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3 Protein Processing in Food and Bioproduct Manufacturing and Techniques for Analysis

Joyce Irene Boye and Chockry Barbana

3.1 INTRODUCTION

Proteins and amino acids are the building blocks of life. They play a critical role as components of blood, muscle, enzymes, hormones and hormone receptors. In addition to their biological role in sustaining the functions of living organisms, proteins also play important roles in foods providing taste, texture and flavor, which are essential criteria for food selection. Due to their versatile functionality and complex molecular structure, proteins have also been explored in many industrial applications. Some recent examples include adhesives, protein plastics, gels, coating, additives and biomaterials. Protein scaffolds and cross-linked networks also hold much promise for use in the pharmaceutical industry.

In nature, proteins exist as components of biological matrices with other compounds such as lipids, carbohydrates, minerals, and other minor components. This composite existence is ideal in foods, as it allows various sources of nutrients to be supplied at the same time. In some instances, however, there are distinct advantages in separating protein fractions in order to obtain enriched or purified fractions for specific food, nutraceutical or industrial application. Thus, for sports nutrition as an example, there may be the need for highly enriched protein beverages that quickly help to build and restore muscle. Engineering of protein scaffolds for pharmaceutical applications may require the use of highly purified proteins as raw materials for production. Preparation of simulated meat products using plant proteins as meat analogs may also require the use of highly purified protein sources.

This chapter provides an overview of the properties of food proteins and details some of the techniques currently used for the extraction and purification of proteins from different food sources. Protein processing for human food use is emphasized. However, most of the described processes are also applicable for feed and other industrial and pharmaceutical applications. As the purity and quality of extracted proteins have to be frequently assessed to determine their suitability for different applications, the chapter also provides a summary of some of the principal techniques used for analyzing the properties of proteins in food and bioproduct manufacturing.

Food and Industrial Bioproducts and Bioprocessing, First Edition. Edited by Nurhan Turgut Dunford.

3.2 GENERAL PROPERTIES OF PROTEINS

Proteins are comprised of amino acids linked by peptide bonds (Figure 3.1). A list of the 20 amino acids most commonly found in nature is presented in Table 3.1. Other rare amino acids found in nature include ornithine, citrulline and cycloleucine. Amino acid exists in the D or L conformation depending on their rotatory optical activity (Figure 3.2). Most amino acids found in nature exist in the L conformation.

Proteins have complex molecular structures. The linear sequence of the amino acids comprising a protein is classified as its primary structure. In different proteins, these linear sequences assume conserved structures along the axis of the polypeptide in the form of alpha-helixes, 3_{10}-helix, beta sheets, or random coils, turns which are described as the secondary structure of the protein. These secondary structures are stabilized primarily by hydrogen bonds. For thermodynamic stability, proteins rearrange themselves into tertiary structures comprising several secondary structures stabilized by van der Waal's, electrostatic, or hydrophobic interactions, hydrogen bonding, as well as disulfide cross-links. Some proteins have a fourth structural level called the quaternary structure in which two or more

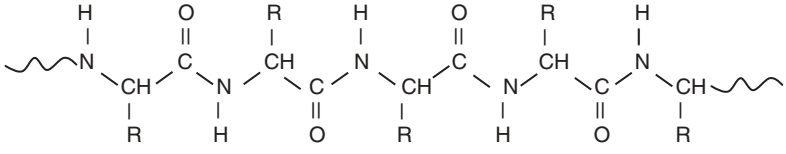

Figure 3.1 Amino acids linked by peptide bonds.

Table 3.1 The 20 amino acids most commonly found in nature and their characteristics.

Amino Acid	Abbrev.	Letter	Type	MW
Alanine	ala	A	Aliphatic	89.1
Arginine	arg	R	Basic	174.2
Asparagine	asn	N	Amidic	132.1
Aspartic acid	asp	D	Acidic	133.1
Cysteine	cys	C	Sulfur containing	121.1
Glutamine	gln	Q	Amidic	146.1
Glutamic acid	glu	E	Acidic	147.1
Glycine	gly	G	Aliphatic	75.1
Histidine	his	H	Basic	155.2
Isoleucine	ile	I	Aliphatic	131.2
Leucine	leu	L	Aliphatic	131.2
Lysine	lys	K	Basic	146.2
Methionine	met	M	Sulfur containing	149.2
Phenylalanine	phe	F	Aromatic	165.2
Proline	pro	P	Aliphatic	115.1
Serine	ser	S	Hydroxylic	105.1
Threonine	thr	T	Hydroxylic	119.1
Tryptophan	trp	W	Aromatic	204.2
Tyrosine	tyr	Y	Aromatic	181.2
Valine	val	V	Aliphatic	117.1

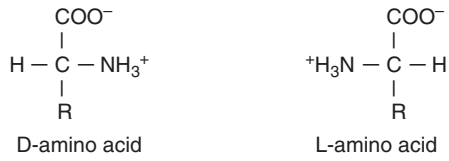

Figure 3.2 Conformation of D and L amino acids.

monomers, sometimes called "subunits", associate to form complex structures stabilized by non-covalent bonds.

Amino acids may be classified as acidic, basic, sulfur containing, hydroxylic, aliphatic, hydrophobic or amidic (Table 3.1). The number and type of amino acids in a given protein determine, respectively, its size and net charge at different pHs. Proteins vary in molecular mass, ranging from <10 000 Da to >1 million Da, depending on the number of amino acids contained within the protein. Environmental conditions, such as pH and the presence of salts, can alter the charge state of amino acid residues and, consequently, the net charge of the protein. The isoelectric point of a protein is defined as the pH at which the net charge on the protein is zero. These two properties of proteins (i.e. charge and size) play a critical role in the technologies and processing conditions used for their separation.

Another property of food proteins that is of interest in processing is their solubility. Osborne (1924) classified proteins based on their solubility as follows: water soluble proteins (albumins), salt soluble proteins (globulins), alcohol soluble proteins (prolamins) and acid and alkali soluble proteins (glutenins). The solubility of proteins under aqueous conditions can be further modified by heat treatment, hydrolysis and the presence of protein structure perturbing chemical reagents. Depending on the extent of heat treatment, proteins can unfold, exposing more hydrophilic amino acids, which can enhance solubilization. In contrast, harsh heat treatment can result in extensive denaturation, association and irreversible aggregation, which can reduce protein solubility. Hydrolysis of proteins using chemical agents, such acid or alkali or enzymes, can break up proteins into smaller fragments, which can increase their solubility.

3.3 PROTEIN SEPARATION PROCESSES IN FOOD AND BIOPRODUCT MANUFACTURING

Proteins can be processed to obtain enriched flours, concentrates or isolates for various food and bioproduct applications. In this chapter, the term bioproduct is used to indicate functional foods, nutraceutical products, cosmetic products and other industrial (non-food) products.

In general, protein flours may contain up to 65% w/w protein on a dry basis (db), whereas concentrates and isolates contain >65% (w/w, db) and >80–90% (w/w, db) protein, respectively. Microbiological and quality standards will vary for different applications.

Proteins used in the food and bioproduct sectors are derived from either animal or plant sources, which vary significantly in their lipid, carbohydrate and protein content. Proteins of interest may be separated using dry or wet processing techniques. Dry processing primarily involves air classification and is frequently applied to plant materials such as cereals (e.g., wheat) and grain legumes (e.g., peas, chickpeas, lentils) containing high amounts of starch and protein. Wet processing has several processing steps. Some of the major unit

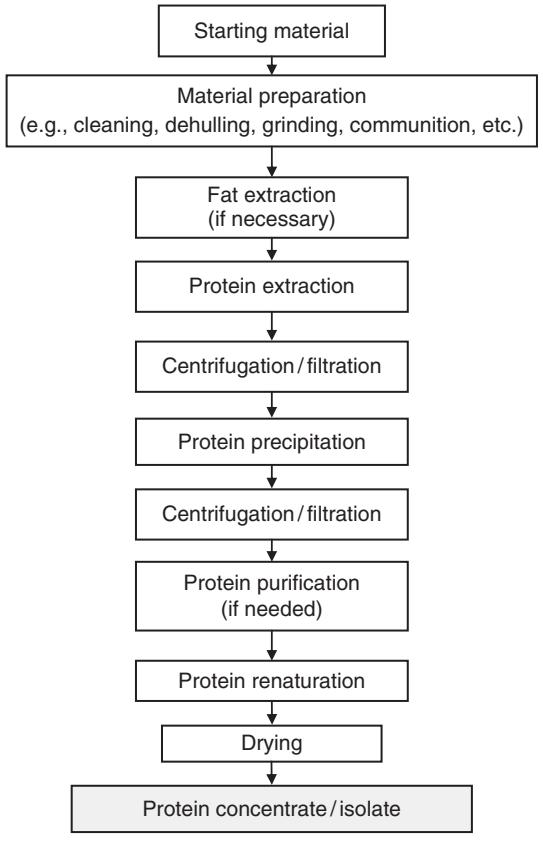

Figure 3.3 General schematic showing some key unit operations for the wet processing of proteins.

operations used for the wet processing of proteins are presented in Figure 3.3. Deviations to these operations occur depending on the starting material and the desired end product. The material preparation step for wet processing will, therefore, vary depending on the source material.

