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Abstract

The use of exogenous proteases to improve meat tenderness has attracted much interest recently, with a view to consistent production of tender meat and added value to lower-grade meat cuts. This review discusses the sources, characteristics and use of exogenous proteases in meat

tenderization to highlight the specificity of the proteases toward meat proteins and their impact on meat quality. Plant enzymes (such as papain, bromelain and ficin) have been extensively investigated as meat tenderizers. New plant proteases (actinidin and zingibain) and microbial enzyme preparations have been of recent interest due to controlled meat tenderization and other advantages. Successful use of these enzymes in fresh meat requires their enzymatic kinetics and characteristics to be determined, together with an understanding of the impact of the surrounding environmental conditions of the meat (pH, temperature) on enzyme function. This enables the optimal conditions for tenderising fresh meat to be established, and the elimination or reduction of any negative impacts on other quality attributes.

Keywords: **Meat; proteases; tenderization; plant; microbial**

Content

Introduction

Meat quality is a multi-dimensional concept that follows to a great deal the general food quality model proposed by Cazes-Valette (2001). Meat is valued based on cultural (e.g.; pork or beef vs. lamb); religious (halal or kosher vs. non-halal); functionality (e.g. table meat cuts such as steaks and roasts vs. manufacturing cuts used for small goods processing) and many other factors (e.g. hygienic, ethical, organoleptic, social, symbolic and nutritional). Organoleptic (sensory) quality is a paramount factor determining meat quality and it plays a major role in meat marketability. For example, the amount of fat and the colour of the meat are the main attributes at the point of sale influencing the purchase decision (Mancini and Hunt, 2005). However, when the meat is cooked, fresh meat colour is of minor importance and flavour and tenderness are the main sensory attributes that determine the consumer's satisfaction.

Meat as a commodity, however, differs from other food items in that the sources of the meat are quite variable and the characteristics of the product within the same species will depend on on-farm factors (such as breed, diet, and handling); physiological factors (such as the genetic background and muscle type and location); and processing factors (such as post-mortem temperature regime, electrical inputs and storage conditions). Biological variation in tenderness among animals from the same genetic and farming background is well known and large tenderness variation within mobs of animals (group of animals farmed together) from the same background have been reported (Bekhit et al., 2003; Devine et al., 2006). Within the same

animal, tenderness from right and left side cuts can vary (Smulders et al., 2006) depending on post-mortem handling and this variation can be found even within the same piece of meat (Devine et al., 2006; Rhee et al., 2004). Consumers understand the inherent tenderness variability in different meat cuts and associate the differences in tenderness with price and cooking methods for different meat cuts. However, inconsistent tenderness within cuts is a problem for the meat industry and consumers (Koohmaraie and Geesink, 2006) that carries financial consequences. The production of consistently tender meat is required to retain consumer confidence in red meat which is competing with other types of meat that intrinsically do not have toughness problems (such as chicken). As a result, there are numerous publications and patents describing knowledge and processes to reduce meat toughness and achieve consistently tender meat. Several publications have reported the willingness of consumers to pay more for meat that has a guaranteed tenderness (Feldkamp et al., 2005; Boleman et al., 1997; Lusk et al., 2001; Miller et al., 1995). Polkinghorne et al. (2008) stated that prime grilling cuts are less than 10% of a carcass. Therefore 90% of the carcass can potentially benefit from the use exogenous enzymes to upgrade the tenderness of meat. This means that tenderizing tougher meat cuts can be economically rewarding for the meat industry as well as improving consumers' satisfaction with red meat. The present article reviews exogenous enzymes that have been investigated to tenderize meat and improve eating quality with the aim of evaluating the potential of available commercial proteases as meat tenderizers and to produce some guidelines for the application of these enzymes.

Variation in meat tenderness

The physiological function of muscles and age of the animals determines the fibre type composition (fast vs. slow twitch; aerobic vs. anaerobic) and the connective tissue content and solubility. Also, after slaughter, the rate of chilling of a muscle or part of a muscle will depend on its location in the carcass. These differences will lead to wide variation in the eating and keeping qualities of the meat. As a result, some muscles are generally tender (*Infraspinatus* (INF), *Psoas major* (PM)), or tough (*Biceps femoris* (BF), *Supraspinatus* (SS)). Other muscles, such as M. *triceps brachii* vary in tenderness, depending on processing conditions (Belew et al., 2003; Torrescano et al., 2003). Variation in the tenderness of a muscle amongst and within animals is well documented (Devine et al., 2006; Shackelford et al., 1995; Rhee et al., 2004). For more detailed information the reader is referred to Lefaucheur (2010).

The toughness of the meat is determined by two structural components. The first component is the connective tissue in the meat, contributing to what is commonly referred to as "the background toughness". Connective tissues are essentially composed of structural proteins that provide support for the muscles at different hierarchies. The structural aspects and the contribution of the connective tissues to the tenderness in raw and cooked meat have been recently reviewed by Lepetit (2008). The contribution of connective tissue to meat toughness depends on the structure and/or the amount of different collagens and elastin in the meat (Lepetit, 2008). This part of meat toughness is mostly influenced by "on-farm" factors such as breed, sex, age, physical activity and so forth. Processing and post-mortem handling practices will have little effect on "background toughness".

The second component contributing to meat toughness is determined by post-mortem changes in the contractile apparatus of the muscle (the sarcomere) which undergoes a shortening phase during the development of rigor mortis. The toughness caused by muscle shortening is primarily influenced by processing conditions. Thus, by manipulating the processing conditions, significant improvement in meat tenderness can be achieved. Shortening induced toughness is resolved to a variable degree by the actions of endogenous proteases during post mortem storage of meat (ageing). This is mainly attributed to the actions of μ-calpain (Geesink et al., 2006), although the involvement of other proteolytic systems has been suggested (Hopkins and Thompson 2002; Dutaud et al., 2006; Ouali et al., 2006; Ilian et al., 2004; Kemp et al., 2010). The role of the endogenous muscle proteases and the degradation of muscle proteins post-mortem will not be covered here. For further information the reader is referred to recent reviews on this topic (Geesink and Veiseth, 2009; Hopkins and Geesink, 2009).

The ever increasing requirements for meat safety and production efficiency in the meat industry have led to the development of efficient chilling systems that minimize microbial activity and weight loss during storage. However, low temperatures during the onset of rigor mortis can induce excessive muscle shortening which is minimal at 15°C and increases at both sides of this temperature (Locker and Hagyard, 1963; Geesink et al., 2000). In addition, the action of proteolytic enzymes is temperature dependent and as a result, the speed of the tenderization process is negatively affected by low temperatures. Collectively, the above factors contribute to the variability in the tenderness of meat and several interventions to ensure a consistent and desirable level of tenderness have been developed.

Interventions to improve the meat tenderness

Techniques to improve the tenderness of meat have been the subject of many studies. Post-mortem interventions can be classified on the basis of the mode of the process into 3 main categories: physical, chemical and enzymatic.

The physical interventions include electrical stimulation of carcasses, aging conditions (function of temperature and time), freeze-thaw cycles, pressure treatments (hydrodyne/shock wave, ultrasound and high hydrostatic pressure) and mechanical tenderization (blade/needle, cubing, flaking and mincing tenderization) and contraction-prevention (stretching/tenderstretch/alternative hanging, tendercut^{TM}, wrapping, rapid crust freezing).

Chemical interventions include infusion, marinating or injection with calcium salts (e.g. chloride, lactate, ascorbate, and carbonate), sodium salts (e.g. chloride, acetate, citrate, and ascorbate), phosphate salts, commercial preparation containing maltodextrin plus starch or combinations of these compounds. The use of these chemical compounds normally leads to a desirable biochemical outcome (e.g. activation of calpains with calcium, solubilisation of proteins with sodium chloride and improving water holding capacity with phosphate salts) that improves meat tenderness.

Enzymatic interventions include infusion, marinating or injection with exogenous proteases from plants (ficin, bromelain, papain, actinidin, zingibain); microbes (collagenase from *Clostridium histolyticum*; aspartic protease from *Aspergillus oryzae*; fungal protease EPg222; thermophile enzyme E A.1 protease from *Bacillus strain E A.1*; 4-1.A protease from *Thermus*

strain Rt4-1.A; caldolysin from *Thermus strain T-351*; elastase from *Bacillus sp. EL31410*, collagenase from *Vibrio B-30*) and animals (porcine pancreatin).

The present review will focus on exogenous enzymatic methods used to improve the tenderness of intact meat cuts. Thus information on physical or chemical intervention methods will not be covered here as this has been covered in other reviews (Hopkins, 2004; Hopkins and Huff-Lonergan, 2004; Taylor and Hopkins 2011). Information on physical disintegration of the meat structure (e.g. mincing, flaking and so on) and restructuring/binding techniques can be found in a recent publication by Sun (2009). The GRAS status of the enzymes is granted by regulatory bodies (e.g. Food and Drug Administration of United States (FDA), see below) based on the available body of knowledge on the compounds and the knowledge evolved by the common use/consumption of the source plants. It is worth mentioning that the handling of the enzymes and their products needs to be strictly in accordance to the products safety sheets as exposure to these products can be potentially hazardous at certain exposure levels.

Sources and use of exogenous proteases for meat tenderization

Numerous publications have documented that meat tenderization is governed by the degradation of key structural proteins and myofibrillar fragmentation. It is generally accepted that the process is enzymatic in nature with endogenous proteolytic enzymes and their inhibitors controlling the process. Several recent theories and excellent reviews on muscle endogenous enzymes and their role in meat tenderness are available (Sentandreu et al., 2002; Ouali et al., 2006; Hopkins and Geesink, 2009; Geesink and Veiseth, 2009; Kemp et al., 2010) and therefore this will not be discussed in the present work.

Specific muscles from old animals can have substantial toughness that is caused by the strength of connective tissues or insufficient endogenous proteolytic enzyme capacity to tenderize the meat post-mortem (Veiseth et al., 2004). As a result, these muscles remain tough even after extended aging. The tenderness of this type of meat can be greatly improved by the use of exogenous enzymes. Even when processing defects occur, exogenous enzymes can be useful. For example, small amounts, as low as 5 ppm, can alleviate some of the extreme toughness caused by cold shortening (Rhodes and Dransfield, 1973). Therefore, there has been considerable interest in the proteolytic activities of several plant (cysteine proteases) and microbial enzymes and their use as meat tenderizers (Kang and Warner, 1974; 1982; Robbins et al., 1986; Teran, 2003; Ashie et al., 2005; Toren, 2007). Schwimmer (1981) cited 35 patents on the use of proteases from plant, microbial and animal sources and this number has increased substantially in recent times due to advancements in molecular techniques and the use of new sources of proteases (Kang et al., 1982; Kang et al., 1982; Robbins et al., 1986; Cason and Lyon, 2000; Teran, 2003; Ashie et al., 2005; Clarkson et al., 2006; Svendsen and Minning, 2007).

