



REVIEW

Endogenous Proteolytic Systems and Meat Tenderness: Influence of Post-Mortem Storage and Processing

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Abstract Meat proteolytic systems play a crucial role in meat tenderisation. Understanding the effects of processing technologies and post-mortem storage conditions on these systems is important due to their crucial role in determining the quality characteristics of meat and meat products. It has recently been proposed that tenderisation occurs due to the synergistic action of numerous endogenous proteolytic systems. There is strong evidence suggesting the importance of μ -calpain during the initial post-mortem aging phase, while m-calpain may have a role during long-term aging. The caspase proteolytic system is also a candidate for cell degradation in the initial stages of conversion of muscle to meat. The role of cathepsins, which are found in the lysosomes, in post-mortem aging is controversial. Lysosomes need to be ruptured, through aging, or other forms of processing to release cathepsins into the cytosol for participation in proteolysis. A combination of optimum storage conditions along with suitable processing may accelerate protease activity within meat, which can potentially lead to improved meat tenderness. Processing technologies such as high pressure, ultrasound, and shockwave processing have been reported to disrupt muscle structure, which can facilitate proteolysis and potentially enhance the aging process. This paper reviews the recent literature on the impacts of processing technologies along with post-mortem storage conditions on the activities of endogenous proteases in meat. The information provided in the review may be helpful in selecting optimum post-mortem meat storage and processing conditions to achieve improved muscle tenderness within shorter aging and cooking times.

Keywords meat, endogenous enzymes, processing, post-mortem storage

Introduction

Meat tenderness is generally considered the most important palatability factor influencing consumer acceptability, particularly for red meat (Lamare et al., 2002). The presence and activity of endogenous enzymes within the muscle cells and the extracellular matrix is an important factor controlling muscle proteins and their

interactions, and therefore is a significant contributor to the development of tenderness (Huff Lonergan et al., 2010). Enzymatic degradation of muscle proteins during post-mortem aging under chilled conditions contributes to the rapid tenderisation of meat (Chéret et al., 2007).

Although there are different viewpoints of how the process occurs, many studies have suggested that the cathepsins, calpains, and proteasome enzyme systems are involved in post-mortem proteolysis and tenderisation of meat. Goll et al. (2003) and Koochmaraie and Geesink (2006) concluded that post-mortem muscle tenderisation is mainly caused by the action of μ -calpain and to a lesser extent the action of m-calpain. The post-mortem pH falls to below 6, which promotes the release of cathepsins from the lysosomes and eventually facilitates meat tenderisation (Geesink and Veiseth, 2008; Moeller et al., 1977). However, as Cathepsin D is active at a pH range from 3 to 5, it has a relatively less important role in muscle tenderisation than other cathepsins at a post-mortem pH of 5.5 (Mikami et al., 1987). Cramer et al. (2018), Ouali et al. (2006) and Sentandreu et al. (2002), on the other hand, proposed that the process is a multi-enzymatic system, which may also involve other proteases such as proteasomes and caspases. Thus, one of the objectives of this paper is to review the recent literature to provide an updated viewpoint on the role of various endogenous proteolytic systems in meat tenderisation.

Various tenderisation technologies, including pulsed electric field (PEF), shockwave processing, and high-pressure processing (HPP), when applied to pre- or post-rigor meat have been suggested to decrease meat toughness (Warner et al., 2016). Electrical stimulation has been observed to accelerate the decline in pH; the release of calcium ions from sarcoplasmic reticulum, activating calpains, and also leading to muscle proteolysis by more rapid release of lysosomal enzymes, thus helping in the development of meat tenderness during the early post-mortem storage period (Sentandreu et al., 2002). Meat tenderisation could possibly be enhanced by employing the action of lysosomal proteolytic enzymes through careful manipulation of the sous vide cooking process by including cooking steps at the highest activation temperature of several enzymes (calpains, 26S proteasome and cathepsins) (Kaur et al., 2020; Myhrvold et al., 2011; Uttaro et al., 2019). Thus, understanding the effects of processing technologies and meat storage conditions on endogenous enzymes is of utmost importance, due to their crucial role in determining shelf-life and quality characteristics of meat and meat products. This review discusses the impacts of processing technologies along with post-mortem storage conditions on the activities of endogenous proteases in meat. Appropriate processing in combination with optimised post-mortem storage conditions is important in attaining optimum levels of proteolysis in meat, achieving desired meat tenderness within shorter aging times. To our knowledge, no review on this topic has been published so far.

Post-Mortem Storage Conditions, Proteolytic Systems and Meat Tenderness

The effect of storage conditions on meat quality is of great interest, as storage temperature plays a crucial role in determining the shelf-life and quality of the meat. The storage of meat under frozen conditions helps to prolong the product shelf-life and this is a crucial factor when meat is exported. However, consumers often have a perception that frozen meat has poor eating qualities as compared to “fresh” chilled meat (James and James, 2010; Madhusankha and Thilakarathna, 2020).