3.3.1 Dry processing

3.3.1.1 *Air classification*

Air classification is a milling technique that allows the fractionation of plant grains/seeds into high starch and high protein flours. The milling process yields flours having particles of two discrete sizes and densities. Air classification exploits this phenomenon to separate the light fine fraction (protein) from the heavy coarse fraction (starch).

During air classification, whole or de-hulled seed is ground into very fine flour, and the flour is subsequently classified in a spiral air stream to separate the starch from the protein. Repeating the process a few times improves separation efficiency, as protein bodies can still adhere to the surface of starch granules after the initial run. Agglomerates present in this initial starch fraction consist of starch granules embedded in a protein matrix, but by repeated pin milling and air classification, further purification can be obtained (Gueguen *et al*., 1984).

For optimal separation efficiency, the cotyledons of seeds must be finely ground to achieve complete cellular disruption and maximum protein and starch separation during air classification (Tyler and Panchuk, 1982). The milling technique used must, therefore, be capable of producing a very fine grind, yet selective enough to break up cells and cell fragments without severely damaging the starch granules (Jones *et al*., 1959). A portion of the protein derived from the membranes and stroma of the choloroplasts in which the starch granules developed cannot be milled free of the starch granules despite repeated milling (Tyler, 1984). The purities of the coarse (starch) and the fine (protein) fractions obtained by air classification are, therefore, often lower than what may be obtained by wet extraction.

Amongst others, high protein flours (up to 75% purity in some cases) have been successfully produced from wheat, soybean, beans, lentils, chickpeas and peas using air classification (Wu and Strongfellow, 1979, 1981; Tyler and Panchuk, 1982; Wolf *et al*., 2002).

3.3.2 Wet processing

3.3.2.1 *Material preparation*

Animal by-products

Animal by-products (e.g., blood, skin, bones and offal) may serve as useful sources of biomass for harvesting high-value proteins. Prior to extraction, proteins are solubilized by disruption of the cells and tissues retaining them. Protein solubilization is a major critical step affecting yield and quality of the extract. Methods available for tissue disruption include grinding, homogenization and sonication. Mechanical disruption using colloidal mills, in which the biomass is fed through a rotor-stator, high speed dispersion mills or bead mills, which break down cell walls through their tumbling action, may also be used. In some instances enzymes (e.g., zymolase, lysozyme, and lysostaphin) may be added to facilitate tissue disruption followed by homogenization, sonication or vigorous vortexing.

Equipment selection will depend on the specific material being processed and the scale (analytical, pilot or industrial). As animal materials frequently contain proteases that can hydrolyze proteins of interest and decrease their functionality, appropriate precautions are required to slow down or prevent these reactions (e.g., thermal inactivation or processing at cold temperatures). After the appropriate level of communition has been attained, a filtration step can be used to recuperate the supernatant containing the desired proteins for further downstream processing.

Plant by-products

Plants contain high amounts of fiber and cellulosic material that needs to be removed prior to protein extraction. Some plant materials are also high in fat, making a defatting step necessary prior to protein extraction. Effectively disintegrating plant materials to obtain particle sizes that allow for maximum fat and protein extraction is an important first step in processing. This is best done by beginning the processing with a milling step. Examples of milling equipment used in research and in industrial settings include centrifugal mills, hammer mills, ball mills, roller mills and disc attrition mills. De-hulling and milling can be done as a single unit operation or as two separate unit operations depending on the type of equipment used and the material being processed (i.e., ease of de-hulling).

Materials such as soybeans seeds, which are high in fat (17–23%), may require a defatting step. The techniques most commonly used are solvent extraction using hexane, mechanical extraction and aqueous extraction. Details of these processes are summarized elsewhere

(Mustakas, 1980; Serrato, 1981). The material obtained for further downstream processing will vary depending on the process used and, in the case of soybean, could be a full fat soybean meal or flour, defatted meal (untoasted, mildly toasted or fully toasted). The toasting process refers to the heat treatment applied to remove residual solvent after hexane extraction. Both mechanical and solvent extraction techniques have their limitations (Russin *et al*., 2010). Mechanical extraction, or pressing, is limited in its applicability, particularly with low oil content oilseeds such as soybean. Elevated temperatures employed during pressing can also have a deleterious effect on the quality of the extracted oil and residual meal (Sugarman, 1956; Nelson *et al*., 1987). Hexane extraction has many economic, environmental and safety limitations. Economically, one of the main concerns is the stability of both hexane supply and price due to fluctuation in the fossil fuel market (Friedrich and List, 1982; Lusas *et al*., 1990; Gandhi *et al*., 2003; Russin *et al*., 2010). Concerns also exist about the environmental impacts of hexane use and its toxicity.

As an alternative, aqueous techniques for fat extraction are being explored. In this case, after communition the full fat soy material is solubilized to perform a solid–liquid extraction/separation. During this step, insoluble compounds are removed, leaving a liquid solution containing both proteins and lipids. This solution is further separated by three-phase centrifugation to yield a solid, an aqueous and an oil/emulsion phase, each of which can be further processed downstream (Russin *et al*., 2010). Enzymes (e.g., lipases, cellulases) may be added during this process to breakdown fat and carbohydrate components to facilitate protein extraction (Russin *et al*., 2010). The main principle of enzyme-assisted extraction is the use of enzymes which damage and/or degrade plant cell walls, so increasing the permeability of the oil in the oilseed (Domínguez *et al*., 1994). The two main approaches include the use of single and mixed enzymatic systems. The latter has increased utility, given that the mixed systems allow for various enzymes to simultaneously act on the cellular structures, leading to a more effective release of oil (Fullbrook, 1984; Domínguez *et al*., 1993; Russin *et al*., 2010). The use of lipases or phospholipases to breakdown fats, particularly in high fat aqueous extraction systems where emulsions are likely to occur, is of interest; however, adequate care must be taken to minimize oxidation during processing, as this could result in the generation of off-flavors in the finished product.

3.3.2.2 *Protein extraction*

Alkaline extraction

Aqueous alkaline extraction is one of the most commonly used techniques for protein extraction; it takes advantage of the solubility of proteins at alkaline pH. In this process the prepared biomass, which may be full fat, partially defatted, or fully defatted, and in the case of plant materials may or may not contain fibrous materials such as hulls, is dispersed in water using flour:water ratios ranging from 1:5 to 1:20. The pH of the mixture is adjusted to alkaline (pH 8–11) and the mixture is continuously stirred for 30 to 180 min to maximize protein solubilization. During this time the pH is maintained at the desired value and the temperature may be elevated (up to 55–65 °C) to further enhance protein solubilization and extraction. The mixture is subsequently filtered to remove any insoluble material to yield a supernatant containing the extracted proteins. Some extraction processes call for a second extraction of the precipitate using similar pH as in the first extraction or higher in order to extract any remaining proteins in the precipitate and increase protein recovery (Boye *et al*., 2010a).

Lawhon and co-workers (1981a) found the extractability of protein in aqueous medium (water) from full fat soy flour to be higher when using higher flour to water ratios (i.e. 1:30

or 1:25 soy flour to water ratio by weight) than when using lower ratios (i.e., 1:12 or using a double extraction at 1:10 followed by 1:6). In the former case, extraction at 60 °C for 30 min at pH 6.6, 8.0 and 9.0 gave nitrogen recoveries of 91.4, 94.2 and 89.3%, respectively. In the latter case (1:12 flour to water ratio) the extraction was done at pH 9.0 and a recovery of 80.4% nitrogen was obtained, whereas for the double extraction (1:10 followed by 1:6) at pH 2.5, 81.8% nitrogen recovery was obtained. The authors indicated that water ratios influenced nitrogen extractability more significantly than pH.

pH and temperatures used during alkaline extraction must be carefully chosen to avoid extensive denaturation as well as the development of by-products, such as lysinoalanine (N6-(DL-2-amino-2-carboxyethyl)-L-lysine), an unusual amino acid implicated as a renal toxic factor in rats. Lysinoalanine has been found in proteins of home-cooked and commercial foods and ingredients and was initially thought to occur in both edible and non-food proteins only after alkali treatment. However, some reports have shown that it can be generated in various proteins when heated under non-alkaline conditions (Sternberg *et al*., 1975).