A summary of the proteases that have been investigated as meat tenderizers is given in appendix Table 1 and a summary of their impact on meat quality attributes is given in appendix Table 2. The proteolytic activities of plant extracts have been observed and documented throughout human history and the historical background of the most commonly used proteases (papain, bromelain and ficin) are covered by Gaughran (1976) and earlier publications by Tappel and co-workers (1956; 1959). Because of the long history of using figs, pineapple and papaya as food, the proteases from these fruits or their trees have been warranted generally regarded as safe (GRAS) status by authorities' decades ago. The acute oral toxicity dose of commercial papain in

mice is 10g/kg body weight and the review on papain granted GRAS status in 1977 (Denner, 1983; FDA, 1995; 1997; 1999; 2009). Similarly, both ficin and bromelain have been accredited GRAS status (Denner, 1983). The rapid developments in biotechnology and protein purification capacities encouraged the use of microbial (bacterial and fungal) sources for the production of proteases with greater specificity toward substrates and this facilitated production in greater amounts and purity. Another obvious advantage is the lack of seasonality and the ability to produce the microorganisms at any time with greater ability to modify the molecular structure with the aim of optimizing the characteristics of the target enzyme. The microorganisms used for the production of these proteases (and other enzymes) are safe to be use for the purpose intended and comply with the required safety conditions set by the FDA and outlined in FAO/WHO (2003). The GRAS notices of several proteases from plant (papain, ficin, bromelain, malt), microbial (*Bacillus subtilis; Bacillus subtilis* var. *amyloliquefaciens*; *Aspergillus Niger; Rhizopus oryzae* and animal sources (pancreatin pepsin, trypsin) are available (FDA, 1995; 1997; 1999).

Papain, bromelain and ficin (also spelled as ficain) are cysteine proteases (EC.3.4.22; Barrett et al., 2004); often referred to as thiol or sulfhydryl proteases, which have a molecular weight range between 21-30 kDa and are the most studied for meat tenderization. They are endopeptidases which have low substrate specificity and are able to catalyse the hydrolysis of a wide range of bonds (peptide, amide, ester, and thiol ester and thiono ester bonds) (Schwimmer, 1981). Papain and papain-like proteases are synthesised in plants as inactive precursors whose maturation requires intramolecular polypeptide cleavage involving two consensus sequences in the N- and C-terminal (Karrer et al., 1993; Wiederanders, 2003). Structural comparison of these cysteine proteases reveals a common configuration in which the monomeric polypeptide is

folded into two distinct domains with the active site cleft in between (Baker, 1980; Kamphuis et al., 1984; Choi et al., 1999). The mechanism of action of cysteine proteases was studied extensively using papain as the model enzyme (Storer and Ménard, 1994). The formation of an ion-pair between Cys25 and His159 residues (Figure 1) is responsible for the enzymatic activity of papain (Storer and Ménard, 1994; Grzonka et al., 2007). Maximum activity of the enzymes is obtained when the thiol group of the enzyme is in the reduced form (Storer and Ménard, 1994). The hydrolysis reaction takes place upon the formation of an S-acyl enzyme moiety intermediate. The regeneration of the cysteine protease occurs when a water molecule reacts with the intermediate, leading to the release of the N-terminal fragment and a new catalytic cycle begins (Storer and Ménard, 1994).

Protease characteristics

Many studies have reported the characteristics of the proteases from information based on the behaviour of pure enzymes whereas many of those reported on the application of the enzymes in meat studies have used commercial or crude extracts. For example, Kang and Warner (1974) demonstrated that commercial papaya enzymes from different suppliers contained different ratios of papain, chymopapain and papaya peptidase A exhibiting distinctive physical, chemical and biological characteristics which led to differing activities. The outcome from these studies is that the enzyme characteristics (e.g. optimum activity pH and temperature; the inactivation temperature) in pure and crude extract are different. Therefore, commercial protease products should be characterized before their use to establish the enzyme characteristics assuming that the extraction and the production conditions remain the same.

Papain characteristics

Papain (EC 3.4.22.2) is a cysteine protease obtained from the latex of the papaya plant (*Carica papaya*) which is thought to play a physiological role in protecting plants against insects (Konno et al., 2004). The three dimensional structure has been determined for papain (Kamphuis et al., 1984). Papain has been shown to have a broad-spectrum enzymatic activity over a relatively wide range of pH (5-8) and a high optimal temperature (65°C) (Smith and Hong-Shum, 2003). The specificity of papain is primarily determined by the substrate P2 position (the position for higher affinity of binding of the enzyme for substrates incorporating residues N-terminal to the cleavage site). Berger and Schechter (1970), using synthetic peptides and inhibitors in mapping the active site of papain, demonstrated that papain has a specificity for amino acids with aromatic side chains such as Phe and Tyr at the P2 position. Within the active site, Cys25 and His159 are two of the essential residues for the protease activity (Polgar, 1973).

Crude extracts contain other proteases (chymopapain and papaya peptidase A). Pure papain demonstrates a maximum activity at pH range of 5.8–7.0 and at a temperature range of 50–57°C when casein is used as the substrate (Grzonka et al., 2007). The activity and stability of papain are preserved for several months when stored at 4°C. Papain can be activated and inactivated by reducing and oxidising agents respectively. On prolonged storage, oxidation of the active site thiol group can lead to inactivation which can be partially reversed by thiol reagents (e.g cysteine; sodium metabisulfite) (Grzonka et al., 2007). Papain is inactivated at a temperature of 90°C (Gomes et al., 1997). The enzyme activity is decreased by pressure, especially at high pressure (800kPa) and temperature (60°C) possibly due to oxidation of the thiolate ion at the

active site, to SO_2 or SO_3 (Gomes et al., 1997). This information can be potentially valuable in designing products using high pressure technology so as to optimize the extent of tenderization.

Bromelain characteristics

Bromelain is a mixture of cysteine proteases, extracted from the stem (EC 3.4.22.32, 24.5 kDa) and fruit (EC 3.4.22.33, 25 kDa) of the pineapple (*Ananas comosus*) plant. Both enzymes are glycosylated single-chain proteins, but they are immunologically distinct. Stem bromelain has lower proteolytic activity and a less specificity for peptide bonds compared to fruit bromelain (Barrett et al. 2004; Grzonka et al., 2007). While only two proteases have been detected in pineapple fruit, chromatographic fractionations have shown the presence of up to six components in the crude extract of pineapple stem. The catalytic activity of most of these proteases has been extensively investigated in multiple studies using various synthetic peptides (Inagami and Murachi, 1963; Murachi et al., 1964; Napper et al., 1994; Rowan et al., 1988; 1990). The enzymatic activity spectrum is slightly less than that of papain with detected proteolytic activity on synthetic peptides at pH 5.0-7.0 and optimal temperature of 50°C (Inagami and Murachi, 1963; Murachi et al., 1964; Napper et al., 1994; Rowan et al., 1988; 1990) although a wider range (optimum at pH 6–8.5 and a temperature range of 50 to 60°C) has been reported by Grzonka et al. (2007). Results from these studies have shown that most of the proteases have distinct specificity which has been attributed to some small sequence differences (Lee et al., 1997). Pure bromelain is stable when stored at −20°C (Inagami and Murachi, 1963; Rowan et al., 1988; 1990) and cysteine is the most effective compound to activate bromelain (Inagami and Murachi, 1963; Murachi et al., 1964; Napper et al., 1994).

Ficin characteristics

Crude ficin latex contains about 10 proteases (Kramer and Whitaker, 1964). Ficin (EC 3.4.22.3, MW= 26 kDa) is obtained from the latex of *Ficus glabrata, Ficus anthelmintica, Ficus laurifolia* (Gaughran, 1976). The maximum activity of ficin is obtained within a pH range of 5-8 and a temperature range of 45-55°C. Similar to other cysteine proteases, ficin requires cysteine or other reducing agents for activation. The enzymes have broad specificity with the acceptance of hydrophobic amino acid residues (Schwimmer, 1981). The enzyme is inactivated irreversibly with powerful oxidizing agents and iodoacetate. The enzyme is inhibited by mild oxidizing agents and divalent metals, but the inhibition can be reversed. While the optimum pH for hydrolytic action lies usually in the region of pH 7; extensive solubilisation of certain substrates like elastin occurs at pH 5 (Gaughran, 1976). The optimum pH seems to be dependent on the substrate concentration. With casein, Whitaker (1957) found two peaks of activity to occur at pH 6.7 and pH 9.5 dependent on the substrate concentration. The half life of ficin at 60°C is 1.5 hrs (Whitaker, 1957).

Actinidin Characteristics

Actinidin (also spelled as Actinidain, EC 3.4.22.14) is obtained from the kiwi fruit (*Actinidai deliciosa*). The enzyme is available from other cultivars but the level of actinidin varies greatly among them. The actinidin levels in 28 cultivars range from non detectable to 10.7 mg/ml juice (Nishiyama, 2007). Commercially, the enzyme is obtained from ripe fruit that has been frozen and thawed to disintegrate the cell walls and maximize the yield.

There are 6 possible actinidin proteases. All have similar molecular weights of 23.5 kDa. The three dimensional structure has been determined for actinidin (Baker, 1980). The actinidin pH stability range is 7-10 with and optimal activity at a temperature of 58-62°C (Yamaguchi et al., 1982). The optimal activity was reported to be in the pH range of 7.3-7.6 (Yamaguchi et al., 1982), but a pH range of 5 to 7 has also been reported (Boyes et al., 1997), reflecting difference in the cultivar and the assay used for estimating the proteolytic activity. Actinidin is **not approved** as GRAS by the FDA, but there is a growing interest in the protease because it has a less intensive tenderizing effect in meat compared with the more traditional proteases described above (Lewis and Luh, 1988; Wada et al., 2002; Bekhit et al., 2007; Christensen et al. 2009; Han et al., 2009; Toohey et al., 2011). Actinidin regulation varies according to country. Actinidin hydrolyze both myofibrillar proteins and connective tissue proteins (Lewis and Luh, 1988; Wada et al., 2002; Bekhit et al., 2007; Christensen et al. 2009; Han et al., 2009) but it appears to have higher proteolytic activity toward collagen (Samejima et al., 1991; Wada et al., 2002).

Zingibain Characteristics

The crude extract of ginger has been reported to contain 2 cysteine proteases (Choi et al., 1999; Su et al., 2009) with a molecular mass of 29 and 31 kDa or 3 enzymes displaying proteolytic activity (Ohtsuki et al., 1995) with the same molecular mass (29 kDa). The three dimensional structure of zingibain has been determined by Choi et al. (1999). Research using crude extracts (appendix Table 1) indicates that the proteolytic activity is stabilized during storage using ascorbic acid (0.2%) and a fraction (MW=34.8 kDa) containing the enzyme had a maximum activity at pH 6-7 and at temperature of 60°C (Thompson et al., 1973; Adulyatham

and Owusu-Apenten, 2005; Bhaskar et al., 2006). The proteolytic activity was decreased (≈15%) by 2% NaCl and 75% of the activity was lost by heating at 70°C (Thompson et al., 1973). The half life of the enzyme at 60°C is about 2.3 min in the absence and 24 min in the presence of 0.2% sodium ascorbate. The GRAS status have been given to several fractions of ginger (GRAS 2520-2523) but not for Zingibain. The protease has been reported to have higher specificity toward collagen compared with actomyosin (Thompson et al., 1973).