Calpains

The two major muscle protein groups affecting post-mortem meat tenderness are the myofibrils and the connective tissue proteins (Kemp and Parr, 2012). The calpains have been widely reported to hydrolyse the myofibrillar proteins (Álvarez et al., 2019; Kemp and Parr, 2012; Lana and Zolla, 2016). Recent research has documented degradation of proteins like desmin,

titin and nebulin, which are substrates for calpains to be highly associated with meat tenderness (Lomiwes et al., 2014; Starkey et al., 2016). This suggests a significant role of calpains, particularly calpain 1 or μ -calpain in post-mortem meat tenderisation (Geesink et al., 2006; Huff Lonergan et al., 2010; Koohmaraie and Geesink, 2006). However, the main component of the connective tissues, collagen, is not degraded by the calpains (Purslow, 2005). The reason is that the typical triple helix structure of native collagen makes it resistant to most common proteases. However, collagenolytic proteases like mammalian cysteine proteases, some types of mammalian matrix metalloproteases (MMPs), and a few bacterial proteases have been reported to degrade native collagen (Zhang et al., 2015). MMPs, also known as matrixins are also responsible for the catabolism of connective tissue. They are a family of structurally related zinc MMPs that are suspected to be implicated in apoptosis (Mannello and Gazzanelli, 2001; Parsons et al., 1997). These peptidases are poorly studied by meat scientists because collagen doesn't go through major changes in meat stored at low temperature (0°C–4°C) (Sentandreu et al., 2002).

Calpastatin, the endogenous inhibitor for both μ -calpain and m-calpain, has been correlated with tenderisation both across and within species (Boland et al., 2019; Chéret et al., 2007). Several groups have proposed that μ -calpains play the most important role in post-mortem muscle proteolysis and meat tenderisation (Bowker et al., 2010; Koohmaraie and Geesink, 2006). Riley et al. (2003) reported that variations in μ -calpain activity are evident during post-mortem proteolysis of myofibrillar proteins. In contrast to the above, a study by Goll et al. (2003) suggested that less than 10% of calpain is activated in the skeletal muscle. The optimal conditions for calpain activity have been estimated to be pH 7.5 at 25°C, with activity still detectable at pH 5. Meat tenderisation is known to occur at approximately pH 6.3, at about 6 h post-mortem in beef as μ -calpain is activated at low calcium concentrations (10 to 50 μ M). The activity of m-calpain is at its optimum in the pH range of 6.5 to 8.0 and in the presence of 1 to 2 mM calcium. m-Calpain exhibits its lowest activity at pH 5.5 and 5°C, which is the typical condition of the beef carcass at 24 to 48 h post-mortem (Bowker et al., 2010). The activity of m-calpain was observed to remain nearly constant throughout post-mortem aging at 1°C for up to 14 d, but a gradual decrease in μ -calpain has been observed for bovine *Longissimus* muscle (Koohmaraie et al., 1987). As activation of calpain leads to autolysis, these researchers concluded that μ -calpain, but not m-calpain, might be involved in tenderisation. Bhat et al. (2018a) reported that amount of both intact and autolysed μ -calpain decreased with aging time in two different muscles (*Biceps femoris* and *Semimembranosus* from culled dairy cows). Both intact and autolysed μ -calpain were detected on the second day of aging, but not after seven days of aging. In contrast, the amount of native m-calpain decreased with aging time, while the amount of autolysed m-calpain increased, with the highest amount observed on the 14th d in both muscle types. Similarly, Biswas et al. (2016) observed an optimal μ -calpain induced post-mortem aging time at 48 and 72 h for *Biceps femoris* muscle of Jhakrana and Jamunapari breeds of goat, respectively. Similar results were reported by Colle and Doumit (2017), who detected only 5.4% of the initial μ -calpain activity in the bovine *Semimembranosus* muscle by 2 d of post-mortem aging while m-calpain remained active in most bovine *Semimembranosus* and *Longissimus lumborum* muscles by the 14 d of aging. These studies proved the contribution of both μ -calpain and m-calpain in the development of post-mortem tenderness, with the former contributing to proteolysis of myofibrillar proteins during the early post-mortem stage while the latter contributed to additional tenderisation with prolonged aging time. Numerous factors such as calcium, pH, temperature, etc., affect the activity of μ -calpain in post-mortem muscle (Mohrhauser et al., 2014).

A high level of calpastatin is associated with a decrease in meat tenderness (Lana and Zolla, 2016; Lian et al., 2013). Calpastatin is a heat-stable, unstructured protein that, in the presence of calcium, can reversibly bind and inhibit four molecules of calpain (Hanna et al., 2008). The exact mechanism for the inhibitory action of calpastatin on calpains is undefined. However, it has been suggested that calpains degrade calpastatin by cleaving the disordered regions between

calpastatin inhibitory domains, forming peptide fragments that are also calpain inhibitors (Lian et al., 2013; Mellgren, 2008). A reduction in calpastatin activity was observed under refrigerated storage (Koohmaraie et al., 1987) and at temperatures above 25°C (Geesink et al., 2000). A reduction in calpastatin activity was found to lead to higher myofibrillar degradation in porcine *Longissimus* muscle (Pomponio and Ertbjerg, 2012). Koohmaraie et al. (1991) have shown that the rates of tenderisation of muscle from different animals (beef<lamb<pork) were inversely related to the ratio of calpastatin to calpains (beef>lamb>pork). De Oliveira et al. (2019) studied the changes in activities of μ - and m-calpains, and calpastatin variants in two bovine muscles (*Longissimus lumborum* and *Triceps brachii*) during post-mortem aging. One of the two calpastatins had a significant effect on μ -calpain activity; and thus their ratio was suggested to be an important contributor determining the extent and rate of post-mortem proteolysis (De Oliveira et al., 2019).