Acid extraction

The solubility of some proteins increases under acidic conditions (i.e., pH <4). This low pH range can, therefore, be used to solubilize proteins prior to their recovery. The principle of acid extraction is similar to that of alkaline extraction, except that the initial protein extraction is conducted under acidic conditions. The acid extraction technique is generally used less frequently than the alkaline extraction technique and, as with alkaline extraction, processing conditions can influence the yield and purity of the finished product. Using this technique, Alli and co-workers (1993) extracted a bipyramidal crystalline protein preparation from dried seeds of white kidney bean (*Phaseolus vulgaris*) by extracting ground seeds with citric acid solution (0.4 N, pH 4.0) followed by refrigeration (4 °C, 18 h) to precipitate the protein material. The isolate obtained contained 95.7% protein.

Salt extraction

The selective extraction of proteins in aqueous solutions having different ionic strengths can be used for their fractionation and separation. The process is based on the salting-in and salting-out phenomenon of food proteins.

At low molarities (0.5–1 M), ions of neutral salts promote the solubilization of proteins ("salting in"). Interactions between the ions and charges of proteins reduce electrostatic attractions between protein molecules enhancing their solubility. Additionally, the hydration of the ions increases the solvation of the proteins contributing to increased solubility. At higher salt concentrations (>1 M), competition between salts and proteins for available water forces the proteins to precipitate ("salting out"). This phenomenon can, therefore, be used for protein recovery as explained later.

For salt soluble globulins as per the Osborne classification, addition of salts to the extracting medium facilitates their solubilization. After extraction, extensive dilution of the solution can cause these globulins to precipitate out of solution especially at low temperatures leading to their fractionation.

Foam fractionation of proteins

Foam fractionation (or separation) is an adsorptive bubble separation technique in which soluble, surface-active substances can be removed from solution by preferential adsorption at the gas–liquid interface (Wang and Liu, 2003). Proteins contain both hydrophilic and hydrophobic amino acid residues that are surface active. During foam formation, bubbles

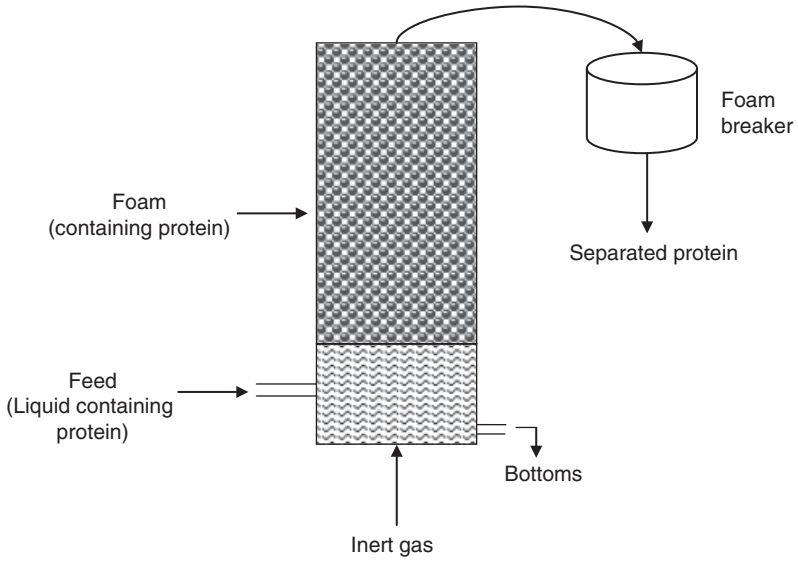

Figure 3.4 Schematic process for foam fractionation.

are created as a result of the passage of gas through a liquid medium. Proteins with greater surface activity orientate themselves and are capable of being adsorbed at the gas–liquid interface created during bubble formation. A schematic of a typical foam fractionation set up is presented in Figure 3.4. Bubbles leaving the surface of the feed solution carry both adsorbed compounds and bulk liquid into the foam; as the interstitial liquid drains back along the lamella of the bubbles, non-adsorbed solutes are returned to the retentate leaving an enriched foam (Wang *et al*., 2009). The remaining liquid contained in the gas phase is, thus, selectively enriched with protein having greater surface activity compared to the proteins retained in solution.

Foam fractionation is a relatively inexpensive technique for protein separation. Most studies in the literature are, however, experimental and very few report on industrial applications. Among other applications, foam fractionation has been used to separate wheat flour proteins, ovalbumin, lysozyme, egg albumin, milk proteins (e.g., beta-casein, bovine lactoferrin, bovine serum albumin, alpha-lactalbumin, and beta-lactoglobulin) and potato protein from potato juice waste water after starch extraction (Weijenberg *et al*., 1978; Keller *et al*., 1997; Hossain and Fenton, 1998; Brown *et al*., 1999; Wang and Liu, 2003; Wang *et al*., 2009).

Parameters influencing efficiency of protein separation include bubble size distribution, bubble rupture and coalescence, fluid drainage and rate of bubble formation. Separation ratio (SR) defines the ratio between the protein concentration in the foam and that in the starting solution (feed). Separation efficiency (SE), on the other hand, is the product of the flow rate of the foam phase and protein concentration in the foam divided by the product of the flow rate of the liquid effluent and protein concentration of the effluent. Separation ratio is affected by the feed protein concentration (FPC), feed flow rate (FFR), gas velocity (GV) and the height of sampling (HS). For a given superficial GV, SR decreases with an increase of FPC (Wang and Liu, 2003). Additionally, the averaged protein concentration in the foam decreases with increasing GV; SR decreases with increasing FFR. Increasing the gas to feed flow rate ratio (G/F) decreases SR up to a critical G/F beyond which a plateau is reached.

SE on the other hand increases with increasing G/F as it enhances the amount of protein molecules transferred from the liquid to the foam phase. At high gas to feed flow rate ratios, however, there is greater likelihood of bubble burst. Additionally, Hossain and Fenton (1998) reported higher enrichment and protein recoveries with continuous processing compared to semi-batch mode.

Wang and co-workers (2009) successfully used foam fractionation to separate lysozyme from chicken eggs and reported that separation efficiency was significantly influenced by pH and sodium chloride concentration. Activity recovery and protein recoveries were highest (40% and 60%, respectively) at pH 7 and 0.3 M NaCl concentration. Addition of sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide, Triton-X and Tween 80, resulted in protein recoveries of 93%, 88%, 68% and 72% and activity recoveries of 30%, 20%, 55% and 58%, respectively.

Gas flow requirements to produce stable foams were found to be lower for bovine serum albumin and beta-lactoglobulin, which had greater surface activity compared to beta-casein and alpha-lactalbumin, and the enrichment factor and percentage volume loss in foam were also higher for the former proteins (Hossain and Fenton, 1998).

Matouq (2008) compared the separation of proteins from yogurt whey and cheese whey. Less foam formation occurred with cheese whey compared to yogurt whey and higher enrichment values were obtained with yogurt whey, although the cheese whey contained higher protein content. As higher concentrations of fat tend to decrease foam formation, the author concluded that the higher fat content of the cheese whey may explain the decreased foam formation and stability.

Various workers have explored ways to enhance the SE of the foam fractionation by using adsorptive ligands, such as Cibacron blue of a triazine dye bound with a polyethylene glycol, kaolin, iron oxide, graphite and SDS (Keiichi *et al*., 1998; Yoshihiro and Toshiro, 2000; Suzuki *et al*., 2002).

Yoichiro (1986) proposed continuous countercurrent foam separation equipment in which samples are introduced into the middle portion of a gas–liquid dual countercurrent flow system. Material having an affinity to the foam is carried with the foam stream whereas other materials are carried with the liquid stream.

As conditions used during foam fractionation can change the molecular structure of target proteins and, consequently, protein function, it is important that these conditions be selected carefully. Changes can occur due to the direct effects of ligands used, unfolding of proteins at the gas–liquid interface, high shear stress rates and chemical damage due to oxidation (Maruyama *et al*., 2007).

Extraction using reverse micelles

Proteins can be extracted from solution using liquid–liquid separation techniques, also known as reverse micellization. The technique involves the use of a biphasic liquid system comprising an aqueous solution of the protein and an organic micellar solution containing monodisperse aggregates of surface-active molecules, usually ranging in diameter from 1 to 10 nm (Asenjo and Chaudhuri, 1996). Shaking of the two solutions results in partitioning of proteins from the aqueous into the micellar phase (Figure 3.5). Synthetic protein chaperones can be added to protect proteins from extensive denaturation and to aid in renaturation especially for pharmaceutical type applications.

A solid state extraction version of the method involves suspension of protein powder in the micellar phase followed by gentle stirring to allow the selective separation of the target proteins into the organic micellar solution.