Characteristics of microbial enzymes

Fungal enzymes

The proteolytic activity of many of the fungal strains has been known for many years and has a long history in traditional food production. The actions of fungal proteases plays a very important role in products such as Nato, Miso and Soy sauce and these products are used as a source of microorganisms for the production of proteases or tenderizing preparations. Ahmed et al. (2006) reported a tenderizing effect for fermented apple juice and Miso, which may in part relate to microbial activity of the culture used. Several proteolytic preparations from *Aspergillus oryzae* are available commercially. The preparation may contain several GRAS (FDA, 1995; 1999; 2009) proteases that have wide pH action such as neutral protease (EC 3.4.24.4), alkaline protease (EC 3.4.21.14) and aspartic protease (EC3.4.23.6). These proteases generally have a mild or no effect on the myofibrillar proteins, but possess a good proteolytic action against both collagen and elastin. The proteases are active over a very wide pH range and have a moderate temperature of denaturation (≤ 70°C). For example, aspartic protease from *Aspergillus oryzae* has an optimal activity at a pH range of 2.5-6 and less than 20% of its activity remains after

cooking at 75°C (Ashie et al., 2002). Aspartic protease has no effect on collagen (Ashie et al., 2002) and limited effect on myofibrillar proteins compared to plant protease with self-limiting hydrolysis at high concentration (Cronlund and Woychik, 1986; Ashie et al., 2002).

Bacterial Enzymes

The proteolytic activity of many bacterial strains has been known for many years. Many bacteria strains (e.g. Bacillus) play a very important role in protein degradation in fermented meat and fish products (Bekhit, 2010). Subtilisin (EC3.4.21.62) and neutral protease (EC3.4.24.28) are the main proteases from *Bacillus subtilis* and *Bacillus subtilis* var. *amyloliquefacien* and have GRAS status (FDA, 1999). Proteases from *Bacillus licheniformis* also have GRAS status (CFR184.1027). Generally, proteases from bacterial sources have relatively specific activity and low inactivation temperatures making them useful for meat tenderization. For example, alkaline elastase from *Alkalophilic Bacillus sp. strain Ya-B* demonstrated an optimum activity in the pH range of 5.5-6.0 and temperature range of 10-50°C (Takagi et al., 1992; Yeh et al., 2002). Bacterial proteases have lower hydrolytic activity toward myofibrillar proteins compared to plant proteases, but their ability to hydrolyze collagen was intermediate to that found bromelain and papain (Yeh et al., 2002).

Measurements of proteases activity

Several methods, spectrophotmetric, colourimetric, fluorimetric, radiometric, holographic, solid phase, chromatographic and more recently cyclic voltametric methods have been used to determine proteolytic activity (Sarath et al., 1989; Baş and Boyaci, 2010). Various materials have been used as substrates (e.g. milk, haemoglobin, gelatine, casein and labelled

casein products, collagen disc and labelled collagen products, and synthetic amino acid substrates). Casein products and synthetic substrates are the most common substrates used in characterization of proteases and for determining the proteolytic activity of the proteases. Native (Kholif et al., 2011) and denatured (Wretlind and Wadström, 1977) casein or casein labelled with chromophoric (Ladd and Snow, 1993; Chang et al., 1981) or fluorogenic probes (Bolger and Checovich, 1994; Farmer and Yuan, 1991) are used to quantify the proteolytic activity of the enzymes. Synthetic substrates containing amino acids (e.g. Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide and CBZ-Lys-*p*-nitrophenol) are commonly used to determine the esterase activity of the enzymes. Elastase activity of the proteases is determined using elastin labeled with chromophoric compound (e.g. elastin-congo red) (Sachar et al., 1955; Wretlind and Wadström, 1977). Collagenase activity of proteases is determined using collagen discs (Smyth and Arbuthnott, 1974) or azo-dye impregnated collagen, Azocoll, (Chavira et al., 1984). While the above methods are useful as means for the quantification and characterization of the proteolytic activities among different enzymes, they do not reflect the actual activity of the enzyme in meat where a more complex protein structure is encountered. There is a dearth of literature on the quantitative characterization of the proteases using meat components.

Meat tenderization using exogenous proteases

Numerous studies have used plant proteases (papain, bromelain and ficin) to improve meat tenderness, dominating the literature before 1980 (appendix Table 2). Other plant proteases (actinidin and zingibain) were investigated a few decades ago (Lewis and Luh, 1988; Thompson et al., 1973; Lee et al., 1986) and interest in their application has revived more recently

(Mendiratta et al., 2000; Naveena et al., 2004; Bekhit et al., 2007; Christensen et al.; 2009; Toohey et al., 2011) because they have a milder tenderizing effect or a greater specificity toward either connective tissues or myofibrillar proteins. The traditional plant proteases tend to have broad specificities and indiscriminately break down connective tissue and myofibrillar proteins (Foegeding and Larick, 1986; Miller et al., 1989; Ashie et al., 2002). This leads to some undesirable attributes in the tenderized meat (mushy texture, bitterness, off flavour) and thus proteases with more specific hydrolyzing activity were needed and this directed the research toward microbial proteases (wild or modified) from non-pathogenic and safe microorganisms. Collagenases from *Clostridium histolyticum* (Foegeding and Larick, 1986) and *Vibrio* B-30 (Miller et al., 1989); and elastases from *Alkalophilic Bacillus* (Takagi et al., 1992) and *Bacillus sp. EL31410* (Qihe et al., 2006) have been used in meat studies. Proteases with activity against both myofibrillar and connective tissue proteins from *Bacillus strain E A.1*, *Thermus strain Rt4-1.A* and *Thermus strain T-351* (Wilson et al., 1992), *Penicillium chrysogenum* Pg222 (Benito et al., 2003), *Pseudomonas perolens* (Buckley et al., 1974), *Aspergillus sojae* (Gerelt et al., 2000), *Aspergillus oryzae* (Gerelt et al., 2000; Ashie et al., 2002), *Rhizomucor miehei* (Stefanek et al., 2002; Ashie et al., 2005) have been studied. Furthermore, several animal proteases (Phillips et al., 2000; Pietrasik et al., 2010) have been recently reported to have limited success (appendix Tables 1 and 2). Other promising sources of plant proteases (soy bean, melon and caper) and microbial proteases (*Aspergillus niger*, *Streptomyces griseus* and *Bacillus subtilis*) have been reported (Schwimmer, 1981), but have not been exploited to improve meat tenderness.

The effective and successful use of any of the proteases mentioned above lies in understanding the characteristics and strength of the protease to be used and the environment and

conditions where it will be used. A main pitfall in the literature is the use of many varying concentrations, sources of materials and assays to determine the enzymes activities. Calkins and Sullivan (2007) discussed the conditions of 8 enzyme assays used with proteases (Milk Clot Unit, Tyrosine Unit, Plant Proteolytic (Papain Unit), Casein Digestion, Gelatine Digestion, Haemoglobin Unit, Proteolytical Unit, Spectrophotometic Acid Protease Unit). The assay pH varied from 4.5-7 and the assay temperature varied from 30-45°C and several synthetic substrates [labelled casein (BODIPY) and labelled collagen (Azocoll)] are used. However, the use of meat fractions [myofibrillar, collagen and elastin] in characterizing the proteases is not commonly used. All of these assays, often not correlated, can be different from the actual activity in meat and make it difficult to relate different published reports. Clearly the need to have a standard method that can be used by different labs and easy for the industry to use regularly is needed.

Tenderization of meat with papain

Papain can hydrolyze almost any peptide bond. Chymopapain seems to play the major role in the tenderization with an optimal pH around 5 whereas the optimal pH for papain and papaya peptidase A is around 7-8 (Schwimmer, 1981). Papain has the ability to degrade connective tissue and myofibrillar proteins. The temperature range for its activity is high with a rapid increase in the enzyme activity above 50°C with maximum activity at 60-65°C (Dransfield and Etherington, 1981). Papain has very low activity at the temperatures that are normally used for the storage of meat post-mortem (90°C for complete inactivation (Dransfield and Etherington, 1981). Rhodes and Dransfield (1973) studied pre-slaughter injection of papain to

overcome the toughness caused by rapid chilling of lamb carcasses. Maximum tenderization was observed at 70°C and the tenderizing effect was increased by increasing the amount of papain.

The injection of papain ante-mortem (10-30 minutes before the slaughter of the animal) to achieve a uniform distribution of the enzyme in the animal carcass and even tenderization process was developed by Swift and Company Ltd (Hogan, 1962; Kang et al., 1974; Kang and Warner, 1974; Kang et al., 1982 a, b) in a process termed (ProTen). Active papain cannot be injected directly in live animals as it will lead to severe shock and stress symptoms (Kang and Warner, 1974; Figure 2 from (Dransfield and Etherington, 1981). An alternative approach involving injection of inactive papain (in which the catalytic cysteine was oxidised using hydrogen peroxide) through the jugular vein before slaughter has been investigated. This inactive form is reactivated in the meat post-mortem due to the anoxic conditions that promote the reduction of the cysteine residue of the active site of the enzyme and protein degradation took place during cooking (Rhodes and Dransfield 1973).

The level of papain required for achieving suitable tenderization is in the range of 2-5 ppm (Dransfield and Etherington, 1981). However, the tenderization with this method had several drawbacks (Bailey and Murdock, 1991) and the tenderizing potential is difficult to predict due to physiological factors in live animals (Kang and Warner, 1974). For example, the texture of the tenderized meat is different from good quality meat; meat can be over-tenderized and may develop objectionable flavours. Also, ante-mortem injection with proteases can lead to extensive degradation of several organs that may be of commercial interest (such as liver, kidney) (Bailey and Murdock, 1991). However, Bradley et al. (1987) concluded that there were

no clinical ante-mortem biochemical and post-mortem morphological changes as a result of the ProTen^{TM} treatment and thus no evidence that pain had been experienced.

The method of delivery is important in achieving an appropriate level of tenderness. Proteases are available commercially in powder and liquid forms. Several commercial preparations that include other ingredients (salt, phosphates or flavourings) are available to be used directly by processors and households. It must be kept in mind that tenderization is time and temperature dependent, so it might be appropriate to surface treat meat at home, but in large processing facilities better incorporation methods such as injection and tumbling, are required (Tappel et al., 1956). Also, depending on the end market, an appropriate preservation method should be included (chilling or freezing). Some of the bacterial proteases appear to have a self-limiting capacity and over-tenderization can be avoided when they are used in meat.