Cathepsins

The role of cathepsins in post-mortem tenderisation is controversial, primarily because they are found in the lysosomes, which limits substrate accessibility. Due to the decline in pH and temperature throughout the post-mortem storage, the membranes of the lysosomes ruptures and causes the release of cathepsins into the cytosol (Bowker et al., 2010; Lana and Zolla, 2016). Cathepsins are acidic lysosomal proteins and they must be released from the lysosomes to participate in post-mortem proteolysis of myofibrils (Bowker et al., 2010; Kemp et al., 2010).

Cathepsin B, D, H, and L are the most abundant in muscle fibres and they have been claimed to be involved in the degradation of proteins during post-mortem aging (Boland et al., 2019; Bowker et al., 2010). Chéret et al. (2007) showed that, in meat, both calpains and cathepsins act synergistically while an earlier study by Hopkins and Thompson (2001) reported that the inhibition of cathepsins B and L was not found to have any effect on meat tenderness. Cathepsin D has been reported to remain active only within a narrow pH and temperature range (Zeece et al., 1986), suggesting that this enzyme might not play a major role in the post-mortem tenderisation process.

Proteasomes

Several studies have indicated that caspases and bovine proteasomes are involved in the proteolysis of myofibrillar proteins, including myosin and actin (Kemp and Parr, 2008). A study conducted by Dutaud et al. (2006) elucidated the physico-chemical characteristics of 20S proteasome in relation to the post-mortem conditions (pH, temperature, osmolarity, etc.). The activity loss of 20S proteasome was found to be less affected by these conditions in post-mortem bovine muscle. Depending on the muscle type, the estimated value of remaining intact proteasome concentration in meat stored for 16 d at 0°C–4°C was about 30%–48%. Consequently, they concluded that under similar conditions, the 20S proteasome was very likely to have more proteolytic activity than μ -calpain.

Caspases

The caspases, which are neutral cysteine proteinases, have been suggested to interact with the calpains/calpastatin enzyme system that might affect post-mortem proteolysis (Bowker et al., 2010; Huff-Lonergan, 2014). In a study conducted by Kemp et al. (2006) using post-mortem porcine *Longissimus* muscle, caspase 3/7 and caspase 9 exhibited the highest activity at 2 h post-mortem and their activity decreased with post-mortem time. In the same study, it was also found that caspase activity was negatively correlated with Warner-Bratzler shear force (WBSF) measurements, thus suggesting a role of caspases in meat tenderisation. Kemp et al. (2009) reported a decline in activities of caspase 3/7 and caspase 9 in three different muscles

including the *Longissimus*, *Semimembranosus* and *Infraspinatus* muscle during post-mortem conditioning period of callipyge and normal lambs. The activity of caspase 9 was declined faster as compared to the caspase 3/7. Additionally, a positive correlation was noticed between the initiator (caspase 9) and executioner (caspase 3 and 7) isoforms. This correlation was consistent with the observation that caspase 9 was responsible for the breakdown and activation of caspase 3/7 downstream.

It seems clear from the above-discussed studies that proteolysis, in part, is one of the major contributors to post-mortem meat tenderisation (Álvarez et al., 2019). An important point to mention is that pH decline and high ionic strength are closely related to the rate and extent of myofibrillar proteolysis (Barbut et al., 2008). Changes in the ionic strength, pH and temperature can change the conformation of the proteolytic enzymes, which can activate them to hydrolyse the protein substrate (Melody et al., 2004). These alterations occur in parallel with the development of rigor and further influence the rate of meat tenderisation (Huff Lonergan et al., 2010; Lian et al., 2013; Simmons et al., 2008).

Effect of the post-mortem storage on the proteolytic systems and meat tenderness

Storage of meat above freezing temperature results in more tender meat (James and James, 2010). In the slaughterhouse, dry aging is carried out by hanging beef carcasses for a period of at least 2 weeks in a controlled environment at a temperature ranging from -1°C to 5°C (James and James, 2010; Lian et al., 2013). The purpose is to provide adequate time for the meat to tenderise by allowing the degradation of intracellular muscle protein by the proteolytic systems. It has been suggested that freezing helps to improve the tenderness in beef even without the aging step. The formation of intracellular ice crystals during frozen storage leads to the physical disruption of muscle cells and the rupture of connective tissue. This phenomenon could possibly be an explanation for the improved tenderness (Faridnia et al., 2015). Ice crystal formation could also contribute to the rupture of lysosomes, which facilitates the release of cathepsins into the cytosol. This would enable cathepsins to participate in post-mortem proteolysis. Shanks et al. (2002) also revealed that freezing could significantly reduce the WBSF values for *Longissimus* beef steaks during various aging periods (post-mortem 1, 2, 3, 4, 6, 7, 10, 14, and 35 d). However, Wheeler et al. (1990) observed no significant differences in tenderness of steaks prepared from fresh and frozen subprimals after comparable aging time periods.