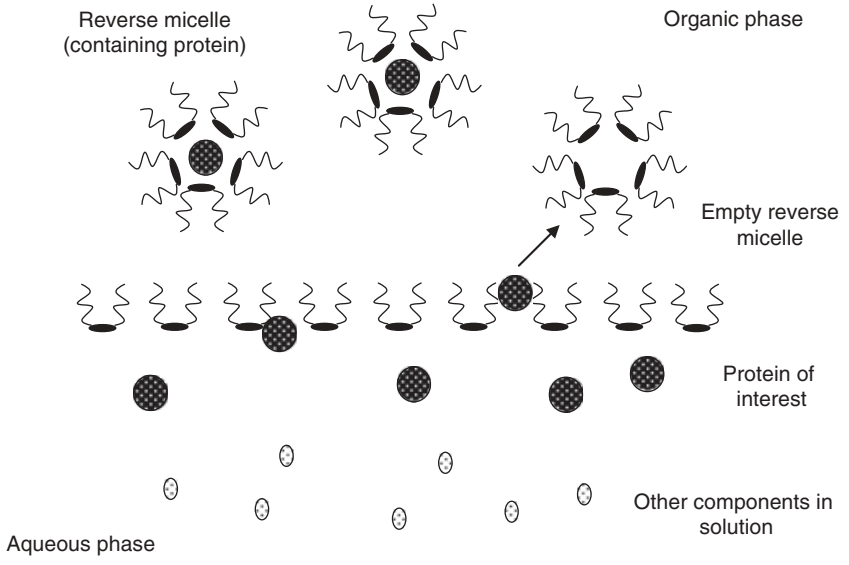

Figure 3.5 Schematic representation of the extraction of proteins using reverse micellization.

Protein solubilized in the reverse micellar solution can be transferred back into an aqueous solution by contacting the micellar solution with an aqueous solution containing a high concentration of salt (e.g., KCl, $CaCl_2$), which has the capability to exchange with protein in the micelles (Asenjo and Chaudhuri, 1996). Reverse micellization has been successfully used to separate a variety of proteins including enzymes (e.g., lysozyme, trypsin and ribonucease).

3.3.2.3 *Protein recovery*

Isoelectric precipitation

Most proteins precipitate at pH values close to their isoelectric point. This property can be used to selectively precipitate different proteins from solution. As most food proteins have their isoelectric point in the range pH 4–5, this pH range is frequently used for protein recovery in the food processing industry. Typically, after alkaline, acid or salt extraction, the pH of the protein extract is adjusted to the desired isoelectric point to induce protein precipitation, followed by centrifugation to recover the protein, washing to remove salts, neutralization and drying.

Salting out

Water from the hydrated protein is removed at high enough neutral salt concentrations (>1 M), leaving the exposed proteins to interact with each other through hydrophobic interactions. This results in protein aggregation and precipitation, a phenomenon known as "salting out". The Hofmeister series promote salting out, aggregation and stabilization or unfolding, dissociation and salting in depending on the ascending or descending order of the ions: $SO_4^{2-} < F^- < CH_3COO^- < Cl^- < Br^- < NO_3^- < I^- < ClO_4^- < SCN^-$, $NH_4^+ < K^+ < Na^+ < Li^+ < Mg^+ < Ca_2^+$ (Cheftel *et al*., 1985). Ammonium sulfate is frequently used for the selective precipitation of proteins because it is relatively inexpensive and has high solubility. Other salts used in protein applications are magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$).

Achouri and co-workers (2010) reported that initial extraction of soy proteins using isoelectric precipitation at pH 6.8 followed by cryo-precipitation yielded 4.2% product recovery for the 11 S soybean protein fraction with 98% protein purity for a control extracted with sodium hydroxide, and average yields of 4.4% and 5.17%, respectively, when sodium sulfate (Na_2SO_4) and ammonium sulfate [$(NH_4)_2SO_4$] were used. Addition of calcium chloride ($CaCl_2$) doubled the extraction yield to 9%.

As some salts may be toxic, an appropriate salt must be selected for the target end-product application (industrial, food, pharmaceutical). The type of proteins precipitated in a given solution will also vary as a function of the salt concentration used.

In a modified version of the salting out process, salt proteins may be preferentially separated out of solution by dilution of the protein extract. In this case, after extraction of protein using an appropriate salt solution at desired ionic strength, the solution is extensively diluted to decrease the solubility of the salt soluble proteins, inducing protein precipitation.

Paredes-López and co-workers (1991) extracted protein from a 10% (w/v) solution of defatted chickpea with sodium chloride (0.5 M, pH 7.0) and obtained a chickpea protein isolate containing 87.8% protein using this method. After concentration of the extract by ultrafiltration, protein was flocculated by the addition of water (4 °C, pH 7, 1:4 v/v ratio of protein extract: water). Márquez and co-workers (1996) reported protein contents ranging from 74.7 to 84.2% for common bean protein extract using similar methods.

Microfiltation and ultrafiltration

Microfiltation (MF) and ultrafiltration (UF) are pressure-driven filtration processes that utilize a porous membrane to selectively retain compounds larger than a nominal molar mass (retentate) while allowing particles of lower molar mass to pass through the membrane (permeate). It is a frequently used alternative to isoelectric precipitation.

During this process, solutions containing extracted or dissolved proteins are subjected to MF and/or UF to concentrate the proteins. MF membranes retain larger molecular weight compounds and are better suited for retaining particles with a molecular mass greater than 300 000 and in the 0.02–10 micron size range. They are, therefore, more useful for the separation of cellular material, such as in biological, biochemical, pharmaceutical and nutraceutical applications. A useful application of MF in food processing is the removal of yeasts and bacteria without the requirement for thermal treatment. In protein purification work, MF could be used to specifically retain larger molecular weight proteins of interest, or to remove these interfering proteins. The permeate can be further processed to obtain a higher purity protein extract.

UF membranes retain smaller molecular mass particles (300–300 000) with sizes ranging from 0.001 to 0.02 microns and are frequently used in protein processing. UF membranes are typically constructed from regenerated cellulose, cellulose acetate, ceramic composites, polysulfone, polyethersulfone, polyamide, polyacrylonitrile or polyvinyl alcohol. The molecular weight cut-off of ultrafiltration membranes is diffuse by about one order of magnitude, which makes it difficult to achieve absolute retention. Thus, a UF membrane with a molecular weight cut-off of 200 000 could retain particles as small as 20 000 molecular mass. In addition to molecular mass the shape of particles will also influence their retention or permeation. Membranes with specific molecular weight cut-offs must be carefully selected in order to retain the desired protein of interest. Other factors affecting efficient separation of proteins by microfiltration or UF include the type of membrane, the molecular weight cut-off, the volume concentration ratio and diafiltration conditions. A major problem

with membrane separation processes such as MF and UF is fouling of the membranes during processing, as this can affect membrane selectivity.

MF and UF have been used extensively to separate proteins from milk, soybeans, peas, chickpeas, lentils amongst others (Romero-Baranzini *et al*., 1995; Cheryan, 1998; Fredrikson *et al*., 2001; Boye *et al*., 2010a).

Cryo-precipitation

The solubility of some proteins decreases significantly as the temperature is reduced below room temperature, a property that can be selectively used for their recovery. Cryo-precipitation refers to the method of protein precipitation under refrigerated conditions or from freezing and cold thawing.

Melcher and Fraij (1980) successfully used cryo-precipitation at –20 °C to separate a zein II extract obtained from corn meal into a methionine-rich polypeptide fraction (cryo-precipitate) and a cryo-supernatant containing predominantly polypeptides with amino acid composition similar to that of the zein 1 proteins. The cryo-precipitate was readily dissolved in 70% ethanol containing mercaptoethanol and precipitated again on cooling, indicating that the cryo-precipitaion was reversible.

In medical terms, a cryo-precipitate is defined as a concentrate of high molecular weight plasma proteins which precipitate in the cold (Pantanowitz *et al*., 2003). Various researchers have used the technique to recover cryoglobulins, a heterogenous group of immunoglobulins (monoclonal and polyclonal) which precipitate or gel at reduced temperature (e.g., 4 °C) and redissolve on heating to 37 °C (Weber and Clem, 1981; Wang, 1988). The purified proteins exhibit crystalline, amorphous or gelatinous structure as a result of their differing primary structure and physicochemical properties. All major immunoglobulin classes (IgM, IgG, IgA) and their subclasses have been found in cryo-precipitates, including monoclonal immunoglobluins, mixed immunoglobulins and polyclonal immunoglobulins. Other proteins found in plasma that are insoluble at cold temperatures include cryofibriniogen, heparin precipitable protein and aggregates of C-reactive protein and albumin (Stathakis *et al*., 1978; Shanbrom, 1980; Wang, 1988; Coelho and Wolf, 1991).

Francis and co-workers (2000) found that frozen saliva samples contained variable amounts of precipitate on thawing. The cryo-precipitates comprised of low molecular weight proteins (<14 KDa), which were either absent or present at reduced concentrations in the supernatant.

Cryo-precipitation is, therefore, a promising technique for the recovery of high value proteins and is a technique that is likely to be increasingly explored in the food, nutraceutical and pharmaceutical sectors.

