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Review

Tenderness – An enzymatic view

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ABSTRACT

One of the most common causes of unacceptability in meat quality is toughness. Toughness is attributed to a range of factors including the amount of intramuscular connective tissue, intramuscular fat, and the length of the sarcomere. However, it is apparent that the extent of proteolysis of key proteins within muscle fibres is significant determinant of ultimate tenderness. The objective of this manuscript is to describe the main endogenous proteolytic enzyme systems that have the potential to be involved in muscle post-mortem proteolysis and whether the experimental evidence available supports this involvement.

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Contents

1. Introduction

Of all the meat traits, tenderness is considered to be the most important with regard to eating quality (Miller, Carr, Ramsey, Crockett, & Hoover, 2001). It appears that the main determinant of ultimate tenderness is the extent of proteolysis of key target proteins within muscle fibres (Koohmaraie & Geesink, 2006; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995a). Given the recognised importance of proteolysis in the tenderisation process this review has focused on the biochemistry of enzyme systems that have a potential role in the process, and some of the experimental evidence either rejecting or supporting their involvement. Our research in this area has focused on the fundamental biochemical aspects of the potential proteolytic systems involved tenderisation in pigs therefore the majority of the research described in this review focuses on this species, however, the scope of this review has been extended to cover fundamental observations made in other species.

2. Post-mortem proteolysis and candidate proteolytic systems

The final tenderness of meat depends on the degree of alteration of the muscle structural and associated proteins (Hopkins & Taylor, 2002). Specific myofibrillar, myofibril cytoskeleton and costamere proteins, such as titin, desmin and vinculin respectively, are subjected to cleavage, with some cleavage of the major myofibrillar proteins such as actin, myosin (Fig. 1; Goll, Thompson, Taylor, & Christiansen, 1992; Hopkins & Thompson, 2002; Koohmaraie & Geesink, 2006; Lametsch et al., 2003; Taylor et al.,

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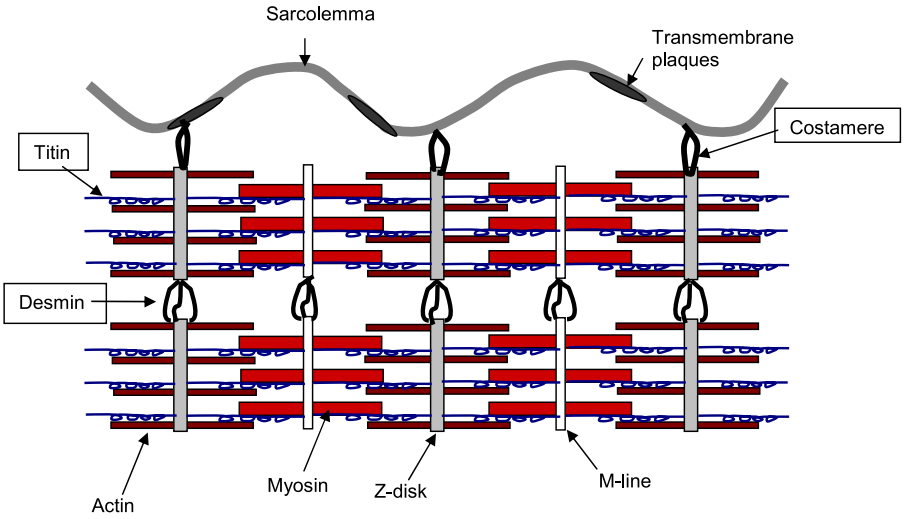

Fig. 1. Schematic representation of muscle myofibrillar proteins showing the major components of the sarcromere. Boxes indicate the cytoskeletal structures and proteins susceptible to post-mortem cleavage (adapted from Taylor et al., 1995a).

1995a). For a protease system to be considered to be involved in post-mortem proteolysis and meat tenderisation it must meet a certain basic criteria as defined by Koohmaraie (1988): firstly, the protease must be endogenous to skeletal muscle cells; secondly it must be able to mimic post-mortem changes in myofibrils *in vitro* under optimum conditions and finally it must have access to myofibrils in tissue. In this review the candidate proteolytic systems considered as having a potential role in the degradation of proteins post-mortem are described and the experimental investigations that have been carried out to determine whether they have a role are discussed.