Specificity toward meat proteins

Generally, all plant proteases can hydrolyze both connective tissue and myofibrillar protein. The indiscrimate effects of papain on muscle proteins during heating was first reported by Tappel et al. (1956) and they recognised the need to have better incorporation methods since papain penetration through the meat was limited. The enzymes do not hydrolyze native collagen in raw meat, but they work on the denatured form during cooking (Wada et al., 2002). On the other hand, microbial proteases exhibit more specific activity toward substrates in meat. For example, aspartic protease expressed in *Aspergillus oryzae* acts only on myofibrillar proteins without any effects on connective tissue (Ashie et al., 2002). Similarly, elastase from *alkalophilic Bacillus* demonstrated very low activity toward myofibrillar proteins and specific

activity toward elastin (Takagi et al., 1992; Yeh et al., 2002). An interesting feature of some of the microbial proteases is the self-limiting activity which eliminates the risk of over-tenderization (Ashie et al., 2002). Aspartic protease from *Aspergillus orayzae* is inactive during post-mortem vacuum storage for 7 and 14 days at 4°C (Pietrasik and Shand, 2006).

Effect of concentration

Bromelain (0.29 mg protease/122.5 mg meat protein) was found to be more active against collagen proteins and less active toward myofibrillar proteins than papain (0.19mg/122.5 mg protein) or ficin (0.09 mg/122.5 mg protein) and effective at improving meat tenderness (Kang and Rice, 1970; Fogle et al., 1982). Foegeding and Larick (1986) compared the efficacy of commercial collagenase (0.2 mg/ml), bromelain (0.2 mg/ml), ficin (0.06 mg/ml) and papain (1.0 mg/ml) on the hydrolysis of collagen and myofibrillar proteins. In disagreement with Kang and Rice (1970) and Fogle et al. (1982), Foegeding and Larick (1986) found the efficacy of collagen solubilisation was in the following order; collagenase > ficin > bromelain > papain, whereas activity with myofibrillar proteins was in the following order; bromelain> ficin > papain > collagenase.

More recently, Calkins and Sullivan (2007) reported the hydrolysis of collagen by papain (9 ppm), bromelain (14.5 ppm) and ficin (9 ppm) to be in the following order; papain > ficin > bromelain. Fogle et al. (1982) also demonstrated that papain is a more effective meat tenderizer than bromelain and that the tenderizing effect was concentration dependent for both enzymes. Clearly, there is no agreement among the reports and it is difficult to achieve any general trends as the concentrations vary among the studies. Crude ginger extracts appear to not have the

concentration-tenderization relationship that was found with papain, ficin and bromelain (Lee et al., 1986).

It is important to keep in mind that the effective concentration of a protease will depend on the meat cut, storage temperature and the use of other interventions. Berry and Cross (1982) demonstrated a synergetic positive effect for the use of a papain-bromelain mixture, blade tenderization and aging combination on the tenderness and the sensory properties of longissimus lumborum (LL) and semimembranosus (SM) muscles from old cows. They also reported that the synergy was better in cold boned LL compared with hot boned LL. However, it seems that freezing the hot boned LL samples (-39°C) in their study caused severe cold shortening as indicated by the very low sensory scores for the untreated control samples. In a more recent study in which a kiwi-fruit juice based solution was injected into the SM from young cattle and the SM was then packaged using SmartStretch™ (Taylor and Hopkins, 2011), it was found that this process produced a significantly more tender meat as measured by shear force (Toohey et al., 2011). Interestingly, there was no effect however on compression of the SM which suggests no impact on the connective tissue of the SM.

Effects of temperature

Using a synthetic substrate (Azocoll), Foegeding and Larick (1986) reported minimal *in vitro* proteolytic activity in the temperature range of 20-40°C for commercial collagenase, bromelain, ficin and papain. The maximum temperature range was 40-60°C for collagenase; > 40°C for bromelain and ficin and > 50°C for papain. Heating at 70°C did not affect the activity of bromelain, ficin and papain, but the collagenase activity was abolished. Because of the high

inactivation temperature for papain (90°C) and bromelain and ficin (≈75°C), there is potential for high residual activity after cooking, especially with medium or rare degrees of doneness, which can lead to over-tenderization. Zingibain has an optimum activity at about 60°C and 75% of the activity is lost at 70°C (Thompson et al., 1973) which explains its mild tenderizing effect. Microbial proteases generally have a low inactivation temperature. As mentioned above, aspartic protease loses more than 80% of its activity during cooking at 75°C (Ashie et al., 2002). It is widely believed that the tenderizing effects of plant proteases occur only during cooking. However, Samejima et al. (1991) demonstrated that the immersion of raw meat in a crude actinidin solution at 4°C caused structural changes. This however, was not the case with proteases from *Aspergillus oryzae* (Pietrasik and Shand, 2006). As with all of the reactions that involve enzymes, it is normally dependent on time, temperature and the enzyme-substrate ratio and these factors need to be considered during the application of and optimization of a protease meat-tenderizing procedure.

Cooking method plays a very important role in achieving the required tenderness in papain and bromelain treated meat. For example, Fogle et al. (1982) reported that higher activity of the proteases and tenderization was achieved in slow cooking compared with fast cooking, and thus lower papain and bromelain concentrations were required in slow cooking than in fast cooking to produce the same level of tenderness. This means that the amount of enzymes to be injected in meat should be tailored according to the destined use of the meat and the cooking method that will be used.

Impact on other meat quality traits

Bromelain generally generates a better flavour than papain (Kim and Taub, 1991). Papain causes the formation of a mushy texture and off flavours (McKeith et al., 1994; Stefanek et al., 2002). High concentration of some microbial proteases can have a similar negative impact on the sensory properties of meat (Wilson et al., 1992) and generate bitterness in treated meat (Qihe et al., 2006). On the other hand, crude ginger extracts can improve the keeping qualities of the meat during storage (decrease lipid oxidation and microbial growth (Mendiratta et al., 2000) and improved the eating quality of the meat (Naveena et al. 2004). Similarly, Bekhit et al. (2007) found that kiwifruit juice decreased lipid oxidation and slightly improved the colour in lamb after 3 weeks of vacuum packaging without over-tenderization of the meat (Figures 3 and 4), whereas Toohey et al. (2011) reported a decrease in colour stability for SM injected with a kiwi-fruit based solution. However, the lack of positive effect on colour stability in the study of Toohey et al. (2011) may well be due to the loss of the antioxidants during preparation filtration, whereas in the study of Bekhit et al. (2007) the solution was not subjected to phases of filtration (Han et al. 2009).

Potential problems associated with the use of exogenous enzymes in meat tenderization

Allergic reactions due to the use of or exposure to papain (Tarlo et al., 1978; Díez et al. 1998), ficin (Díez Gömez et al., 1998; Focke et al., 2003), bromelain (Nettis et al., 2001) and actinidin and thaumation-like protein from kiwifruit (Pastorllo et al., 1998; Bublin et al., 2008) have been documented. On the other hand several health claims for ginger extracts have been reported (Butt and Sultan, 2011). Patients with kiwi allergy show immunological reactivity to papain from papaya and bromelain from pineapple (Gall et al., 1994), indicating a similar level

of risk to those patients regardless which of the above proteases is used. Heat and digestion can affect the reactivity of actinidin but not thaumation-like protein and this later was found in processed foods (Bublin et al., 2008) indicating its stability during progressing. Information on the effect of processing conditions and digestion for papain, ficin and bromelain are not available. Also, information on the allergenicity of microbial proteases is not available. The information on the potential allergic effects of the proteases should not be alarming since a large number of vegetables and fruits are have the same level of allergenicity (Breiteneder and Radauer, 2004), many permitted food additives (Taylor and Hefle, 2001) and even meat itself could be allergenic for certain individuals (Restani et al., 2009). As with all other potential allergens-containing foods; education and appropriate labeling would help meat treated with proteases to be common products.

Regulation of the proteases activity

Cysteine proteases contain several cysteine residues of which one (Cys 25 in the case of papain) is part of the catalytic triad in the active site and is thus essential for catalysis carried out by these enzymes. The activity of these proteases is regulated by the oxidation/reduction status of the catalytic site and they share similar effects in the presence of activator and inhibitor compounds due to their structural homology. Generally speaking, EDTA, reducing compounds (cysteine, sodium thiosulfate, DTT) and calcium ions can activate the proteases (Tables 1-5). Several natural and synthetic compounds can inhibit the proteases, but no information is available on the impact of some possible natural inhibitors present in meat.

Free cysteine, a reducing agent, has been shown in various studies to be able to activate papain. An early study by Fruton and Bergmann (1940) using synthetic peptides showed that addition of free cysteine to the reaction mix enhanced papain activity by at least 20 fold. The addition of free cysteine to papain can reverse the enzyme inactivation caused by metal ions (Sluyterman, 1967). Papain exhibited maximum activity in the presence of cysteine at 200 mM (Homaei et al., 2010). In the presence of cysteine the optimal temperature for papain activity was increased by 20°C (from 60°C to 80°C) and the pH range of papain activity was extended toward both pH extremes. Interestingly, the study of Sluyterman (1967) also found that papain was inactivated in the presence of air and a low concentration of free cysteine, indicating that the activation mechanism of papain by free cysteine is concentration-dependent. Beside cysteine, other compounds such as EDTA, DTT, KCN and HCN have also been identified as activators of proteases of the papain family (Fruton and Bergmann, 1940; Ohtsuki et al., 1995; Sluyterman, 1967).

In contrast, oxidising agents such as nitric oxide (or its donors) are capable of inhibiting the proteolytic activity of papain and papain like proteases in a reversible manner. Five different S-nitroso compounds inhibited papain in a time and concentration dependent manner (Xian et al., 2000). This study also reported that the incubation of a fluorescent probe (S-nitroso-5-dimethylaminonaphthalene-1-sulfonyl) with papain resulted in the appearance of fluorescence of the enzyme indicating the formation of a thiol adduct. These results collectively verify the mechanism of papain inactivation in which disulfide bonds are formed between the Cys 25 in the enzyme active site and a highly reactive thiolate in the inhibitor. Interestingly, ascorbic acid, which has been regarded as a highly active antioxidant and powerful reducing agent, exhibits an

inhibitory effect on papain activity. A study by Ockerman et al. (1993) has shown both L- and iso-ascorbic acid inhibited papain activity by over 87% and the inhibitory effect increased with concentration and with incubation time. Ascorbic acid exhibits pro-oxidant properties in the presence of metal ions such as $Fe^{3+}$and Cu^{2+} by interacting with the ions to generate hydroxyl free radicals (OH) which are strong reactive oxygen species (Halliwell, 1996). Other inhibitors of cysteine proteases include natural inhibitors such as cystatins and exogenous inhibitors such as Cu^{2+}, KCl, NaCl and potato proteins (Morimoto et al., 2006; Ockerman et al., 1993; Ohtsuki et al., 1995). A key important characteristic of any potential inhibitor to be used successfully in meat applications to control the proteases excessive tenderization is to be food grade. Future research should be focused on investigating food grade compounds as potential protease inhibitors with the aim of controlling the level of tenderization of meat.