Electrodialysis using bipolar membranes

Proteins can be preferentially precipitated from solution using a technique known as electrodialysis coupled with the use of bipolar membranes. The application is based on the specific properties of electrolysis (redox reactions at the electrodes) coupled with membrane action (Bazinet *et al*., 1998) resulting in either electro-acidification or electro-alkalinization. A bipolar membrane is a composite membrane consisting of a cation exchange layer and an anion exchange layer which can split solvents (water and methanol, so far) into H^+ and OH^-/CH_3O^- at the interface under reverse potential bias (Xu and Huang, 2008).

For a solution circulated into an electrodialysis cell on the cationic side of the bipolar membrane, where the H^+ are generated, the pH of the solution will decrease. Similarly, a solution circulated on the anionic side of the bipolar membrane, where OH^- are generated,

will experience an increase in pH (Bazinet *et al*., 1998). During processing, protons generated by the bipolar membrane come into contact with proteins circulating in the electrodialysis cells on the cationic side of the bipolar membrane. At the isoelectric point when the net charge on the protein is zero, the proteins aggregate/precipitate allowing for their selective separation. Proteins can be subsequently recovered by centrifugation or filtration as is done in other conventional processes for protein separation.

Using this technique, Bazinet and co-workers (1998) lowered the pH of a soy protein solution from 8.0 to 4.5 in a cell of 100 cm^2 effective electrode surface, at a constant current of 25 mA/cm^2 and obtained a precipitate with 95% protein purity. Mondor and co-workers (2004a, 2004b) also successfully applied bipolar membrane electrodialysis to separate soy proteins.

Application of bipolar electrodialysis for the simultaneous removal of inhibitory acetate and pH control during *E. coli* fermentation was investigated by Wong and co-workers (2010). The final biomass and recombinant protein concentrations obtained increased by up to 37 and 20%, respectively.

There has been limited application of bipolar membrane electrodialysis for protein separation at the commercial scale due to cost, complexity of use and lack of availability of units for large scale processing.

Protein precipitation using organic solvents

Organic solvents such as acetone and ethanol can be used to induce protein precipitation. Similar to the salting-out phenomenon, addition of organic solvents removes water from the hydration spheres of the protein allowing electrostatic forces to bring oppositely charged regions of the protein together. Water is, thus, removed both by bulk replacement by the organic solvent and by structuring of the water around the organic molecules (Singh, 1995). Acetone, ethanol, acetone–methanol, chloroform–methanol, tricholoroacetic acid–ethanol are examples of solvents and solvent mixtures frequently used for protein precipitation. Modifications in protein functionality, safety of solvent handling and miscibility are some of the challenges associated with organic solvent protein precipitation.

3.3.2.4 *Protein Purification further downstream*

Washing

After recovery, proteins can be further purified by repeated washing with an appropriate solvent followed by centrifugation and/or filtration. This extra unit operation allows the removal of non-protein soluble components trapped within the interstitial spaces of the precipitate. The process can be further optimized to remove contaminating soluble proteins prior to drying (e.g., by selecting an appropriate pH of the water used for washing).

By washing the acid precipitated soy protein with pH 4.5 wash water, then resolubilizing and washing at pH 9.0 culminating with a final pH 4.5 precipitation, fat content was further decreased from 7–9% to 3–6%, while increasing the protein content from ~81–85% to ~90–92% without the added washing step (Mattil *et al*., 1979).

Lawhon and co-workers (1981b) also reported that solubilizing full fat soy flour in water under controlled conditions (1:12 w/w solid:water ratio, pH 9 at 60 °C for 30 min), separating the aqueous phase from the slurry by centrifugation and washing the residue with water (1:5 solid:water ratio) at pH 9.0, precipitating the combined aqueous phase at pH 4.5. Washing the curd with acid water (pH 4.5) then decreased the fat content to 3.2%, while ensuring a protein content approaching 90%.

The addition of extra washing steps ultimately increases the efficiency of oil and protein extraction. To maintain desired functionality, the pH of the precipitate must be appropriately adjusted prior to drying.

Dialysis

Dialysis removes small molecular weight contaminants by selective and passive diffusion through a semi-permeable membrane. At the analytical scale, proteins can be purified using dialysis membranes with frequent water changes and constant stirring to drive the removal of contaminating smaller molecular weight compounds. Temperature, pore size, surface area, stirring speed and concentration gradient are factors influencing the rate of microsolute removal.

In general, dialysis proceeds faster at room temperature than at 4 °C. However, depending on the dialysis time, it may be prudent to conduct dialysis at lower temperature to prevent spoilage and loss of functionality. Thermal stability of the molecule should also be considered in temperature selection. Pore size of dialysis membranes must be carefully selected in accordance with the protein of interest. Although dialysis is an efficient way to increase protein purity at the analytical scale, it is less practical at the industrial scale, and therefore other techniques, such as diafiltration and chromatography, may be of greater interest.

Diafiltration

Diafiltration is typically performed as part of the membrane separation process described above. It refers to the process of adding water to the retentate during MF and/or UF and continuing the elimination of membrane-permeating species along with the water removed in the permeate (Cheryan, 1998). This allows removal of (contaminants) unwanted soluble materials such as smaller molecular weight sugars, alcohols, acids (e.g., phytic acid), and minerals from solution resulting in further protein purification.

Diafiltration can be performed in the continuous or discontinuous (batch) mode. In the discontinuous mode, the volume of the retentate is reduced, followed by redilution with water and repeating the UF in consecutive steps. In the continuous mode, water at the processing temperature and pH is added to the feed tank at the same rate as the permeate flux, resulting in a constant feed volume.

To enhance microsolute removal, UF/diafiltration can be carried out in a dialysis mode where water is pumped into the permeating membrane at three to ten times greater flow rate than the flux, while keeping the net pressure in the permeate channel lower than the feed pressure (Cheryan, 1998). The significant decrease in the concentration of microsolutes in permeate compared to the feed establishes a concentration gradient which enhances microsolute permeation. Reverse osmosis, a filtration technique which allows the removal of very low molecular weight compounds, can be used to subsequently concentrate the solute while producing the water for the UF dialysis.

3.3.2.5 *Chromatographic techniques*

Chromatography offers a wide variety of techniques that could be used to analyze as well as purify proteins. Many of these techniques can be used at relatively large scales to purify and collect proteins from biological mixtures for further analysis or for final use. In contrast, analytical chromatography is primarily used for qualitative and quantitative characterization purposes. Short descriptions of the most widely used methods are detailed in the following sections.

Gel filtration chromatography

Gel filtration chromatography has been widely used to purify biomolecules due to its simplicity and efficiency (Pastorello and Trambaioli, 2001). The technique separates proteins on the basis of their molecular size independent of the composition of the buffer used as eluent. Different media are used to perform the molecular exclusion in packed columns. The inertness and physicochemical stability of the chosen media are key requirements in column selection. Details of the technique can be found elsewhere (Amersham, 2002). Gel filtration is generally used in combination with the other chromatographic techniques described (Neyestani *et al*., 2003).

Ion exchange chromatography

Ion exchange chromatography separates biomolecules according to their net chemical charge under determined experimental conditions. The degree of interaction between the charged proteins and the opposite charges of the ionic groups of the ion exchange medium, as well as differences in the charge properties of the proteins being separated, are key factors influencing separation (Ishihara and Yamamoto, 2005). The elution method generally involves a gradient of at least two buffers, such that the elution of the proteins is performed after the disruption of the electrostatic bonds between the biomolecule and the ionic groups of the chromatographic medium resulting from an increase in ionic strength or pH change (Levison, 2003). Two ion exchangers are commonly used: anionic exchange columns are generally used to separate acidic proteins, whereas cationic columns are more widely utilized for the purification of basic proteins (Pastorello and Trambaioli, 2001).

Reversed phase chromatography

Reversed phase chromatography is a widely used chromatographic technique which offers high resolution in separating proteins on the basis of their hydrophobicity (Pastorello and Trambaioli, 2001). The stationary phase is generally composed of an *n*-alkyl hydrocarbon that is able to interact with the proteins through hydrophobic interactions. The separation is often performed using a gradient elution. Thus, after adsorption to the immobilized *n*-alkyl hydrocarbon, the injected proteins are eluted due to the disruption of their hydrophobic bonds with the stationary phase after decreasing the polarity of the mobile phase (Pastorello and Trambaioli, 2001). Reversed phase chromatography has been extensively used in analytical analysis as well as at the preparative scale for the purification of food and recombinant proteins (Olson *et al*., 1994).

Affinity chromatography

Affinity chromatography separates proteins according to their specific reversible interactions with a chemical ligand attached to the chromatographic matrix; it might be a substrate analogue, enzymatic inhibitor or activator, or a specific ligand of the targeted protein (Chaiken, 1986). Hence, proteins could be separated based on their biological function and specific structure.

Immunochromatography refers specifically to the immobilization of antibodies onto the gel matrix for affinity chromatography and is a technique that has been widely used for the isolation of high purity proteins (Riggin and Sportsman, 1993).

Proteins separated by chromatography using any of these techniques can be detected using UV spectroscopy, refractive index, evaporative light scattering, fluorescence or mass spectrometry detection.