3. Cathepsins and other muscle peptidases

Cathepsins are a group of enzymes comprised of both exo- and endo-peptidases and are categorised into cysteine (cathepsins B, H, L and X), aspartic (cathepsins D and E) and serine (cathepsins G) peptidase families (Sentandreu, Coulis, & Ouali, 2002). Many research groups have discarded the contribution of cathepsins to meat tenderisation on the basis of a number of observations. Firstly, there is not large scale actin and myosin degradation in the post-mortem conditioning period; these being primary substrates for cathepsins (Koohmaraie, Whipple, Kretchmar, Crouse, & Mersmann, 1991). Secondly, cathepsins are located in the lysosomes and must therefore be released for them to have access to myofibril proteins and to add to meat tenderness (Hopkins & Taylor, 2002). However, low pH levels and high carcass temperature can enhance the disruption of the lysosomal membrane (O'Halloran, Troy, Buckley, & Reville, 1997) and failure of ion pumps in the membranes as the carcass enters rigor, consecutively to ATP depletion, could overcome this (Hopkins & Thompson, 2002). Thirdly, there is little association between cathepsins' activities and the variation in tenderness in meat samples (Whipple et al., 1990). However, cathepsins B and L activities at 8 h post-mortem have been found to positively correlate with tenderness in beef (O'Halloran et al., 1997). Cathepsin L hydrolyses the largest number of myofibrillar proteins, including troponin T, I and C, nebulin, titin and tropomyosin; which are degraded during the post-mortem conditioning period as well as myosin and actin, in rabbit, beef and chicken myofibrils (Mikami, Whiting, Taylor, Maciewicz, & Etherington, 1987).

More recently it has been reported that activity of serine peptidases inhibitors was a good predictor of meat toughness (Zamora et al., 2005). Serine peptidases form the largest group of peptidases in mammalian systems. The most best characterised are those associated with digestion (trypsin and chymotrypsin) and blood clotting (thrombin). There are reports of serine peptidases being expressed in skeletal muscle, although there is debate whether the activity identified in muscle homogenates originates from muscle itself or the associated cells (for a review see Sentandreu et al., 2002). In the study by Zamora et al. (2005), the activity of semi-purified serine peptidase inhibitor was found to be positively related to toughness, and when combined with 6 other variables, which included micro-calpain activity, was predictive of cattle meat toughness after 6 days post-mortem storage. Such reports re-enforce the observations that inhibitors of the peptidases have a better predictive value of meat quality than the enzyme directly involved in the proteolysis, an example of this is calpastatin, the calpain proteolytic enzyme inhibitor (see below).

4. Proteasomes

The proteasome is a multicatalytic protease complex involved in the regulation of a number of basic cellular pathways, by their degradation of proteins in the cytosol and nucleus (Coux, Tanaka, & Goldberg, 1996). Proteasomes are ubiquitously expressed in the body and are abundant in skeletal muscle (Robert, Briand, Taylor, & Briand, 1999). The proteasome (26S) consists of a 19S regulatory subunit and the 20S multicatalytic structure containing the proteolytic enzyme activities. The 20S proteasome, also known as the multicatalytic proteinase complex (MCP), is the catalytic core of these proteasome complexes (Dahlmann, Ruppert, Kloetzel, & Kuehn, 2001). Proteolysis by the proteasome is an ubiquitin-dependent process, at least four ubiquitin proteins must attach to the lysine residue of the target substrate. The poly-ubiquitinated proteins are subsequently recognised by the proteasome, which removes the ubiquitin chain and degrades the substrate (Taillandier et al., 2004). This process is ATP dependent and once this is depleted the 26S proteasome dissociates into the 19S subunit and the 20S proteasome, the latter not requiring ATP or ubiquitin (Peters, Franke, & Kleinschmidt, 1994). This latter observation along, with its relatively high level of expression in skeletal muscle, has led several groups to examine the potential role of the 20S proteasome in post-mortem proteolysis. Initial studies reported that proteasome extracted from ovine muscle was not involved in myofibril proteolysis (Koohmaraie, 1992). However, a number of subse-

Table 1
Estimates of the calcium concentration (μM) required for activation, autolysis and interaction with calpastatin. The figures are the concentrations required for half maximal activity, binding or rate of autolysis (adapted from Goll et al., 2003).

Calpain property	Autolysed μ-calpain	μ-calpain	Autolysed m-calpain	m-calpain
Proteolytic activity	0.5–2	3–50	50–150	400–800
Calpastatin binding	0.042	40	25	250–500
Autolysis−phospholipids		50–150		550–800
Autolysis + phospholipids		0.8–50		90–400

quent studies have provided evidence supporting the possibility that the proteasome could contribute to meat tenderisation. Taylor et al. (1995b) and Robert et al. (1999) found that bovine proteasomes were capable of causing proteolysis of myofibril proteins including nebulin, myosin, actin and tropomyosin in bovine myofibrils. Proteasome activity is maintained during the post-mortem conditioning period, with substantial activity still detectable at 7 days post-mortem and at pH levels of less than 6 (Lamare, Taylor, Farout, Briand, & Briand, 2002). Additionally Dutaud, Aubry, Sentandreu, and Ouali (2006) showed specific structural changes including an increase in the Z-disks' width, stretching into the I-band in muscle fibres incubated with 20S proteasome, an observation that is not found in fibres treated with either calpain or cathepsin. Likewise, using an anti-proteasome inhibitor and a proteome approach, Houbak, Ertbjerg, and Therkildsen (2008) demonstrated that there was a lack of degradation of proteins commonly seen proteolysed in post-mortem muscle such as troponin T and nebulin. However, as emphasised by Koohmaraie and Geesink (2006), the degradation pattern of myofibrillar proteins in incubations with 20S proteasome are not the same as that seen in post-mortem muscle, although this does not appear to exclude the proteasome from making a contribution to the process of post-mortem proteolysis (Houbak et al., 2008).