Methods to reduce the over-tenderization effect of plant proteases

The use of inhibitors to reduce the activity of plant proteases has been investigated with some success (Tables 1-5). Funaki et al. (1991) found that a good degree of tenderization, without the over-tenderizing effect of papain, could be achieved by incubating meat with a 2% papain solution (for 2 hours at 8°C followed by 22 hours of incubation with a cysteine protease inhibitor (oryzacystatin from rice seeds). Cystatin from egg white (4% egg white can reduce 87% of the activity) can also reduce the activity of papain (Weerasinghe et al., 1996a,b). Ascorbic acid (at 2.5×10^{-3}M) inhibited papain activity in meat, but induced off-flavours (Ockerman et al., 1993). Potato powder (1%); whey protein concentrate (3%) and natural cysteine proteases can inhibit papain activity (Table 1). New emerging technologies (such as pulsed electric field) may

play an important role in limiting the over-tenderizing effect of proteases (Yeom et al., 1999). Several protease inhibitors are available in blood plasma (kininogen, protease inhibitor 1-4, postalbumin 1A and 1B, and α_2- macroglobulin) that proved to be useful in the fish industry (Seymour et al., 1997; Kang and Lanier, 2005), The use of plasma proteins from bovine, pork and chicken as protease inhibitors in the fish industry has the negative side of imparting undesirable reddish colour, which is not a problem if used in red meat. Several plants, especially legumes, have protease inhibitors (Garcia-Carreno et al. 1996; Kang and Lanier, 2005) which could be potentially useful. Clearly any successful use of these compounds as future inhibitors will be dictated by the safety of the effective level of use, consumer acceptability (e.g. fraction from serum of taboo animals in certain societies), effects on the meat sensory quality (specifically flavour) and cost associated with the use of compounds.

Conclusions

Several plant; microbial and animal proteases are available for meat tenderization. Despite the abundant information regarding the *in vitro* kinetics and mechanism of action of plants proteases available in literature, a meaningful use of this information is hindered by several factors. For example, the variation in the source of the enzymes and the degree of purity of the commercial preparations will mean that the composition of the enzymatic preparation will vary with source, especially when several isoforms of the enzyme exists. This has led to conflicting information among different labs with regard to effective enzyme concentrations and the most suitable enzyme available for tenderization. Also, the kinetics of proteases generated using synthetic substrates does not reflect the expected enzymatic reaction rates and behaviour

with meat proteins due to the complexity of structure and diversity of proteins in meat. The actions of the enzymes in meat will be dependent on the concentration (enzyme:substrate ratio) and the time-temperature profile, therefore the concentration of enzyme used, temperature of treated meat and storage temperature. The literature lacks information on the effects of exogenous proteases at different post-mortem times and the effect of cooking (rate of cooking and endpoint temperature) on the tenderization rate and this requires further research for optimal use of these proteases. The method of application (coating, marination, injection, or tumbling) could have a dramatic effect on the level of tenderization obtained. Other issues that remain to be resolved are the level of proteolysis that takes place during post mortem storage at refrigerated temperatures, the effect of post mortem injection time and whether any benefits can be gained from pre-rigor vs post rigor application of exogenous proteases.

Since, the composition of commercial preparations may vary depending on the source and the preparation methods used, characterization of the enzymes is required before successful use in meat application to gain some insight about the activity and purity of the enzyme preparation. The challenge for the meat research community therefore is to design a universal and simple assay to assess the activity of enzyme preparations against both connective tissue and myofibrillar proteins which can be translated into expected outcomes in regard to the tenderizing effects.

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Appendix Table 1. The characteristics and activity of different plant, microbial and animal proteases investigated in meat tenderization.

Enzyme	Source	Purification/ Characteristics	Activity/ Substrates	Activators	inhibitors	Refere
Actinidin (EC3.4.22.14)	Kiwifruit (*Actinidia deliciosa*)	-Purified from kiwifruit (Hayward cultivar). Ammonium sulfate (60% saturation) precipitation from Kiwifruit extract. The precipitate was redissolved in 50 mM citrate buffer (pH 5.5) and dialyzed overnight against this buffer. -DEAE-Sepharose Fast Flow column and the adsorbed fractions were eluted with 0.0–1 M linear gradient of NaCl in the buffer.	-Higher collagen hydrolysis at higher pH (moderate at 5.5, high at 7 and 8.5)	-	-	Mostafa et al. (2
Alkaline elastase	alkalophilic Bacillus sp. strain Ya-B	-pH range of 5.5-6.0 and the temperature range of 10-50°C.	-Elastolytic activity of the new elastase was			Takagi al. (199 Yeh et a (2002)

	strain Ya-B or mutant of it	range of 10-50°C.	60-200 times greater than that of papain and bromelain. -Lower hydrolyzing activity toward casein and myofibrillar proteins at a lower pH. Mild tenderizing action.			(2002)
Capparin	Caper *(Capparis spinosa)*	-The purified enzyme (MW= 46 kDa) has an optimum activity at pH=5.0 and 60°C.	-The enzyme (0.05 mg) digested $5.4*10^{-3}$ mg of chicken and $9.4*10^{-3}$ mg of cow meat in 1 min. The best digestion at 60 for chicken meat and 50°C for cow meat. -Activity with gelatine > azocasein > casein.	-Ca^{2+}, $Mg^{2+}$and Zn^{2+} activated the enzyme at lower conc.	-Low Conc. Hg^{2+} while high Conc. Co^{2+} for complete inhibition.	Demir e (2008)
Placental protease	bovine	-Enzymes often exist in latent form require activation by addition of p-aminophenylmercuric acetate (APMA) to a final concentration of	-No proteolytic activity detected with casein, actin, or myosin heavy-chain substrates. The enzymes are	-	-	Phillips al. (200

		0.5 m*M* followed by 4 h incubation at 37°C.	specific for collagen and gelatine myofibrillar protein hydrolysis. -The enzymes may be incorporated in a mixture targeting connective tissues for tenderizing meat.			
Papain (EC 3.4.22.2) Bromelain (EC 3.4.22.4) Ficin (EC 3.4.22.3)	Papaya latex Pineapple stem Fig latex	-The crude extracts maybe a mixture of papain, chymopapain and lysozyme with pH range 5-9pH (5-8) broad specificity (≈ 21-23 kDa) pH (5-8) broad specificity (28-33 kDa) pH (5-8) broad specificity (26 kDa)	-	-	-	Adler-Nissen (1993) Knee et (1991)
Collagenase (Sigma type VII)	Produced by *Clostridium histolyticum*	-Commercially obtained	-*Collagen solubilisation* Collagenase (0.2 mg/ml) > Ficin (0.06mg/ml)> Bromelain (0.2 mg/ml)> Papain	-	-Collagenase was activated by CaCl2[@]	Foegedi and Lar (1986)
Bromelain						
Ficin						

Papain			(1.0 mg/ml) *-Salt-soluble proteins* Bromelain>Ficin > Papain* > Collagenase			
Capparin	Caper *(Capparis spinosa)*	-The purified enzyme (MW= 46 kDa) has an optimum activity at pH=5.0 and 60°C.	-The enzyme (0.05 mg) digested $5.4*10^{-3}$ mg of chicken and $9.4*10^{-3}$ mg of cow meat in 1 min. The best digestion at 60 for chicken meat and 50°C for cow meat. -Activity with gelatine > azocasein > casein.	-Ca^{2+}, $Mg^{2+}$and Zn^{2+} activated the enzyme at lower conc.	-Low Conc. Hg^{2+} while high Conc. Co^{2+} for complete inhibition.	Demir e (2008)
Pancreatin	Ox or pig	-Mixture of trypsin, chymotrypsin, elastase and carboxypeptidase (commercially available)	-	-	-	Pietrasil al. (200

Zingibain	ginger *(Zingiber officinale)*	-Crude extract [Freshly diced ginger] was homogenized with 4 parts (w/v) cold Tris-HCl buffer (0.05 *M*, pH 8) filtered through 4 layers of muslin and the filtrate centrifuged (12000 × *g*) at 5°C for 20 min. The supernatant was stored at 5°C. (GP) -Ginger acetone powder [Diced ginger was frozen at –30°C], homogenized with 4 parts cold acetone (–30°C) for 90 s and allowed to stand for 15 to 20 min. The precipitate formed was collected by vacuum filtration through Whatman No. 1 paper. Suction was continued with further washes with 5 parts (v/w) cold acetone followed by air drying.](GPAC) -Higher activity can be obtained with ethanolic extraction -Enzyme (MW= 34.8 kDa) maximum activity at 6-7 pH and at 60°C.	-Negligible effect on GP stability or specific activity: EDTA (1 m*M*), DTT (0.3 m*M*), and Ca Cl_2 (100 m*M*). -Crude GP extract from ginger was unstable. Half life ($t_{1/2}$) decreased from 2.1 d at 5°C to 17 min at 30°C. -At 60°C, $t_{1/2}$ for GP was 2.3 min or 24 min in the absence or presence of 0.2% (w/v) ascorbic acid, respectively. At temperatures of <50°C, GP was stabilized by ascorbate. -Sodium ascorbate (0.2% w/v) stabilized GP and no activity loss occurred after 14 d at 5°C.	-	-	Adulyat and Ow Apenter (2005); Bhaskar al. (200

		-Cysteine protease. Maximum activity of crude extract (obtained from acetone precipitate) was when extraction buffer (phosphate buffer, 0.1 M) was at pH 6. -At least two enzymes with maximum activity at pH 5 and 5.6 with BSA. -Heating at 70°C reduced the activity by ≈75%)	-Higher specificity toward collagen compared with actomyosin. -Solulibilization at 5°C occurred at for both actomyosin and collagen -Meat tenderness (≈45% reduction in WBSF) (BF injected with 0.2mg/ml and stored for 20 hrs at 5°C) was reported and mushiness was mentioned where		-2% Salt reduced the activity by ≈15%. -Higher concentrations resulted in more inhibition toward BSA compared with collagen.	Thomps et al (19

*The assay temperature was too low for Papain activity

@ the results seems not confounded with activation with Calpains as the results was based on collagen substrate assay

Appendix Table 2. Summary for the impact of using plant, microbial and animal proteases on meat tenderness and quality.