Several studies have been conducted to evaluate the effects of storage conditions on different endogenous proteases and their inhibitors (Table 1). Pomponio and Ertbjerg (2012) investigated the effects of post-mortem storage temperature (2°C , 15°C , 25°C , and 30°C) on calpain activity for porcine *Longissimus* muscle. It was discovered that μ -calpain was activated earlier than m-calpain at all temperatures. Autolyzed m-calpain was reported after 5 d at 2°C storage temperature. The experimental results also indicated that the activity of calpastatin and the myofibril particle size (myofibrillar fragmentation was analysed using a Malvern Mastersizer) decreased with increasing incubation time (2 h post-mortem to 120 h post-mortem) and temperatures (2°C – 30°C). From these observations, the authors suggested that both μ - and m-calpain are involved in proteolytic tenderisation of meat (Pomponio and Ertbjerg, 2012). In contrast, at refrigerated temperatures (4°C), the autolysis of m-calpain during aging has been observed in neither bovine (Camou et al., 2007) nor ovine muscle (Veiseth et al., 2001). In another study by Xu et al. (2012), the μ -calpain activity in porcine *Longissimus dorsi* muscle was undetected after 1d post-mortem storage at refrigerated conditions (0°C to 4°C).

Meat Processing Technologies, Meat Tenderness and Proteolytic Systems

Several techniques to improve the meat tenderness have been proposed in many studies and their effects on meat are elaborated in the following sections (Table 2).

Table 1. Some studies showing the effects of post-mortem aging/storage conditions on endogenous proteases in meat from different animal sources

Source	Muscle type	Post-mortem storage conditions	Results	References
Beef	<i>Longissimus</i> muscle	14 d at 1°C	- m-Calpain activity remained nearly constant - μ -Calpain activity decreased gradually	Koohmaraie et al. (1987)
	<i>Semimembranosus</i> and <i>Longissimus lumborum</i> steaks muscles	84 d at -75°C	- Only 5.4% of the initial μ -calpain activity remained in bovine <i>Semimembranosus</i> muscles after 2 d of post-mortem aging - m-Calpain was activated in most bovine <i>Semimembranosus</i> and <i>Longissimus lumborum</i> steaks muscles by the 14 d of aging - The procaspase-3 activity was noticed in the bovine muscles up to 7 d of storage	Colle and Doumit (2017)
	<i>Longissimus thoracis</i>	7 d at 4°C	- Active caspase-3-isoforms and their levels decreased with post-mortem aging	Huang et al. (2016)
	<i>Semimembranosus</i> , <i>Longissimus lumborum</i> , <i>Longissimus thoracic</i> , <i>Psoas major</i> , and <i>Triceps brachii</i>	7 d at 4°C	- No autolysis of m-calpain was noticed during the aging period	Camou et al. (2007)
Pork	<i>Longissimus</i> muscle	5 d at different temperatures (2°C, 15°C, 25°C, and 30°C)	- μ -Calpain was activated earlier than m-calpains at all temperatures - Autolyzed m-calpain was reported after 5 d at 2°C storage temperature - The activity of calpastatin and myofibril particle size decreased with increasing incubation time (2 h post-mortem to 120 h post-mortem) and temperatures (2°C to 30°C)	Pomponio and Ertbjerg (2012)
	<i>Longissimus dorsi</i>	1 d post-mortem storage (4°C and 25 °C)	- μ -Calpain activity was undetected after 1 d post-mortem storage	Xu et al. (2012)
	<i>Longissimus</i> muscle	192 h after slaughter (temperature not mentioned)	- Caspase 3/7 and caspase 9 exhibited the highest activities at 2 h post-mortem, and their activities decreased with post-mortem time	Kemp et al. (2006)
Lamb	<i>Longissimus</i> , <i>Semimembranosus</i> and <i>Infraspinatus</i> muscles	21 d post-mortem storage at 4°C	- The activity of caspase 9 was observed to decline faster in contrast to caspase 3/7 in lamb <i>Longissimus</i> , <i>Semimembranosus</i> and <i>Infraspinatus</i> muscles during post-mortem storage	Kemp et al. (2009)
Goat	<i>Biceps femoris</i>	96 h post-mortem storage at 4°C	- The optimised μ -calpain mediated aging was achieved after 48 to 72 h post-mortem storage	Biswas et al. (2016)
Chicken	Chicken <i>Pectoralis superficialis</i> muscle	72 h post-mortem storage at 4°C	- After 6 h post-mortem, μ -calpain activity in the chicken <i>Pectoralis superficialis</i> muscle was hardly detectable	Lee et al. (2008)

High pressure processing (HPP)

In the meat industry, high pressure processing (HPP) is applied to a product at or above 100 MPa using a liquid transmitter (Simonin et al., 2012). HPP has been reported to alter the texture and gel-forming properties of myofibrillar proteins, and thus it has been proposed as a physical and additive-free tenderiser for meat products (Buckow et al., 2010). Application of high

Table 2. Some highlights of the effects of different processing technologies on the activities of endogenous enzymes

