). Protein separation on SDS-PAGE, followed by LC-MS/MS analysis, was successfully used for identification of *Aspergillus niger* secretome associated with growth of this fungi (Braaksma et al., 2010), for examination of protein secretion in *A. niger* microsomes upon D-xylose induction (Ferreira de Oliveira et al., 2010) and for the analysis of *Fusarium graminearum* secretome (Ji et al., 2013). A variation of SDS-PAGE, Blue Native PAGE, was used for the identification of *Trichoderma harzianum* secretome (da Silva et al., 2012). Two-dimensional (2D) electrophoresis followed by

MALDI-TOF-MS is also an excellent method for the detection of *Penicillium chrysogenum* extracellular proteins (Jami et al., 2010) while the secretomes of *Aspergillus fumigatus* (Liu et al., 2013), *Phanerochaete chrysosporium* (Manavalan et al., 2011), and *Trichoderma reesei* (Adav et al., 2012) were efficiently quantified by use of a quantitative isobaric tags for relative and absolute quantitation (iTRAQ) approach. Metabolic labeling in cell culture, such as the use of the amino acids that are labeled by stable isotopes (SILAC), an alternative to isotope-assisted quantification labeling methods, is, despite its potential, a rarely used technique for the examination of filamentous fungi (Bianco and Perrotta, 2015). Finally, label-free MS quantification methods were introduced in fungal proteome research, for example, normalized spectra abundance factor (NSAF) spectral counting method was used to determine relative protein quantification in *Uromyces appendiculatus* (Cooper et al., 2007).

29.5 Marine Biotoxins

Marine algae toxins are a heterogeneous group of toxic compounds produced by phytoplankton forming microalgae and they can be metabolized by many enzymatic systems in nature. A possible threat to human health therefore occurs through the consumption of marine food directly or indirectly upon toxin accumulation in marine organisms due to the blooms of toxic algae under favorable environmental conditions (Vilarinho et al., 2013). Indeed, marine biotoxins accumulate in shellfish. Since the natural detoxification of shellfish in a growing area is significantly slower than the toxin accumulation period. If such food is consumed by humans, intoxication may occur due to inappropriate monitoring tools and mechanisms (Ronzitti et al., 2008). So far, major types of algae-related poisonings were classified as amnesic shellfish poisoning (ASP), neurologic shellfish poisoning (NSP), diarrheic shellfish poisoning (DSP), and paralytic shellfish poisoning (PSP) (Nicolas et al., 2014). From a medical point of view, these intoxications are similar to microbiological poisoning by bacteria or viruses from food, even though biotoxin-related symptoms occur fast and usually as early as 30 min up to 4 h after consumption (Hess, 2010). Therefore, development of methods for the detection of marine toxins has been continuously pursued with the aim to provide a means to decrease biotoxin-related food poisoning risks. This is of particular importance due to the globalization of marine food production and marine products trade where unexpected toxin groups may need to be detected. Several authority bodies provided expert opinion on marine biotoxin foodborne disease risk and guidelines on methods for biotoxin identification and characterization (FAO, 2004; EFSA, 2009a,b). Current marine biotoxin classification encompasses eight groups on the basis of their chemical structures, namely okadaic acid and dinophysistoxin (OA group), azaspiracid (AZA), saxitoxin (STX), pectenotoxin (PTX), yessotoxin (YTX), brevetoxin (BTX), cyclic imine (CI), and domoic acid (DA) groups (Li et al., 2016). The mouse bioassay (MBA) is acknowledged as the official reference method for lipophilic