Animal	Treatment Tenderizing Infused compounds	Experimental parameters	Impact on meat					Reference
			tenderness	Colour	Sensory Flavour/acceptability	Other	outcome	

Cattle	Bromelain (0.29mg) Collagenase (1 mg) Ficin (0.09 mg) Papain (0.19 mg) Rhozyme P-11 (RP-11; 14.4 mg) Trypsin (0.4 mg)	Water, salt soluble proteins and insoluble fraction were treated with the enzyme.	- Hydrolysis of connective tissue Collagenase >Bromelain> Trypsin>ficin > RP-11>Papain - Hydrolysis of myofibrillar proteins: Papain > Ficin> RP-11 > Trypsin > bromelain > collagenase.	-	-	-	Combination may lead to better systems for meat tenderization	Kang & Rice (1970)

	Commercial preparation containing papain (Griffith 77-A) or bromelain(Griffith 77-C	Brisket meat cores (from 3-5 years old cows) were treated individually with solution of the preparations at different enzyme level and either cooked fast or slow	Tenderization rate was ↑ with ↑ enzyme level. Papain slow> papain fast>bromelain fast> bromelain slow.	-	-	-	The amount of enzyme to be used should be tailored to cooking method to be used	Fogel et al. (1982)

	Blade (BT) Papain-bromelain (Enz) Aging (days)	LD and SM muscles from old cows were assigned to one of the following treatments: 1) film wrap, freezing at -39°C, BT, ENZ; 2) film wrap, aging for 7 days at 3°C, freezing at -39°C, BT, ENZ; 3) VP, aging for 14 days at 3°C, freezing at -39°C, BT, ENZ; 4) Film wrapped and frozen at -39°C.	Synergetic effect for the combined interventions with aging the treated samples produced the best tenderizing effect.	-	-	Higher or no effect on Juiciness in treated samples depending on the muscle and the cooking method.	Tenderization of LD and SM from utility grade carcasses can be achieved by combined interventions.	Berry and Cross (1982)

	Crude Ginger extract	Steaks and sliced beef (LD, BF and SM) marinated with different levels of GCE (0-0.4 ml/6.5 cm^2 for steaks; 0-3.0 for QF).	↑Tenderization in LD, SM and BF with >0.1 ml/6.5 cm^2 and 0.5 ml/6.5 cm^2 for QF.	-	-	-	Low concentration can tenderize the meat. Higher concentration will not add further value.	Lee et al. (1986)
	Papain (0.1%) Bromelain (0.1%)	Beef round samples were mixed with the enzyme and left at 24°C for 0, 10, 30 and 60 min at pH 5.8.	More proteolysis by both enzymes. Bromelain is less selective toward actin.	-	Flavour better with bromelain	-	Less effect on meat texture may make bromelain a better candidate protease.	Kim and Taub (1991)

	Thermophile enzymes E A.1 protease (from Bacillus strain E A.1), 4-1.A protease (from Thermus strain Rt4-1.A) and caldolysin (from Thermus strain T-351)	The proteases were tested on meat powder and beef patties	E A.1 and 4-1.A proteases were more active on collagen than on meat powder at cooking temperatures (70-90°C), whereas caldolysin was more active on meat powder. The best protease concentrations (of those used) were found to be 0.75 U/g meat for E A.1 protea	-	-	At high concentrations the proteases had a detrimental effect on the mouth feel of the patties.	E A. 1 protease, had low activity against meat powder and high activity against collagen which suits the requirements of a good meat tenderizer.	Wilson et al. (1992)

	Bromelain or Papain	ST steaks injected, dipped or tumbled to contain 50 or 100 ppm. ST roasts injected or tumbled with 10 0r 20 ppm of bromelain or 2.5 or 5 ppm papain	Increased tenderness.	-	More intense off flavour with papain than bromelain.	More bound water in raw state but not in cooked. Mushy texture with papain than with bromelain. The off	Mushy texture with the higher 100 ppm compared with 50 ppm. Injected were more mushy, less connective and had more weight loss than tumbled.	McKeith et al. (1994)

	proteases from Aspergillus sojae and Aspergillus oryzae Papain	meat was osmotic dehydrated by contacts sheets for 18 hrs, dipped for 3 hrs in a solution containing papain or proteases from Aspergillus and stored at 3-4°C for 24, 48 and 168 h	Improved instrumental and sensory tenderness in the meats treated with proteolytic enzymes.	-	The papain-treated meat received the highest score in tenderness, but the juiciness and taste scores were lower than that of the control. Bitterness in papain treated meat but not other proteases.	Increased myofibrillar fragmentation and proteolytic removal of Z-lines. Disruption of the endomysium structure	Mild tenderizing effect from Aspergillus proteases makes them potentially useful for the meat industry.	Gerelt et al. (2000)

	Ficin, bromelain or NCT	-Inside rounds (n=5 for each) injected (20 and 40 ppm for bromelain and ficin, and 0.003 AU/100g meat for NCT). - Injection to 103% weight, VP for 36 hrs (4°C). The tenderizing effects of the high level of NCT and ficin were examined in repeated trials. - The roasts were cooke	Overall tenderness NCT= ficin > bromelain. WBSF did not show differences over storage time. Sensorial tenderness and scores for myofibrillar tenderness and amount of connective tissues: NCT= ficin > control.	-	Less flavour and juiciness in protease treated samples Flavour: NCT= Ficin< control Juiciness: NCT= Ficin< control	-	-	Stefanek et al (2002)

	Aspergillus oryzae expressed Aspartic protease (AP) Papain	Top rounds and briskets injected with enzymes (0.002 to 0.05 AU/100g meat) to 5% increase in weight. Tumbling (5rpm under vacuum) WB shear force	↑ Tenderness with increasing the Papain dose. Small increase in tenderness (<20%) with AP at 0.05 AU/100 g meat and further improvement at higher concentrations.	-	-	-	-AP has limited impact on tenderness (<20% compared with 70% for papain at 0.05 AU) for brisket. -0.01 AU had 25% and 75% improvement for AP and Papain, respectively. -Papain degraded myofibrillar and collagen proteins, whereas AP is not affecting collagen. The change in the tenderness was not increased with storage time in AP unlike Papain.	Ashie et al. (2002)

	Solution containing Papain (2,000 and 20,000 U/100 ml) and elastase (3 U/100ml)	- Freeze-dried meat cuts (5X5X3 cm) from the foreleg of cull cow dipped (5:1) in the test solutions or water (control). - Incubated at 4°C for 1 day (shear force) or 3 days for sensory.	Higher tenderness with proteases. Papain 20000 > 2000≥ elastase >>control.	-	Elastase exhibited better juiciness than control and papain, and better flavour than control.	Higher bitterness with elastase	The degradation of elastin and collagen being specific avoid the over tenderizing effects caused by papain	Qihe et al. (2006)

Cattle	Aspergillus oryzae Protease	**Exp1** SM was injected (105% of green weight) with 0.001, 0.0025 or 0.005 % in product, VP and stored at 4°C for 1, 7 and 14 days. **Exp2** SM muscles were injected 0.0005, 0.001 or 0.0015, VP, stored at 4°C over night and cooked at 71 or 79°C either dry or moist.	Tenderness improved with 0.001 % (≈25% reduction in WBSF). The tenderness increased with the AP protease concentration in the range 0.005-0.0015 % with higher tenderness in moist cooked samples.	-	-	-	0.005% produced mushy and creamy texture. The enzyme was inactive during the VP storage for 14 days at 4°C and it functions during cooking.	Pietrasik and Shand (2006)

	porcine pancreatin	beef SM (2days at 0-2°C, VP for 7 days at 3°C, frozen at -30°C, thawed at 3°C for 3 days) injected (15%) with 0.02% liquid porcine pancreatin, or with 0.02% liquid porcine pancreatin +0.5% sodium chloride +0.25 % tripolyphosphate. The samples were halved and either blade tenderi	Enhancement solution or BT treatments were effective in tenderizing SM, but not the enzyme (both sensory and WBSF).	-	Juiciness and flavour improved with enhancement solution. No effect for the enzyme.	Slight reduction in drip loss but increased cooking loss with enhancement solution. BT increased cooking losses.	At 0.02% porcine pancreatin is not effective tenderizer.	Pietrasik et al. (2010)

Mutton	Crude Ginger extract	Mutton chunks were incubated with (0, 1, 3 or 5% CGE) for 24 h at 4°C. Cooked samples were stored at 4°C for 15 days.	↑Tenderization in CGE treated mutton samples.	-	CGE improved all the sensory attributes of mutton	↓ lipid oxidation and microbial counts with CGE treated samples	3% seems to be optimal for improving the sensory attributes and the keeping quality characteristics.	Mendiratta et al. (2000)
Lamb	Kiwifruit juice Actinidin	LD and leg chops from pre-rigor infused animals (n=6 each)	Very tender meat was obtained after 12 hours PM in kiwifruit infused carcasses.	Slight improv after 3 weeks VP.	-	Decreased lipid oxidation after 3 weeks VP and display for 6 days.	Kiwifruit juice may contain beneficial compounds other than actinidin.	Bekhit et al. (2007b)
Buffalo	Water Cucumis 2% W/V Ginger 5% (V/V)	BF muscles sprayed with the treatm	-An increase in collagen solubility,	-	- Improvement in flavour, juicine	- Ginger extract - treated meat sample	-	Naveena et al. (2004)

	(V/V) Papain 0.2% (W/V)	ents, stored for 48h at 4°C.	sarcoplasmic and myofibrillar protein solubility, and reduction in shear force values were observed in all enzyme-treated samples compared to control.		ss, tenderness and overall acceptability scores were observed in all enzyme-treated samples compared to controls.	s received better scores for appearance, flavour, tenderness and overall acceptability.		
Pork	Neutral marinade (81% maltodextrin+ 19% starch) plus Kiwifruit extract containing actinidin (0-11g/L)	BF muscles 24h PM, 1 day VP at 2°C. Injected to a target weight of 116% of the initial green weight	↑ rate and final tenderization increased with increasing actinidin content	-	- Lower textural characteristics with increasing the actinidin concentration -No differences in flavou	-No differences in juiciness or pH - Increased myofibrillar proteins (desmin) and collag	-Actinidin can be applied as a meat tenderizer without affecting other sensory attributes.	Christensen et al. (2009)

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Table 1. Summary for natural and synthetic compounds acting as inhibitors or activators for papain.