Processing technologies	Proteolytic system	Effect on endogenous proteases	References
High pressure processing	Lysosomal proteases	- Releases and increases the activities of lysosomal proteases - Increased cathepsin D activities observed in pressure treated (520 MPa, 10°C for 260 s) 2 d post-rigor bovine (<i>Biceps femoris</i> and <i>Longissimus dorsi</i>) muscles throughout storage at 4°C for up to 20 d post-mortem - Pressure induced higher endogenous proteolytic activity due to the release of enzymes from lysosomes (between 100–200 MPa), denaturation of muscle proteins and enhanced susceptibility of these proteins to proteolysis	- Jung et al. (2000), Kubo et al. (2002) - Jung et al. (2000) - Ohmori et al. (1991)
	Calpains	- Activates calpains under moderate pressure and with the release of calcium ions from the sarcoplasmic reticulum	- Bessiere et al. (1999), Homma et al. (1996)
Pulsed electric field	Lysosomal proteases	- Releases lysosomal proteases from lysosome	- Faridnia et al. (2015)
	Calpains	- Releases calcium ions which activates μ-calpain - Promotes the autolysis of calpains which enhances the proteolysis during aging	- Alahakoon et al. (2016) - Bhat et al. (2018c), Bhat et al. (2019)
Shockwave processing	Cathepsins	- No improvement in the cathepsin and peptidase activities	- Bolumar et al. (2014)
Ultrasound processing	Calpains	- Releases calcium ions, which activate μ-calpain - Increases calpains autolysis and enhance proteolysis during maturation	- Alarcon-Rojo et al. (2015), Roncalés et al. (1993) - Roncalés et al. (1993), Wang et al. (2018)
	Cathepsins	- Releases cathepsin from lysosomes	- Roncalés et al. (1993)
Thermal processing (Sous vide cooking)	Cathepsins	- Mild heating promotes the activity of cathepsins by rupturing of lysosomes - Cathepsins B+L are most active when being held at 55°C, remain active at 63°C for 19.5 h - Cathepsin H has highest activity at 20°C and lost most of its activity at temperatures above 40°C - Cathepsin B+L activity increased at 50°C after one hour of cooking	- Dominguez-Hernandez et al. (2018), Ertbjerg et al. (2012) - Christensen et al. (2013), Ertbjerg et al. (2012), Wang et al. (2013) - Wang et al. (2013) - Kaur et al. (2020)
	Calpains	- Calpains starts to be inactivated from 55°C and there was no extractable activity at 60°C	- Ertbjerg et al. (2012), Wang et al. (2013)
Electrical stimulation	Calpains	- Early activation of calpains which accelerate muscle proteolysis	- Abbasvali et al. (2012), Ferguson et al. (2000), Lee et al. (2000), Li et al. (2012), Pouliot et al. (2014), Uytterhaegen et al. (1992)
	Lysosomal proteases	- Increases the activity of lysosomal enzymes such as β-glucuronidase, cathepsin C and cathepsin B+L & cathepsin D, in most of the cases	- Dutson et al. (1980), Li et al. (2012), Pommier et al. (1987)

pressure has been reported to possibly induce membrane damage, which may affect enzymatic reactions in both positive and/or negative way (Sikes and Warner, 2016). The synergistic action of proteolytic systems, particularly cathepsins, could be responsible for the meat tenderisation under pressure. The pressure treatment (100–500 MPa at ambient temperature for 10 min) of beef rounds caused pressure-induced endogenous proteolytic activity due to the release of enzymes from lysosomes, the denaturation of muscle proteins and the increased susceptibility of these proteins to proteolysis (Ohmori et al., 1991). The

magnitude of pressure inducing the release of cathepsins from the lysosomes of bovine liver was different for different enzymes. High pressure such as more than 200 MPa is required to release cathepsins B and H, whereas cathepsins D released at a lower pressure of 100 MPa (Ohmori et al., 1992). Pre-rigor *Longissimus thoracis* rabbit muscles treated at 100 MPa caused the disruption of lysosome membranes and consequently the release of cathepsins into the cytosol (Kubo et al., 2002). As such, cathepsins become accessible to the myofibrils and can participate in post-mortem proteolysis (Buckow et al., 2010; Kubo et al., 2002). It has been suggested that certain combinations of temperature and pressure accelerate the activity of the cathepsins (Buckow et al., 2010). The activities of cathepsin D and acid phosphatase have also been found to increase in pressure-treated (520 MPa, 10°C for 260 s) 2 d post-rigor bovine muscles (*Biceps femoris* and *Longissimus dorsi*) throughout storage at 4°C for up to 20 d post-mortem (Jung et al., 2000).

The release of calcium ions from the sarcoplasmic reticulum during HPP of rabbit meat at 200 MPa resulted in the activation of calpains and inactivation of the inhibitor calpastatin (Homma et al., 1996). On the contrary, in an *in vitro* study, using an in-house built bioreactor, the activity of calpain purified from rabbit skeletal muscle was observed to be enhanced at a moderate pressure of 50 MPa (for μ -calpain) and 75 MPa (for m-calpain). Both μ - and m-calpains were inhibited at pressures above 100 MPa, with m-calpain being more pressure-resistant than μ -calpain (Bessiere et al., 1999). Similar observations have been reported where the level of μ -calpain activity in HPP-treated meat was markedly reduced during aging. Both μ -calpain and m-calpain were reported to be partially inactivated at 200 MPa and completely inactivated at 400 MPa due to pressure-induced denaturation (Cheftel and Culioli, 1997). However, the increased catheptic activity was not adequate to compensate for the loss of calpains and structural changes in myofibrils at higher pressure (>400 MPa), resulting a reduced effect on tenderness. In a recent study, Morton et al. (2018) have found that HPP of bovine pre-rigor muscles at 175 MPa caused substantial increases in tenderness but with a decrease in μ -calpain activity, evidence that the primary effect of HPP on pre-rigor meat may be physical rather than enzymatic.