biotoxins (azaspiracids, okadaic acid group toxins, pectenotoxin and yessotoxin group toxins) with some objective pitfalls that include variability in results, insufficient detection capability, and limited specificity. Parallel chemical monitoring tests are therefore suggested in marine biotoxin monitoring that are fast, sensitive, or enable multitoxin detection (Vilarinho et al., 2013). Herein, reference methods are HPLC methods for STX-group toxins and DA, and ELISA methods for determination of the DA. However, a number of alternatives to reference methods have been developed so far, in particular those that tackle the issues of superior separation, identification, and quantification of relevant marine biotoxins in a sensitive and high-throughput manner (Ronzitti et al., 2008; Christian and Luckas, 2008). LC-MS is therefore identified as an analytical method of choice for chemical detection of marine biotoxins and is already accepted as the official reference method for the identification of lipophilic toxins in many countries. LC-MS enables highly sensitive and selective detection of multiple toxins by use of small sample amounts and by simple sample preparation steps. Among available MS technologies, electrospray ionization with triple quadrupole mass analyzer operated in MRM mode provides good targeted identification and quantification of marine biotoxins (Vilarinho et al., 2013). However, such targeted analysis allows detection of known and previously specified biotoxins, while unknown biotoxins remain undetected (Blay et al., 2011). Multiclass screening of marine biotoxins has been successfully achieved using the high-resolution Orbitrap MS system (Blay et al., 2011). Indeed, high-resolution MS instruments, such as Orbitrap, are indispensable tools for confirmatory quantitative analysis of multiple marine biotoxins in complex samples, such as food matrices. The high-throughput method based on QuEChERS strategy and UHPLC-Q-Orbitrap-MS proved reliable approaches for analysis of lipophilic marine biotoxins in shellfish samples, where accurate mass data and good quantification may be achieved (Rubies et al., 2015). Still, there is a lack of tools for complete structural elucidation of all biotoxins analogs within a same chemical group (Vilarinho et al., 2013; Škrabáková et al., 2010). Research is therefore directed to development of even more sensitive and reliable MS-based protocols for discovery, characterization, and quantification of multiple biotoxin sources. A highly specific and sensitive MS-based method has been developed for the determination of azaspiracid analogs in mussel *Mytilus galloprovincialis* tissue extracts, where analogs lacking commercially available standards were successfully assessed (Škrabáková et al., 2010). Specifically, a hybrid LTQ-Orbitrap mass spectrometer was employed for simultaneous nontargeted and targeted analysis of AZA analogs. The ultrahigh-resolution full-scan Fourier transform MS analysis and collision-induced dissociation tandem MS were used for quantitative analysis of contaminated shellfish. In addition, reliability of analyte identity was enhanced with automatic generation of higher energy collision dissociation (HCD) MS/MS spectra (Škrabáková et al., 2010). This is an important achievement as the group of AZA biotoxins induce neurotoxic symptoms along with similar symptoms caused by DSPs and the need for their timely assessment is a prerequisite of modern food control requirements (Nicolas et al., 2014).

However, it should be stated that LC-MS methods do not provide direct estimation of the sample's toxicity potential. Additional approaches and tools will be required to address this issue (Vilaríñó et al., 2013).

In the last decade, proteomic methods were introduced for the investigation of marine biotoxins even though complete genome sequences of some algae and shellfish species have still not been elucidated (Giacometti et al., 2013; McLean, 2013). Proteomic-based studies are used for the characterization of algae proteome where identification of toxicity biomarkers (Giacometti et al., 2013) or biomarkers of biotoxin contamination in aquatic organisms (Zhou et al., 2012) can be readily performed. Dinoflagellates are causative agents of harmful algal blooms along the coastal regions of the world, an ecological event where biotoxins are produced in mass, accumulated in marine organisms, and may thus have a negative impact on human health (Wang et al., 2013a). Therefore, toxicity biomarkers deduced from proteome analysis for different dinoflagellate genera have been pursued (Chan et al., 2006; Wang et al., 2013b). Wang et al. (2013b) compared protein profiles of *Alexandrium catenella*, a neurotoxin-producing dinoflagellate at different toxin biosynthesis stages, and detected 102 significantly altered spots by use of two-dimensional gel electrophoresis (2-DE) coupled to MALDI-TOF/TOF MS. As a result, 53 differentially expressed proteins involved in a variety of biological processes were identified. These findings suggested that the proteins methionine S-adenosyltransferase, chloroplast ferredoxin-NADP + reductase, S-adenosylhomocysteine, adenosylhomocysteine, ornithine carbamoyltransferase, inorganic pyrophosphatase, sulfo-transferase, alcohol dehydrogenase, and arginine deiminase are involved in the biosynthesis of PSP toxins in *A. catenella*. Interestingly, the same proteins were already found in the PSP toxin-producing cyanobacteria. Similarly, proteomic variation and synthesis of PSP toxins in *Alexandrium tamarense* CI01 under different nutrient conditions was investigated by Jiang et al. (2015). Comparative proteomic approach by use of 2D-DIGE (two-dimensional difference gel electrophoresis analysis) coupled to MALDI-TOF/TOF MS identification revealed changes in the amino acid metabolism proteins and photosynthesis during highly toxic periods (Jiang et al., 2015). Proteomics can also be used for the assessment of protein changes occurring in shellfish tissue, such as the identification of proteins involved in shellfish response to biotoxin contamination or products originated from the toxin-producing algae (Ronzitti et al., 2008). Huang et al. demonstrated that the protein profiles in *Perna* considerably changed after exposure to DSP toxins from dinoflagellate *Prorocentrum lima*. Analysis of results based on functional annotation workstation Blast2GO assessment showed that identified proteins are involved in various biological activities, in particular in metabolic processes, cytoskeleton, signal transduction, response to oxidative stress, and detoxification (Huang et al., 2015). Proteomic techniques may also be used for the elucidation of precise marine biotoxins toxicological mechanisms in vitro and in vivo (Giacometti et al., 2013). Proteomic investigation of okadaic acid (OA) acute toxicity on mice intestines revealed that the majority of altered proteins were seriously downregulated after a single oral administration of OA. OA inhibits