3.3.2.6 *Drying Technologies*

Drying represents one of the most important costs in protein processing. Three techniques most commonly used are freeze drying (lyophilization), spray drying or drum drying.

Freeze drying

Freeze drying involves the removal of water from frozen materials by sublimation under high vacuum. The technique consists of three main stages: freezing, sublimation and desorption. The sample to be dried (i.e., example wet protein precipitate), is first frozen at a temperature below 0 °C, which should be sufficient to completely solidify the sample. The frozen sample is then placed under vacuum with pressures lower than 610 Pa at a high enough temperature to stimulate the sublimation of the free ice crystals. Water vapor is mechanically removed from the environment by vacuum pumps or steam jet ejectors (Mujumdar and Devahastin, 2000). To facilitate desorption of remaining water adsorbed by the sample, the temperature of the sample is raised at the latter stages of freeze drying to between 40 and 60 °C, which is sufficient to break any remaining bonds between the water molecules and the sample.

Freeze drying is costly and requires considerable time and energy. However, it has several advantages (Baker, 1997). It has a very low impact on the quality of the processed products and enables the creation of a powder with a quick rehydration rate. Freeze drying may be best suited for proteins targeted for the pharmaceutical and nutraceutical sectors, and perhaps for high end food applications.

Spray drying

Spray drying is one of the most widely used industrial drying processes in the food industry. It can transform low concentration emulsions, solutions or suspensions into powder and is frequently used in the agri-food industry because it is quick and can be completed in a single step. The product to be dried is pumped into the spray dryer and passes through an atomizer, which sprays the suspension in tiny droplets. There are many atomizer models on the market, each having specific advantages and disadvantages (e.g., vanes, nozzles, etc.). Hot air fed into the drying chamber dries the atomized droplets. On contact with the air, the water present in the droplets is quickly vaporized and the powder that forms drops out of the drying chamber. More recent spray dryers offer the possibility of lowering the pressure in the drying chamber, which allows products to be exposed to lower temperatures. This minimizes the impact of temperature on heat-sensitive products, such as proteins, which can denature at relatively low temperatures (80 °C) (Mujumdar and Devahastin, 2000).

Drum drying

Prior to the development of spray dryers, drum drying was one of the most commonly used techniques for drying. Drum drying can be used to dry a variety of liquid materials and is particularly effective for drying viscous liquids. Drum dryers consist of one or two horizontally mounted hollow cylinder(s) made of high-grade cast iron or stainless steel, a supporting frame, a product feeding system, a scraper and auxiliaries (Tang *et al*., 2003). During processing, steam at very high temperatures (up to 200 °C) heats up the interior of the drums while the liquid material to be dried is applied uniformly as a thin layer onto the exterior surface(s) of the drum(s). The heat evaporates the water from the material leaving a dry product that is scraped off as the drum rotates towards the scrapers. The rate of drying and final moisture content depend on the type of material being dried, residence time, feed rate,

thickness of material, steam pressure and temperature, and roll speed of the drum. Drum dryers have been successfully used to dry a variety of protein extracts.

Although drum dryers are easy to operate and have high energy efficiency, they have relatively low throughput compared to spray dryers. Materials may also be unevenly dried and become scorched, resulting in the generation of unacceptable colors or flavors, or the loss of functionality. Thus, drum dryers may be better suited for the drying of lower cost protein extracts (e.g., food and feed applications) than for pharmaceutical or nutraceutical products.

3.4 CALCULATING PROTEIN YIELDS AND RECOVERY

Protein extraction efficiency is an important measure of the effectiveness of protein separation. Calculating the amount of protein recovered is, therefore, important to assess the efficiency of the technique used. Protein recovery may be defined as the ratio of the total mass of protein recovered in the dried extract divided by the total mass of protein present in the starting material as shown in Equation 3.1 below:

$$\text{Protein recovery (\%)} = \frac{\text{Mass of protein extracted (g)}}{\text{Total mass of protein in starting material (g)}} \times 100 \quad (3.1)$$

Thus, the closer the mass of the protein extracted to the original amount present in the starting material, the more efficient is the protein recovery.

Protein extraction efficiency is sometimes simply reported as protein yield defined as the ratio of the total mass of protein recovered in the dried extract divided by the total mass of the starting material (Equation 3.2):

$$\text{Protein yield (\%)} = \frac{\text{Mass of protein extracted (g)}}{\text{Total mass of starting material (g)}} \times 100 \quad (3.2)$$

Another frequently used measure of protein extraction efficiency is protein purity, defined as the concentration of protein in the final extract as measured by the Kjedhal or Dumas methods or a method analyzing for true protein (e.g., Lowry, Bradford or BCA methods). Protein purity may, therefore, be defined as the ratio of the mass of protein in the dried recovered extract divided by the total mass of the dried extract as shown in Equation 3.3:

$$\text{Protein purity (\%)} = \frac{\text{Mass of protein in extract extracted (g)}}{\text{Total mass of recovered extract (g)}} \times 100 \quad (3.3)$$

3.5 PROCESSING EFFECTS ON YIELD AND PROTEIN QUALITY

Protein purity, yield and quality are easily affected by processing conditions (e.g. temperature, time, flour to solvent ratio, condition and protein solubility of the starting material, type of equipment and process used, *g* forces used for centrifugation, laboratory vs. pilot scale extraction, batch vs. continuous extraction, etc.).

Russin and co-workers (2007) reported that by decreasing the average particle diameter of the starting raw material (i.e. soy flour) from 223.4 to 89.5 μm increased total solids recovery from 23 to 32%, while protein recovery increased from 40 to 52%. Final protein content (i.e. purity) of the soy protein isolates was, however, not significantly impacted by average particle size. The results demonstrate that protein recovery can be significantly increased by decreasing the average particle size of the starting raw material (i.e. defatted soy flour), without having any detrimental effect on the purity of the final soy protein isolate.

In the processing of pulse proteins, protein contents of fractions extracted using different techniques have been variable, probably due to differences in the processing conditions used (Boye *et al*., 2010a). Chakraborty and co-workers (1979) extracted pulse proteins at pH 8.5 (30 min, 40 °C) followed by precipitation at pH 4.5 and obtained isolates containing 88.3–90.5% protein for chickpea, pea, Great Northern bean, lentil protein, lima bean, and mung bean isolates. Paredes-López and co-workers (1991) used similar conditions and obtained a chickpea isolate containing 84.8% protein. In the latter study, chickpea protein isolates were obtained from defatted chickpea flour using an initial aqueous extraction at 10% (w/v) solution and pH 8.5, followed by precipitation at pH 4.5, centrifugation and freeze drying. In other studies, de-hulled faba bean flour and pea flour were dispersed in water (1:5 w/v ratio, pH 9, 20 min, room temperature) followed by centrifugation and protein precipitation from the supernatant (pH 4, room temperature, 20 min). The extracts obtained contained 81.2 and 84.9% protein, respectively (Fernández-Quintela *et al*., 1997).

In addition to yield and purity, conditions used during processing (pH, temperature, extraction times, solvents) can modify protein structure and affect protein thermal stability and functionality. These conditions, therefore, need to be properly selected taking the final product application into consideration.

3.5.1 Protein characterization

3.5.1.1 *Biochemical methods*

Slab gel electrophoresis

Electrophoresis is widely applied for protein separation and characterization. The technique involves the separation of proteins on the basis of their mobility in an electric field. Proteins move with different speeds depending on their charge, shape and size. Polyacrylamide gels (PAG), which are formed by the copolymerization of acrylamide monomers with a cross-linking agent such as bisacrylamide, are generally used as the matrix for electrophoresis (Dunn, 1989). Different types of gels containing a linear or non-linear concentration gradient of polyacrylamide can be used to optimize protein separation (Dunn, 1989).

A range of electrophoretic techniques are available which can separate proteins on the basis of their properties, such as size, net charge and relative hydrophobicity. However, the most used electrophoresis techniques for the characterization of food and bioproduct proteins are: native PAGE, SDS-PAGE, immunoelectrophoresis and isoelectric focusing. Protein detection for these different methods is often done by staining with Coomassie Brilliant Blue in a fixative solution or, after fixation, with silver, gold and fluorescence or chemiluminescence probes. More detailed information on protein staining can be found elsewhere (Amersham, 1999a).

Electrophoresis separation can be monodimensional (1D) or bidimensional (2D). In 1D electrophoresis, proteins are separated in one dimension on the basis of only one property, such as charge (isoelectric point) or size. 2D electrophoresis combines the 1D electrophoresis

with a second property in the orthogonal direction (Pastorello and Trambaioli, 2001). 2D electrophoresis is a powerful method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples, and can resolve hundreds to thousands of individual protein species detected as spots or bands after staining (Friedman *et al*., 2009).