Thermal processing (sous vide cooking)

Sous vide is a popular form of low temperature long time (LTLT) cooking, where the temperature is often close to or lower than 60°C and the product is cooked for an extended period of time (Dominguez-Hernandez et al., 2018). The sous vide cooking temperature in achieving optimum meat tenderisation should be high enough to solubilise the collagen and inactivate microorganisms while having minimum myofibrillar shrinkage (Boland et al., 2019; Zhu et al., 2018). Some studies have reported that cooking at 60°C for 4 h improved the tenderness of bovine *Semimembranosus* muscle (Dominguez-Hernandez et al., 2018) and a consensus was reached that LTLT cooking has a positive impact on meat tenderness (Dominguez-Hernandez et al., 2018). Cathepsins have been demonstrated to be thermally stable at sous vide cooking temperatures (below 60°C), thus they were suggested to be involved in the proteolysis of collagen during LTLT treatment (Dominguez-Hernandez et al., 2018). Thus, their proteolytic action may contribute to the tenderising effect during sous vide cooking of meat.

According to the research studies, the cathepsins have the ability to destabilise native collagen and to breakdown thermally weakened collagen into peptides, which may be further hydrolysed by other enzymes (Solvig, 2014). Hence in LTLT treatments, proteolysis could act synergistically with heat denaturation to cause enhanced weakening of collagen and tenderisation. Collagen denaturation has been suggested to be a heating rate-dependent, multistep process that can occur at 55°C–60°C in slow heating regimes. Wang et al. (2013) examined the relationship between duck breast meat tenderness, actomyosin degradation and endogenous enzyme activities (calpain, cathepsin B, L, and D) at cooking temperatures ranging from 30°C to 90°C. It was reported that the shear force decreased from 50°C to 70°C. At 60°C, calpains lost most of their

extractable activity whereas cathepsin B and L remained active. There was no significant change in cathepsin D activity at temperatures below 70°C and this observation was strongly correlated with the degree of actomyosin degradation. The authors suggested that cathepsin D could contribute to actomyosin degradation and thus improve the tenderness of duck meat during the cooking process (He et al., 2019; Wang et al., 2013).

Ertbjerg et al. (2012) documented that cathepsins B+L achieved their maximum activity in porcine *Longissimus* muscle after heating for 1.5 h at 55°C, while calpains were rapidly inactivated at this temperature. The authors suggested that part of cathepsin B and L may exist in the form of a proenzymes that are activated by heat. Cathepsin B+L activity was also detected in the *Semitendinosus* muscle from cows and young bulls after 19.5 h of cooking at 63°C by Christensen et al. (2013), suggesting that cathepsin B and L play a major role in tenderisation during extended cooking at lower temperature (53°C–63°C).

The influence of thermal activation of enzymes on shear force and deformation of bovine *Supraspinatus* and *Rectus femoris* muscles was evaluated by Uttaro et al. (2019), by treating the muscles with different cooking treatments: the single- and multistage sous vide cooking and water bath cooking. The cooked samples were stored at two different storage conditions (one week at 2°C and two weeks at –1.5°C) before reheating the meat at 55°C. A 17%–21% reduction in shear force was observed after a single stage sous vide cooking process (at 59°C for 4 h). This was suggested to be due to the activation of cathepsins B & L and 20S proteasome by heat that might affect both myofibrillar and collagen components of meat. Multistage sous vide cooking (1 h at 39°C, 1 h at 49°C and 4 h at 59°C) caused a further 5%–6% decrease in shear force that was suggested to be due to degradation of primarily myofibrillar proteins possibly through activation of the m-calpain. No significant effects of post-cooking storage were reported (Uttaro et al., 2019).

In a recent study on beef brisket, cathepsin B and L were observed to be more heat stable under sous vide temperature conditions in contrast to Cathepsin H (Kaur et al., 2020). An increase in the cathepsin B+L activity at 50°C after 1 h of cooking suggested that these enzymes could exist as pro-enzymes that were activated during heating. Therefore, higher activities of these enzymes (Cathepsin B+L), at the above-mentioned temperature are likely to contribute to proteolysis and tenderness in sous vide cooked brisket meat.

Ultrasound treatment

Ultrasound is a form of mechanical vibration energy in a solid or fluid at a frequency of 20 kHz and above and can be applied to foods either in a non-destructive (low intensity ultrasound) or a destructive way (high intensity ultrasound) (Alarcon-Royo et al., 2015; Jayasooriya et al., 2004). The low intensity ultrasound is mainly used as an analysis tool whilst the high intensity ultrasound is used to modify the properties of food.