the broad-acting protein phosphatase types 1 (PP1) and 2A (PP2A), which can produce hyperphosphorylation of some proteins, leading to deleterious effects on organisms. Two proteins, villin 1 and hnRNP F, were recognized as potential biomarkers for acute OA toxicity due to their involvement in interrupting cytoskeleton reorganization and small intestines cells' apoptosis, which results in diarrhea (Wang et al., 2012). Similarly, Opsahl et al. (2013) analyzed phosphorylation events and translocations after exposure of cells to the OA. Okadaic acid-regulated proteins associated with cytoskeleton and cell adhesion were determined by use of SILAC in combination with LTQ-Orbitrap MS. The authors suggested that OA activates general cell signaling pathways that trigger breakdown of the cortical actin cytoskeleton and cell detachment.

In conclusion, LC-MS/MS is the method of choice for the assessment of lipophilic biotoxins due to high specificity, sufficient limits of detection, and greatest potential to replace animal-based assays. The best results in the assessments of toxicology risks and control of foodborne disease related to marine algae biotoxins can be achieved by use of different assays, including biological and analytical ones. Animal methods provide useful information on toxicity potentially related to the emerging bioactive compounds in marine organisms exposed to different environmental conditions.

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Proteomics in Food Science

From Farm to Fork

Edited by

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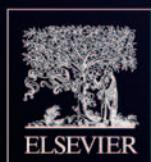
Proteomics in Food Science: From Farm to Fork introduces the current state of play, the predicted trends for the future and elaborates on the promise of proteomics to wide-ranging applications in the food science arena. The application of proteomics to understand changing environmental factors such as drought, flood, salinity and response to abiotic stress, pests, and pathogens is also addressed. The depth of coverage achieved in this book serves as both the starting point for those wishing to embark on proteomics studies in the food sciences and enables those in one food discipline to become familiar with the concepts and applications of proteomics in other disciplines of food science.

Key Features

- Addresses quality issues such as the elucidation of quality traits for a wide variety of foods including meat, fish, dairy, eggs, wine, beer, cereals, legumes, nuts, and fruits.
- Describes a variety of analytical platforms, ranging from usage of simple electrophoresis to more sophisticated mass spectrometry and bioinformatic platforms.
- Includes the identification and characterization of bioactive peptides and proteins, which are important from a nutritional perspective.
- Covers a range of methods for elucidating the identity or composition of specific proteins in foods or cells related to food science, from edible components to spoilage organisms.

About the Editor

Dr. Michelle L. Colgrave is the molecular analysis team leader and proteomics research scientist in CSIRO Agriculture and Food, based at the Queensland Bioscience Precinct in Brisbane, Australia. She employs mass spectrometry and proteomics to help identify key proteins that will benefit Australia's livestock and plant industries and improve human health. Dr. Colgrave is working to identify novel proteins and characterize their function and posttranslational modifications.



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