Enzyme	Inhibitors	Commentary	Reference Source
Papain (papaya)	Chicken egg white cystatin	Papain activity decreased proportionally with increased egg white, reaching 13% residual activity with 4% egg white.[1,2] One molecule of inhibitor reacts with one molecule of papain as shown by activity determinations[3] $^{\backslash}K_i$ value for the inhibition of papain was <1 x 10^{-11} M	Weerasinghe et al. (1996a;b)[1] Lindahl et al.(1988) Fossum and Whitaker (1968)[3] Anastasi et al. (1983)[4]

	Ascorbic Acid	While 2.5 × 10^{-3}M ascorbic acid inhibited papain in meat, this quantity of ascorbic acid induced off-flavour which was unacceptable to sensory panellists. A lower level of 2.5 × 10^{-4} M would retard but not completely eliminate papain activity while producing more acceptable sensory results	Ockerman et al. (1993)
	S-Nitrosothiols	Five *S*-nitroso compounds exhibited different inhibitory activities toward the enzyme in a time-and concentration dependent manner with second-order rate constants (*ki*/*KI*) ranging from 8.9 to 17.2 $m^{-1}s^{-1}$. The inhibition of papain by *S*-nitrosothiol was rapidly reversed by dithiothreitol, but not by ascorbate, which could reverse the inhibition of papain by $NOBF_4$.	Xian et al. (2000)
	Beef plasma protein (BPP)	At 1% BPP, residual activity was 26% of control, which decreased gradually and reached a plateau at 3%.[1]	Weerasinghe et al. (1996a;b)
	Potato powder[1] / crystals[2]	The inhibitory efficiency of potato powder was similar to that of egg white, although inhibition at 1% potato powder (68%) was higher than that for egg white (88%).[1] Quantitative evaluation of inhibitory activity against papain indicated that the inhibitor formed a complex with papain with a molar ratio of 1:4. The calculations that led to this conclusion were based on the inhibitor/enzyme mass ratio at the 50% inhibition level.[2]	Weerasinghe et al. (1996a;b)[1] Rodis and Hoff (1984)[2]
	Cysteine protease inhibitor (CPI) from chicken plasma	Cysteine protease inhibitor from chicken plasma was successfully fractionated using 200–400 g PEG/l. The CPI fraction obtained showed high inhibitory activity against papain.	Rawdkuen et al. (2007)
	Whey Protein Concentrate (WPC)	The most effective inhibition of autolytic protease activity was found at 3% WPC. WPC reduced papain activity linearly with lowest residual activity of 8.7%.	Weerasinghe et al. (1996b)

	Pig plasma protein (PPP)	PPP effectively inhibited papain, with the ability of PPP to inhibit papain increasing proportionally with the increase in concentration (Residual activity of < 20% with 10mg/ml PPP).	Benjakul and Visessanguan (2000)
	Carica papaya latex inhibitor	Three fractions with inhibitory effect of the esterase activity of the papain were isolated from fresh latex.	Monti et al. (2004)
	Pulsed electric fields (PEF)	Papain was irreversibly inactivated by PEF	Yeom et al. (1999)
	Soybean Cystatin Cysteine Protease Inhibitor	A protein of approximately 12 000 molecular weight was isolated from soybean seed hypocotyl-rich extract. The soybean CPI inhibited papain nearly 100%	Hines et al. (1991)
	Cysteine protease inhibitor (Eel-CPI-1)	Eel-CPI-1 inhibited papain (Ki=18 nM) competitively	Saitoh et al. (2005)
	Cysteine Protease Inhibitors from Koji Mould	*Apergillus oryzae* O-1018 separated from industrial koji for brewing sake was found to produce 5 papain-inhibitory compounds in the culture supernatant. The five isolated inhibitors were named CPI-1 to CPI-5.	Yamada et al. (1998)
	Cysteine Protease Inhibitor from Corn Endosperm	K_i value for the inhibition of papain was 2.3 x 10^{-8} M	Abe and Arai (1991)
	Extracellular Cysteine Protease Inhibitor from *Chlorella* sp (ECPI)	With azoalbumin and Bz-Arg-pNA as a substrate of papain, the Ki values of the inhibitor were estimated to be 2.4 nM and 25nM, respectively. One molecule of papain was inhibited by one molecule of inhibitor, so that ECPI forms a 1: 1 stoichiometric complex with this enzyme.	Ishihara et al. (2000)
	Thiol Inhibitor from *Aspergillus niger*	Papain was totally inhibited by filtrates from *A. niger* cultures	Walker and Chaplin (1983)
	Activators		

	EDTA	The activity of immobilized papain is increased by the addition of EDTA with a definite concentration. Immobilized papain could be more effectively activated with the addition of both EDTA and cysteine in an aqueous system. In addition, the enzymatic activity still increased even if the immobilized papain was incubated for 7h at 80°C or 90°C. [1] The addition of ethylenediaminetetraacetate (EDTA) is required for maximum activity.[2]	Zhuo et al. (1998)[1] Baines et al. (1982)[2].
	Cysteine	The maximum specific activity was observed when papain was immobilized with 200 mM cysteine.[1] The activity of immobilized papain is increased by the addition of cysteine with a definite concentration. Immobilized papain could be more effectively activated with the addition of both cysteine and EDTA in an aqueous system. In addition, the enzymatic activity still increased even if the immobilized papain was incubated for 7h at 80°C or 90°C.[2]	Homaei et al. (2009)[1] Zhuo et al. (1998)[2]
	Dithiothreitol (DTT)	The reversibly inactivated papain can be activated by reducing agents such as cysteine or DTT.	Yeom et al. (1999)
	Papain Activation Factor in the Dialyzable Fraction of Soybean Extract	Soybean extract contains a dialyzable fraction which activates papain.	Bahadur and Saxena (1964)
	Phosphorothioate	Phosphorothioate (PSH) was found to activate papain in an equimolar ratio. One molecule of PSH is reversibly incorporated into the protein molecule with the formation of one free sulfhydryl group, which is directly related to the enzymatic activity of papain, as was demonstrated by the reaction of iodoacetic acid with the above protein.	Neumann et al. (1967)
	Sodium Thiosulfate	Sodium thiosulfate was found to be a potent activator of papain. Thiosulfate reduced the clotting time of milk using papain from 1.25 min to 0.57 min	Jaffé (1945)

Table 2. Summary for natural and synthetic compounds acting as inhibitors or activators for bromelain.

Enzyme	Inhibitors	Commentary	Reference Source
Bromelain (pineapple)	Bromelain protease inhibitors from crude bromelain	Seven closely related protease inhibitors were isolated from commercial bromelain acetone powder. The inhibitors are proteins of MW 5000-6000, which inhibit competitively the bromelain catalyzed hydrolysis of CLN ($K_i \approx 10^{-7}$ M).	Perlstein and Kezdy (1972)
	Trehalose/ sucrose	Bromelain in the presence of 1 M trehalose/ sucrose was destabilized under thermal stress. The enzyme was also found to inactivate faster at 60°C in the presence of these osmolytes.	Habib et al. (2007)
	Potato cysteine protease inhibitor (PCPI)	The potato cysteine protease inhibitor variant of p1 8.3 (PCPI 8.3)	Rowan et al. (1990)
	Cysteine Protease Inhibitors	*Apergillus oryzae* O-1018 separated from industrial koji for brewing sake was found to produce 5 bromelain-inhibitory compounds in the culture supernatant. The five isolated inhibitors were named CPI-1 to CPI-5.	Yamada et al. (1998)
	Iodoacetamide & DTT	Solutions containing 10 mg/ml bromelain in PBS, pH 8.0 could be essentially totally inactivated by reduction with 10 mM DTT and alkylation with 50 mM iodoacetamide without precipitation.	Hale et al. (2005)
	Thiol Inhibitor from *Aspergillus niger*	Bromelain was totally inhibited by filtrates from *A. niger* cultures	Walker and Chaplin (1983)
	Activators		
	Thiosulfate	Thiosulfate reduced the clotting time of milk using bromelain from 15.00 min to 6.30 min	Jaffé (1945)
	EDTA	Full activation of the enzyme was obtained with cysteine and EDTA.	Ota et al.(1961)

	Cysteine	Full activation of the enzyme was obtained with cysteine and EDTA.	Ota et al.(1961) Balls et al. (1941)

Table 3. Summary for natural and synthetic compounds acting as inhibitors or activators for ficin.

Enzyme	Inhibitors	Commentary	Reference
Ficin (Ficus)	Soybean Cystatin Cysteine Protease Inhibitor	A protein of approximately 12 000 molecular weight was isolated from soybean seed hypocotyl-rich extract. The soybean CPI inhibited ficin nearly 100%	Hines et al. (1991)
	Potato crystals	Inhibitor activity was demonstrated against ficin, but the inhibition curve data for ficin was considered less reliable than those of papain since this enzyme did not have as high a degree of purity as papain.	Rodis and Hoff (1984)
	Ascorbic acid and Cu^{2+}	The simultaneous presence of ascorbic acid, Cu^{2+}ions and oxygen causes irreversible ficin inactivation. The degree of inactivation is dependent on the concentration of inhibitors.	Fukal et al. (1986)
	Cystatin (egg white)	Under the conditions used, 0.05 ml of egg white in a reaction mixture of 2 ml produced about 50% inhibition of ficin.	Fossum and Whitaker (1968)
	Cysteine protease inhibitor (Eel-CPI-1)	Eel-CPI-1 inhibited ficin (Ki=120 nM) competitively	Saitoh et al. (2005)
	Cysteine Protease Inhibitors from Koji mould	*Apergillus oryzae* O-1018 separated from industrial koji for brewing sake was found to produce 5 ficin-inhibitory compounds in the culture supernatant. The five isolated inhibitors were named CPI-1 to CPI-5.	Yamada et al. (1998)
	Cysteine Protease Inhibitor from Corn Endosperm	K_i value for the inhibition of ficin was 8.3 x 10^{-8} M	Abe and Arai (1991)

	Extracellular Cysteine Protease Inhibitor from *Chlorella* sp (ECPI)	For the proteolytic activity of ficin with azoalubumin and Bz-Arg-pNA, the Ki values were estimated to be 2.5 nM and 27 nM, respectively.	Ishihara et al. (2000)
	Activators		
	Phosphorothioate	Phosphorothioate (PSH) was found to activate ficin in an equimolar ratio. One molecule of PSH is reversibly incorporated into the protein molecule with the formation of one free sulfhydryl group, which is directly related to the enzymatic activity of ficin, as was demonstrated by the reaction of iodoacetic acid with the above protein.	Neumann et al. (1967)
	Sodium Thiosulfate	Thiosulfate reduced the clotting time of milk using ficin from 2.75 min to 0.42 min	Jaffé (1945)
	Cysteine	Cysteine reduced the clotting time of milk using ficin from 2.75 min to 0.46 min	Jaffé (1945)

Table 4. Summary for natural and synthetic compounds acting as inhibitors or activators for actinidin and zingibain.

Enzyme	Inhibitors/activators	Commentary	Reference
Actinidin (kiwifruit)	**Inhibitors:** Cu^{2+}; Cystatin; KCl (0.5-0.8 M); NaCl (0.5-1.2M), Zn^{2+}		Morimoto et al. (2006) Auerswald et al. (1996) Sugiyama et al. (1996)
	Activators: Dithiothreitol		Sugiyama et al. (1996)
Zingibain	**Inhibitors:** Cu^{2+}; Hg^{2+}, Cd^{2+}, Zn^{2+}	Complete inhibition with Cu^{2+}; Hg^{2+}; 50% inhibition with Cd^{2+} and 20% inhibition with Zn^{2+}	Ohtsuki et al. (1995)

(ginger)	**Activators:**Dithio threitol, EDTA, Ca^{2+}	20% activation with DTT and about 16% and 12% with EDTA and Ca2+	

Table 5. Summary for natural and synthetic compounds acting as inhibitors for protease from *microorganisms*.