For meat and meat products, the application of ultrasound to induce physical and chemical changes has been a subject of interest over previous few decades (Jayasooriya et al., 2004). Ultrasonic treatment is a physical method that could be an alternative to chemical and thermal treatment. The disruption of the cellular membranes of the muscle due to ultrasonication could release calcium into the extracellular space, increasing its availability for the activation of calpains (Alarcon-Royo et al., 2015). Wang et al. (2018) observed a significant increment in the degree of autolysed 76 kDa calpain subunits in ultrasonicated (intensity of 25 W/cm² at 5±1°C for 20 and 40 min) bovine *Semitendinosus* muscles after one day of post-ultrasonication storage at 4°C. This was accompanied by an enhanced desmin and troponin degradation during the subsequent aging process at 4°C for up to 7 d. Roncalés et al. (1993) documented the appearance of 30 kDa peptides with an increase in proteolytic activity in lamb muscles treated with ultrasound (57 and 62 W for 10–180s). Thereby, a strong correlation was

noticed between these peptides and meat tenderness (Roncalés et al., 1993). The authors suggested that this may be a result of the mechanical effects of cavitation that release the cathepsins from lysosomes and/or calpain activation by increased calcium release from the sarcoplasm upon ultrasound treatment (Roncalés et al., 1993). Lysosomes have been reported to be damaged by slow freezing or the use of low frequency-high power ultrasound treatments (McGann et al., 1988; Weiss et al., 2011). Cathepsin D was released from the lysosomes following multiple freezing-thawing treatments, high-power ultrasound treatments and mechanical homogenisation in case of fish muscles (Szymczak, 2016). These treatments led to an increase of 170%–300% in its activity. In another study, significant changes in collagen characteristics were observed after ultrasound treatment (40 kHz; 1,500 W; 10–60 min) of bovine *Semitendinosus* muscle (Chang et al., 2012). Collagenous fibres were disordered and staggered loosely, and with an increase in the ultrasound exposure times, granulation and aggregation of denaturing collagen fibres were found in the extracellular space. These observations suggested that low frequency and high power ultrasonication resulted in a significant effect on collagen characteristics and meat texture (Chang et al., 2012). Various studies have documented that the application of power-ultrasound favourably enhanced the tenderisation of meat from beef (Stadnik and Dolatowski, 2011; Wang et al., 2018), chicken (Chen et al., 2015), pork (Ozuna et al., 2013), and goose breast (Zou et al., 2018). Contrary to the above-mentioned studies, no significant improvement in meat tenderness was observed after low intensity ultrasound treatment for bovine *Semitendinosus*, *Biceps femoris* and *Pectoralis* muscles (Lyng et al., 1997; Pohlman et al., 1997a; Pohlman et al., 1997b).

Electrical stimulation

Electrical stimulation is a post-slaughter treatment used in preventing carcass cold-shortening and facilitating muscle maturation processes (Allahodjibeye, 2019). This process leads to an increase in the rate of pH fall, due to increased muscle glycolysis, accelerating the onset of muscle rigor mortis before reaching a temperature that is low enough for cold shortening to occur (Devine et al., 2014). Electrical stimulation has also been observed to result in physical modification of muscle structure, such as the formation of stretched contracture bands and disruption of sarcomeres, which is likely to play an important role in meat tenderisation (Bekhit et al., 2014a; Kadim et al., 2009; Li et al., 2012; Zhang et al., 2019).

Several studies have shown that electrical stimulation resulted in early activation of calpains, accelerated proteolysis of the muscle proteins and increased muscle tenderness in *Longissimus dorsi* muscle of fat-tailed sheep (Abbasvali et al., 2012), and *Longissimus lumborum* muscle of cattle (Ferguson et al., 2000; Li et al., 2012) and lamb (Pouliot et al., 2014). However, Kim et al. (2013) reported that tenderness and proteolysis of the *Longissimus dorsi* muscles from calves stimulated by low voltage remained unaffected. These conflicting observations might be due to the differences in the voltages applied, the muscle types, and the age of the animals at slaughter. Interestingly, electrical stimulation of bovine *Longissimus dorsi* muscle at a very early stage of post-mortem (3 min) reduced the effectiveness of tenderisation due to significant reduction in the early levels of activity of μ -calpain, which was negatively correlated to the tenderness (Hwang and Thompson, 2001).

The activity of lysosomal enzymes such as β -glucuronidase, cathepsin C and cathepsin B+L in the muscles has been reported to be enhanced significantly after electrical stimulation (Li et al., 2012). Uytterhaegen et al. (1992) reported an improvement in tenderness in electrically stimulated bovine *Longissimus dorsi* along with increased activity of the calpains, but not cathepsin B+L. Pommier et al. (1987) found no improvement in the tenderness of electrically stimulated calf *Longissimus dorsi* muscle despite an increase in the activity of cathepsin D. Thus, the improvement in tenderness might not be directly correlated to the activity of lysosomal enzymes in electrically-stimulated muscles.