Native Polyacrylamide Gel Electrophoresis (PAGE)

PAGE under native conditions has been used to separate soluble proteins which retain their biological and enzymatic properties. Factors affecting the migration of proteins in native PAGE are: size, shape, and native charge (Dunn, 1989). A derivative method from native PAGE developed by Schägger and Von Jagow (1991), known as Blue Native PAGE, was originally used for the qualitative and quantitative analysis of mitochondrial protein complexes and proteins ranging in molecular weights from 10 to 10 000 kDa (Dresler *et al*., 2010).

SDS-PAGE

Sodium dodecyl sulfate (SDS) is an anionic detergent that denatures proteins through specific binding to the hydrophobic tail around the polypeptide backbone, giving a net uniform negative charge per molecule (Dunn, 1989). The binding of SDS unfolds the protein as a consequence of hydrogen bond cleavage, and blockage of hydrophobic interactions. Consequently, electrophoretic separation is only dependent on the molecular weight and proteins migrate in the anodal direction. Moreover, proteins can be denatured by the addition of strong reducing agents, such as dithiothreitol (DTT) or β-mercaptoethanol, which disrupt disulfide bonds between cysteine residues, allowing a more detailed characterization of proteins (Dunn, 1989).

Lately, the discontinuous Laemmli system buffer (containing 0.1% w/v SDS) is most commonly used for SDS-PAGE. The method has been widely used for the characterization of the protein profiles of processed whey, meat, cereal, legume, and seed proteins (Roy *et al*., 2007; Miyamoto *et al*., 2009; Boye *et al*., 2010b; Sikes *et al*., 2010; Warchalewski and Gralik, 2010) as well as the analysis of membrane proteins (Braun *et al*., 2009). Further information on SDS-PAGE electrophoresis can be found elsewhere (Amersham, 1999a).

Immunoelectrophoresis

Immunoelectrophoresis is a specific method characterized by the presence of polyclonal monospecific antibodies against a specific protein in the agarose gel. The running buffer used in this method should have a pH corresponding to the isoelectric point of the antibody. The fixation of the latter in the gel leads to the formation of a precipitate comprised of the antigen and the specific antibody, which may be visualized by using a Coomassie blue stain or some other suitable technique (Westermeier and Scheibe, 2009). Immunoelectrophoresis has been used for the qualitative and quantitative analysis of individual proteins in complex mixture (Hansen and Larsen, 2008).

Isoelectric focusing

Isoelectric focusing (IEF) is an electrophoretic method that separates proteins based on their isoelectric points (pI, neutral charge state). The migration is performed in the presence of a continuous pH gradient generated using carrier ampholytes or immobilized pH gradients gels (Dunn, 1989; Amersham, 1999a). After migration according to their charge, proteins attain a steady sate when they reach the pH value corresponding to their pI. The technique is commonly used in combination with SDS-PAGE in 2D electrophoresis. IEF can also be performed under native conditions (Dunn, 1989; Friedman *et al*., 2009).

Capillary electrophoresis

New developments in the field of capillary electrophoresis (CE) offer a powerful tool for the rapid separation of proteins (Mattiasson and Hatti-Kaul, 2003; Huck and Bonn, 2008). Separation of proteins by CE is based on electrophoretic migration driven by an electroosmotic flow (EOF) and occurs in capillary tubes, filled with background electrolytes (BGE) (Tagliaro *et al*., 1998) following similar principles as for standard electrophoresis. CE is characterized by high efficiency, short time of analysis, low consumption of samples and running buffers, and possible connectivity to detectors originally designated for liquid chromatography, including mass spectrometry. Examples of CE use in protein analysis include successful quantification of 7 S and 11 S fractions in several soybean varieties (Blazek and Caldwell, 2009), characterization of cereal proteins (Piergiovanni, 2007; Salmanowicz, 2008; Salmanowicz *et al*., 2008; Di Luccia *et al*., 2009), and the analysis of casein/caseinate addition in processed cheeses (Miralles *et al*., 2006). Further information on the use of CE to purify proteins and study their conformations can be found in the work by Mattiasson and Hatti-Kaul (2003).

Thermal analysis by differential scanning calorimetry

Heat treatment is one of the most commonly used techniques in food processing, and is frequently responsible for the transition of proteins from the native folded state to the denatured unfolded state. Thermal denaturation of proteins involves a structural change which affects the nutritional quality of foods (Plum, 2009).

Differential scanning calorimetry (DSC) is a suitable method for the characterization of thermal and thermodynamic stabilities of the protein. DSC provides several tools for the study of the thermal properties of proteins under controlled heating and cooling rates and can be used to determine the apparent specific heat of proteins (O'Brien and Haq, 2004).

In brief, a DSC instrument comprises two cells fixed in an adiabatic chamber. One cell contains the sample to be tested, the second cell contains a reference solution or an empty DSC pan. The adiabatic chamber is maintained under pressure to avoid the evaporation of the sample (Plum, 2009). A DSC-thermogram represents the plot of heat capacity difference ΔC_p (between the sample and the reference) as a function of temperature. Thermodynamic parameters, such as T_m, ΔH and ΔS, could be determined by the DSC curve analysis. T_m is the temperature at which the concentration of denatured and native forms of the protein are equal. This specific temperature is also called the midpoint of the thermal transition. ΔH represents the enthalpy of thermal transition determined from the integration of the DSC curve. The entropy (ΔS) of the thermodynamic transition of the protein may be calculated from the integrated area under the curve of $\Delta C_p/T$ vs. T. The free energy (ΔG), which gives an indication of the protein stability, can also be determined at any temperature from the values of ΔH and ΔS (O'Brien and Haq, 2004; Plum, 2009). Thermal and thermodynamic properties of proteins analyzed by DSC are greatly affected by the experimental conditions used, such as pH, salts, alcohols, and the presence of other food components (e.g., lipids, polysaccharides) (Grinberg *et al*., 2009).

3.5.1.2 *Biophysical methods*

Biophysics offers a variety of accurate methods that enable kinetic resolution in the submillisecond time scale as well as high protein structural resolution. This section summarizes some of the most frequently used techniques for the biophysical analysis of proteins.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a powerful tool used to monitor changes in protein and polypeptide secondary structure during processing. After exposure of a protein to infrared light, its secondary structure can be determined from the spectra obtained from the absorption of different wavelengths corresponding to specific vibration frequencies of the amide bonds (Jackson and Mantsch, 1995a).

Two kinds of the vibrations, known as amide I and amide II vibrations, are directly correlated to the molecular structure of proteins and polypeptides. The stretching of the carbonyl C = O double bonds is linked to the amide I vibration, and the deformation of the N–H bonds causes the amide II vibration. FTIR determines the amount of light absorbed corresponding to each of the vibrations over a wide range of frequencies (Kumosinski and Farrell, 1993). Empirical correlations between the frequency of the amide I and amide II absorptions of the protein and the secondary structure composition in helix, extended β-sheets, loops and unordered structures have been determined (Jackson and Mantsch, 1995).

The amide I absorption region (1700–1600 cm^{-1}) in the FTIR is widely used for the determination of the secondary structure of proteins (Álvarez *et al*., 2008). Parallel and antiparallel β-sheets are attributed to bands at 1622 and 1632 cm^{-1}, respectively, whereas, α-helix structures correspond to the band at ~1655 cm^{-1}. β-turns and unordered structures can be detected respectively at band positions of 1692 and 1645 cm^{-1} (Jackson and Mantsch, 1995).

Circular dichroism

Circular dichroism (CD) is a powerful method for the study of protein structure and folding in solution under various conditions. The technique has been widely utilized to monitor structural transitions of food and bioproduct proteins resulting from changes in different processing conditions (Martin and Schilstra, 2008). CD is a spectroscopic technique based on the difference in protein interactions with left (L) and right (R) circularly polarized light (Sreerama and Woody, 2004). Hence, CD analysis of proteins is based on the measurements of the difference in absorbance between L and R circularly polarized components expressed as ellipticity (θ) in degrees (Kelly *et al*., 2005). CD spectra thus result from the chirality of some structures, such as the carbon atom bound to four different substituents, the C–S–S–C structure and molecular asymmetric environments (Kelly *et al*., 2005). Table 3.2 shows the spectral region and contributing protein chromophores. The determination of the relative proportions of α-helix, β-sheets, β-turns and random coil structures, as well as the overall features of the secondary structure, could be determined by CD measurements in the far UV region (Sreerama and Woody, 2004). All α-helix rich proteins, such as α-lactalbumin and lysozyme, are characterized by an intense negative band with two peaks at 208 and 222 nm, and a strong positive band at 191–193 nm (Barbana *et al*., 2006), whereas the β sheets rich proteins are distinguished by a negative band at 210–225 nm and a stronger positive band at

Table 3.2 Contributing chromophores in CD spectra of proteins.