Enzyme	Inhibitors/activators	Commentary	Reference Source
Protease produced from *Aspergillus oryzae*	**Inhibitors:** Serum		Bergkvist (1963)
Protease preparation derived from *Bacillus subtilis*	**Inhibitors:** Chymotrypsin Inhibitor I from Potatoes	The bacterial proteases were stoichiometrically inhibited by the chymotrypsin inhibitor.	Ryan (1966)
	Inhibitors: PMSF & DFP	Enzyme activity was inhibited strongly by phenylmethyl sulphonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP) but was not inhibited by ethylene diamine tetra acetic acid (EDTA), while a slight inhibition was observed with iodoacetate, *p*-chloromercuric benzoate (*p*CMB), and β-mercaptoethanol (β-ME).	Adinarayana et al. (2003)
	Activators: Ca^{+2}, Mg^{+2}, and Mn^{+2}		Adinarayana et al. (2003)
Protease produc	**Inhibitors:** Cu^{2+}, Hg^{2+}, EDTA and sodium azide		Devi et al. (2008)

produced from *Aspergillus niger*	**Activators:** Divalent cations (Fe^{2+}, Zn^{2+}, Mn^{2+}, $Ca^{2+}$and Mg^{2+})	By the activating effect of divalent cations (Fe^{2+}, Zn^{2+}, Mn^{2+}, $Ca^{2+}$and Mg^{2+}) and inhibiting effect of chelating agent (EDTA) and Hg^{2+}, the enzyme was found to be a metalloprotease.	Basu et al. (2008)

Figure captions

Figure 1. Enzymatic mechanism of protein hydrolysis by cysteine proteases from Grzonka et al. (2007) with permission (License No. 500608865).

Figure 2. Schematic presentation for the impact of using active or inactive papain on the live animal and meat (post-mortem). Modified from Dransfield and Etherington (1981).

Figure 3. Effects of vascular infusion treatments on the shear force values of (A) LD muscles and (B) leg chops during post-mortem storage at 2°C (means ± SEMs)

Figure 4. The effects of water and kiwifruit juice infusion on TBARS (mg MDA/kg of fresh tissue) values of LD steaks and leg chops at 1 and 21 days post-mortem time display for various times at 4°C.

Figure 1.

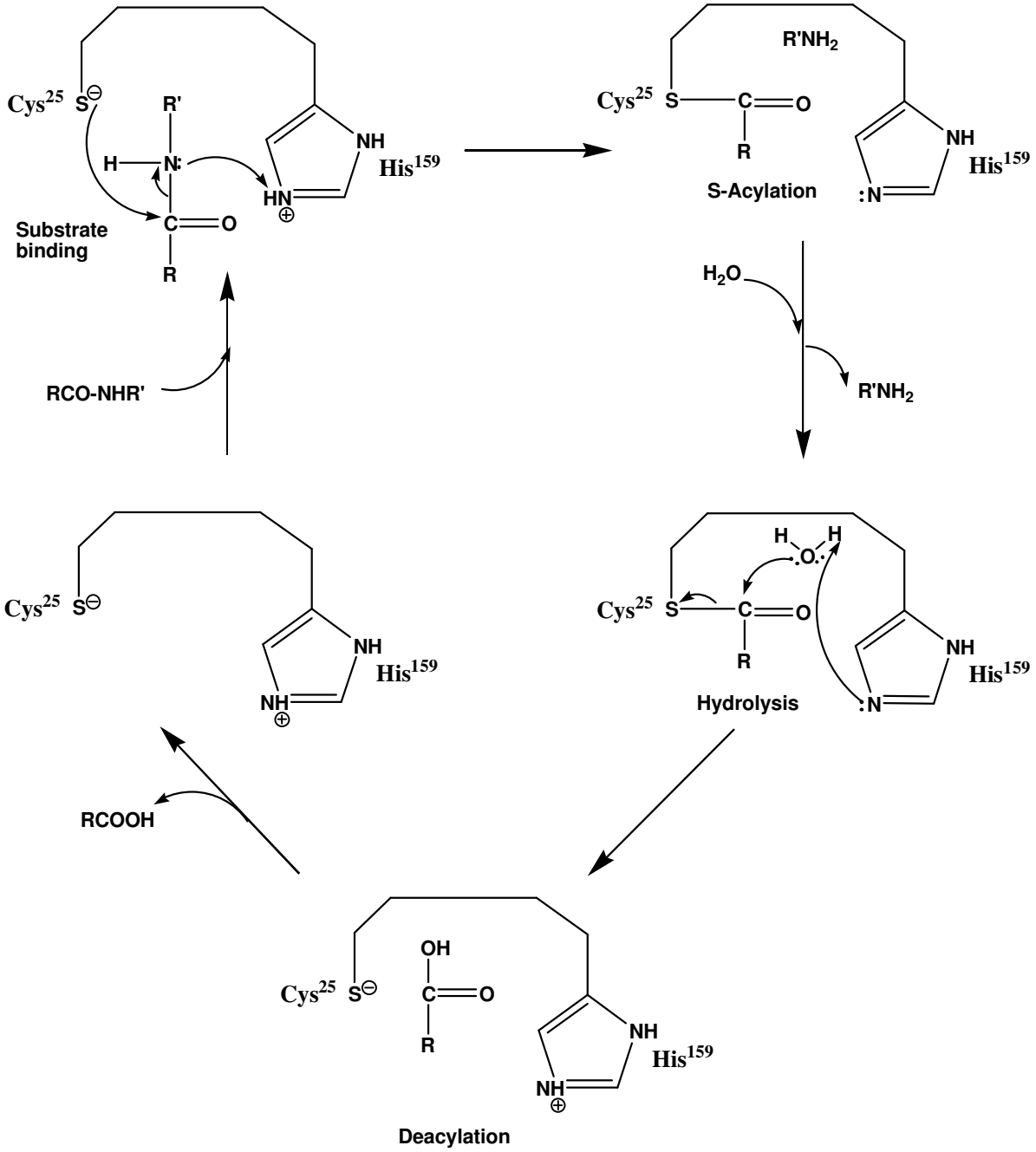

Figure 2.

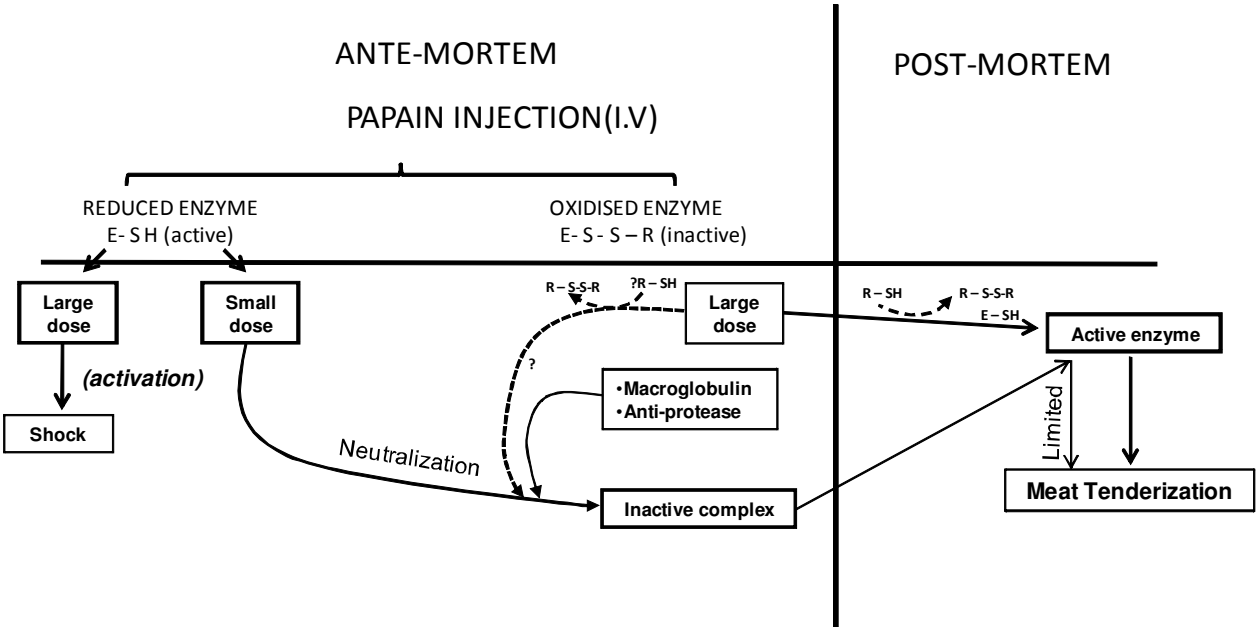

Figure 3

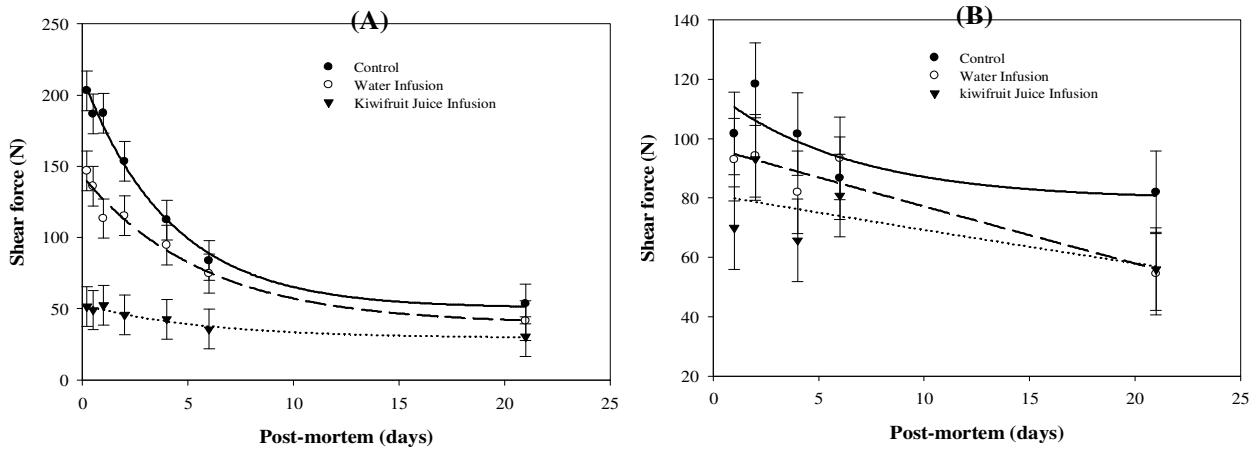

Figure 4