Pulsed electric fields (PEF)

PEF is a non-thermal technique that permeabilises cell and organelle membranes by the application of high-voltage pulses on food using two conductive electrodes (electroporation), which has been explored for meat tenderisation (Bhat et al., 2018b; Warner et al., 2017). PEF treatment could potentially improve meat tenderness by causing the physical disruption of myofibrils, the early activation of the calcium-dependent μ -calpain by releasing of calcium ions from the cellular organelles, and/or facilitating the release of proteolytic enzymes (such as cathepsins B and L) from the lysosomes. Moreover, PEF has been hypothesised to facilitate glycolysis (generally identified through the ultimate muscle pH, pH_u) in pre-rigor meat, which is associated with enhanced proteolysis (Bekhit et al., 2014b). However, the effect of PEF on meat tenderness reported in the current literature varies. This might be due to variation in the processing parameters (electric field strength and specific energy), the properties of meat samples (muscle cuts, hot or cold-boned, and dielectric properties) and the conditions of pre- (freezing) or post- (aging) PEF treatments (Alahakoon et al., 2016). For instance, Suwandy et al. (2015a) noticed an increase in toughness in the hot-boned bovine *Longissimus lumborum* and a decrease in shear force of the hot-boned bovine *Semitendinosus* muscles after PEF treatment. On the other hand, PEF treatment tenderised cold-boned bovine *Semitendinosus* muscles but did not affect the tenderness of cold-boned *Longissimus lumborum* muscles (Suwandy et al., 2015b). Both PEF experiments was conducted using the same processing parameters (Suwandy et al., 2015a; Suwandy et al., 2015b). These observations suggested that the tenderising effect of PEF varies between muscle cuts and post-mortem handling of muscles. Different muscle cuts have different protein (myofibrillar and collagen) and fat compositions which could affect the tenderising effect of PEF treatment (Alahakoon et al., 2016). The hot-boning treated muscles are removed from the carcass in a pre-rigor state and thereby experiences a higher degree of contraction and shortening than cold-boned muscles and produces a tougher meat (White et al., 2006). Faridnia et al. (2015) reported that freezing and thawing prior to PEF improved the tenderness of bovine *Semitendinosus* muscles but PEF treatment alone had no effect. The reasons could possibly be the physical disruption of muscle cells by freezing and rupturing of connective tissue, leading to tenderisation, and the disruption of the lysosomes due to freezing, leading to the release of cathepsins for participation in proteolysis. PEF treatment has been reported to enhance the autolysis of calpains (both μ and m types) and improve proteolysis during the aging process of cold-boned beef, but opinions on the cause of the tenderising effect has been non-unanimous (Bhat et al., 2018c; Bhat et al., 2019). The effect of PEF on the activity of calpains in hot-boned meat and on the activity of lysosomal proteases in meat has not been reported.

Shockwave processing

Shockwave hydrodynamic processing (HDP) involves the generation of pressure waves up to 1 GPa in fractions of milliseconds by either explosive or electrical discharge (Bolumar et al., 2013). It has been reported that HDP improved the meat tenderness by up to 70%, where the electrical HDP treatment showed a milder effect with only 10 to 30% shear force reduction (Bolumar et al., 2013; Hopkins, 2014). The mechanism to explain this observation has not been established. Hopkins (2014) suggested the tenderisation effect of HDP was due to the physical destruction of the muscles and the release and activation of endogenous enzymes caused by disruption of the muscle structure. In contrast, Bolumar et al. (2014) speculated that the tenderising effect was mainly due to the disruption of muscle structure, as no activation of the cathepsins or peptidases was observed in the electrical discharge HDP-treated muscle. The tenderisation effect of HDP might be due to an enhanced aging process as a result of disordered muscle structure, which facilitates the contact of endogenous proteases with their substrate. The effect of explosive HDP treatment on endogenous enzymes in meat has not been reported. The more

intense treatment from explosive HDP will presumably have had more impact on muscle structure, which might aid in the release and activation of lysosomal proteases.

Conclusion

Various studies have suggested that the endogenous proteases act synergistically in the proteolytic tenderisation of meat. The activity of m-calpain remains nearly constant throughout post-mortem aging at refrigerated temperatures but a gradual decrease in μ -calpain has been observed for bovine, ovine and porcine muscles. There is increasing evidence to suggest that the caspases and the calpain system may interact throughout post-mortem aging, indicating the role of caspases in post-mortem proteolysis. The proteasome has been found to be less susceptible to post-mortem meat storage conditions and therefore has been suggested by some studies to have more proteolytic activity than μ -calpain.

Post-slaughtering treatments and processes such as electrical stimulation have been reported to cause early activation of calpains and increase the activity of many lysosomal proteases. Similarly, HPP (at relatively low pressures), PEF, and ultrasound processing have been reported by many studies to help release and increase the activities of lysosomal proteases such as the cathepsins and acid phosphatase and to activate m-calpain through the release of calcium ions from the sarcoplasmic reticulum. Mild heating has been shown to increase the activity of cathepsins, particularly cathepsins B+L (when held at 55°C), whereas calpains start to be inactivated from 55°C. The information reviewed in this paper may be used to design optimum post-mortem meat storage and processing conditions in order to achieve improved muscle tenderness within shorter post-mortem aging and cooking times. However, more research is required to address the effect of different animal species, muscle cuts, age and hot/cold boning, etc., on the achievement of meat tenderness through the use of different processing technologies.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Author Contributions

Conceptualization: Kaur L. Investigation: Kaur L, Hui SX, Morton JD, Kaur R, Chian FM, Boland M. Writing - original draft: Hui, SX, Kaur, L. Writing - review & editing: Kaur L, Hui SX, Morton JD, Kaur R, Chian FM, Boland M.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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