Chromophore	CD spectra region
Backbone amides	<250 nm (far UV)
Aromatic groups	250–300 nm (near UV)
Extrinsic groups	Above 300 nm (near UV–visible region)

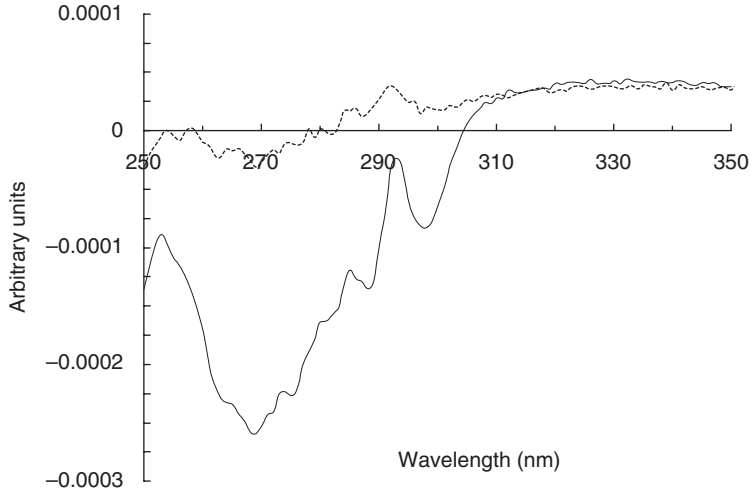

Figure 3.6 Circular dichroism spectra in the near UV region of bovine holo- (solid line) and apo- (dashed line) α-lactalbumin. Circular dichroism spectra were recorded at a protein concentration of 70 mM in 5 mM Tris buffer, pH 7.0, at 20 °C. (Reproduced from Barbana *et al.*, copyright 2006, with permission from Elsevier.)

190–200 nm. Unordered proteins are generally characterized by a negative band at 195–200 nm, and a much weaker signal between 215 and 230 nm (Martin and Schilstra, 2008).

CD spectra analysis at the near UV region provides important information about protein tertiary structure. Spectra at the near UV region arise from the aromatic amino acids, and depend on their number, mobility, spatial disposition and their environment (Kelly *et al.*, 2005). CD signals could also be useful for the study of the conformational changes in proteins caused by ligand binding (Barbana *et al.*, 2006, 2008). Figure 3.6 shows the near UV CD spectra of bovine α-lactalbumin in its holo and apo forms. As observed, apo-α-lactalbumin has very low signal in the near UV region of the CD spectrum, indicative of loss of the native tertiary structure with rapidly rotating aromatic side chains after the release of Ca^{2+}.

Fluorescence spectroscopy

Fluorescence is the light emitted subsequent to absorption of UV or visible light due to electronic state transitions from excited singlet to several vibrational levels (Lakowicz, 1999; Hof *et al.*, 2005). The emission spectrum provides information for both qualitative and quantitative analysis.

Fluorescence spectroscopy is a widely used biophysical tool for the study of the protein structure based mainly on tryptophan fluorescence, which can only be excited by light of specific wavelengths. The fluorophore that is able to emit fluorescence in the trypotophan structure is the indole group. Generally, the fluorescence spectra recorded between 300 and 400 nm after excitation of the tryptophan fluorescence at 298 nm could be very useful for understanding the effect of processing on protein structure. A shift in the maximum of tryptophan fluorescence emission suggests an exposition of the tryptophanyl residues to a polar environment. However, an increase in fluorescence intensity corresponds to an increase in solvent polarity and reduced quenching of some tryptophanyl residues (Barbana *et al.*, 2006, 2008). Figure 3.7 shows the fluorescence spectra of α-lactalbumin before (holo) and after (apo) the removal of Ca^{2+} by chelating agent. The fluorescence intensity of the spectrum of

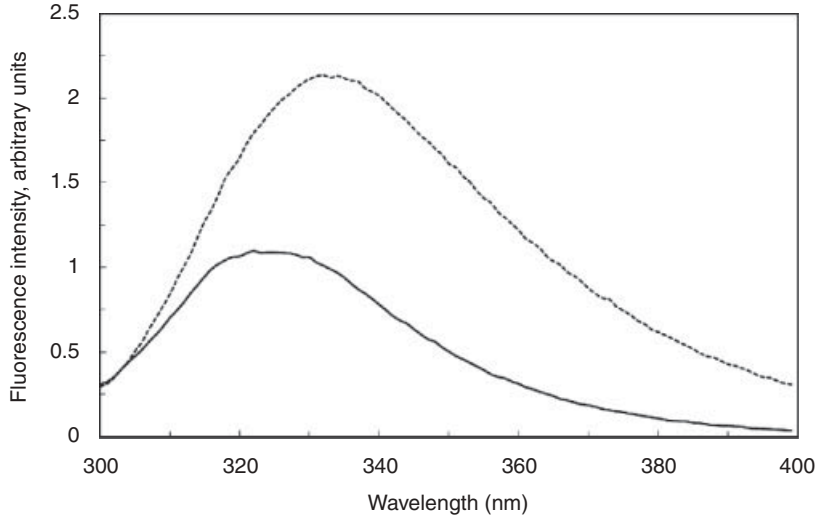

Figure 3.7 Fluorescence spectra of human holo- (solid line) and apo- (dashed line) α-lactalbumin recorded for 20 mM protein solution in 10 mM Tris buffer, pH 8.5, at 20 °C. Fluorescence excitation was at 287 nm (Barbana *et al.*, 2008). Reproduced with kind permission from Springer Science and Business Media.

holo-α-lactalbumin reaches its maximum at 325 nm. The removal of calcium bound to the protein causes an increase in fluorescence intensity and shifting of the wavelength maximum to a significantly higher value (336 nm). These results indicated that tryptophan residues are more exposed to solvent in the apo form, reflecting a conformational change in the protein caused by the release of bound calcium (Barbana *et al.*, 2008). Fluorescence properties of proteins, however, depend on experimental conditions, such as temperature, pH, concentration, polarity of the environment and quenching (intra- or inter-molecular interactions) (Andersen *et al.*, 2008).

3.5.1.3 *Immunochemical methods*

Immunochemical methods are highly sensitive techniques for the detection and identification of protein targets by antigen–antibody specific reactions. Radial immunodiffusion and western blotting are among the most used techniques for the quantitative estimation of processed food and bioproduct proteins based on their immunoreactivity with specific antibodies.

Radial immunodiffusion

Radial immunodiffusion is used extensively for the quantitative estimation of antigens. In a uniformly thin layer of agar containing a specific antibody, the area of the radial diffusion of the corresponding antigen from wells cut in the gel is directly proportional to the concentration of the antigen employed. The method is very accurate and sensitive, with lower detection limits corresponding to an antigen concentration of 1.25 μg/ml (Mancini *et al.*, 1965). This technique could be used for the determination of antigens and evaluation of the effect of processing on the denaturation of food proteins (Wehbi *et al.*, 2005).

Western Blotting

After PAGE electrophoretic separation, protein bands can be transferred to an activated thin support membrane, generally nitrocellulose membrane, by electro-transfer techniques.

The second step involves the immobilization of the bands to the surface of the membrane in the same pattern as in the original PAGE gel. Subsequently, the selected proteins could be analyzed by immunoassays after the treatment of the membrane with specific antibodies which bind to the antigens in the membrane and can be detected by a variety of techniques involving treatments with secondary antibodies and staining (Phillips, 1992). Prior to antibody binding and staining the remaining protein binding sites on the membrane must be blocked (e.g., by bovine serum albumin (BSA) or ovalbumin) to avoid non-specific interaction with the antibodies. Additionally, the removal of unbound reagents by a series of washes between each binding step is necessary to avoid non-specific binding.

The detection of the antigen–antibody complexes on the membrane could be performed by a variety of techniques. The most common method is the application of a secondary antibody which binds specifically to the primary antigen–antibody complex. Hence, radiochemicals, fluorescent compounds, colloidal gold, enzymes, or biotin labels could be easily bound to the secondary antibody, and protein detection could be deduced from the intensity of the colored, fluorescent or chemiluminescent end product. Further details on blotting can be found elsewhere (Amersham, 1999).

3.6 CONCLUSION

Proteins play a key role in food, feed, and in many health and industrial applications. Due to their versatility, the market for semi-purified and highly purified proteins is likely to grow. There has been significant effort to identify novel sources of healthy and functional proteins. Examples of such new sources of proteins include canola and flax proteins that are currently in industrial production for the food, feed and pharmaceutical industries.

As consumers and the food and industrial products sectors become more environmentally conscious, techniques that are more energy efficient, sustainable and have a lower carbon foot print will be required. In this regard novel non-thermal technologies, such as high pressure processing, pulse electric field and ultrasonication, will be preferred for protein extraction and/or improving protein functionality. Since the most expensive unit operation in protein processing is drying, new innovative drying techniques that are cheaper, easy to use and do not adversely alter protein functionality need to be developed. Additionally, optimization of the processing conditions for the current techniques and the development of new processes that could enhance protein functionality while increasing its health benefits and nutritional properties would be extremely useful.

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