5. The calpain system

Calpains are probably the most extensively researched protease family with regard to meat science and it is widely accepted that proteolytic calpain activity does contribute to meat tenderisation (Koohmaraie & Geesink, 2006; Sentandreu et al., 2002). Calpains are a large family of intracellular cysteine proteases. To date 14 members have been identified, which are expressed in a ubiquitous or tissue-specific manner (Goll, Thompson, Li, Wei, & Cong, 2003). In skeletal muscle, the calpain system consists of three proteases, ubiquitously expressed isoforms μ-calpain, m-calpain, and p94 (or calpain 3). The ubiquitously expressed μ- and m-calpain are calcium-activated proteases, requiring micro- and millimolar concentrations of Ca^{2+} for activation, respectively (Table 1) (Goll et al., 2003). Associated with the calpain proteolytic enzyme family is the calpain-specific endogenous inhibitor, calpastatin (Wendt, Thompson, & Goll, 2004).

There is now considerable evidence linking the calpains to tenderisation in beef, lamb and pork. Correlations have shown that the different tenderisation rates between species (beef < lamb < pork) relate inversely to the ratio of calpastatin:calpain (beef > lamb > pork) (Koohmaraie et al., 1991). Other evidence comes from observations on the effects of β-adrenergic agonists, which significantly reduce muscle protein degradation (Bohorov, Buttery, Correia, & Soar, 1987). In treated animals there is elevated calpastatin activity and mRNA expression, which has led to the suggestion that calpain activity is involved in muscle protein turnover (Bardsley et al., 1992; Higgins, Lasslett, Bardsley, & Buttery, 1988; Killefer & Koohmaraie, 1994; Parr, Bardsley, Gilmour, & Buttery, 1992). Associated with the growth effect of β-adrenergic agonists is tougher meat (Dunshea, D'Souza, Pethick, Harper, & Warner, 2005). Likewise the muscle hypertrophy found in callipyge lambs is associated with the high levels of calpastatin, greatly reduced post-mortem proteolysis and a significant decrease in meat tenderness (Geesink & Koohmaraie, 1999). Although these observations indicate a central role of calpains in post-mortem proteolysis they do not indicate which of the calpain proteolytic isoforms is responsible for the post-mortem degradation.

Calpain 3/p94 is expressed almost exclusively in skeletal muscle (Sorimachi et al., 1989) and was originally of interest to meat scientists because it binds to the giant myofibrillar protein titin, at the N_2 line (Sorimachi et al., 1995), a site where proteolysis has been linked to meat tenderisation (Taylor et al., 1995a). Parr et al. (1999a) investigated calpain 3 in post-mortem proteolysis and meat tenderisation in pigs and found that there was no association between calpain 3 expression and 8 day shear force in porcine *longissimus dorsi* (LD) muscle. However, in sheep, variations in calpain 3 mRNA and protein levels have been reported to strongly correlate to variations in tenderness (Ilian et al., 2001) and to be involved in myofibrillar protein degradation in ovine LD muscle (Ilian, Bekhit, & Bickerstaffe, 2004). In order to directly examine the effect on p94 muscle function p94 knockout mice (Kramerova, Kudryashova, Tidball, & Spencer, 2004) were used to examine the effects of the absence of calpain 3 on post-mortem muscle (Geesink, Taylor, & Koohmaraie, 2005). Post-mortem proteolysis occurred in a similar fashion in calpain 3 knockout mice in comparison to control wild type mice, with no differences detected in desmin, nebulin, troponin-T or vinculin degradation, suggesting that calpain 3 is not involved in meat tenderisation. Although these studies were carried out in mice, the body of experimental evidence available suggests that the muscle-specific calpain 3 does not appear to have a major role in post-mortem proteolysis and the associated development of tenderness. This interpretation is further supported by the observation that calpain 3 is not inhibited by calpastatin (Ono et al., 2004) as animals with very high calpastatin do not produce tender meat.

Myofibrils incubated with calpains have produced similar degradation patterns to those observed in post-mortem muscle, with calpains degrading key myofibrillar proteins including nebulin, titin, troponin-T and desmin (Huff-Lonergan et al., 1996.). Since both μ- and m-calpain target and cleave the same myofibrillar proteins experimental investigations have tried to determine which isoform is primarily involved in post-mortem proteolysis. μ-Calpain is activated in early post-mortem (within 3 days of slaughter); during the period where post-mortem proteolysis of key myofibrillar proteins is known to take place (Taylor et al., 1995a). The m-isoform persists longer than the less stable μ-isoform in aging muscle from all species studied, including pig (Sensky, Parr, Bardsley, & Buttery, 1996), suggesting that it is not activated early post-mortem. Additionally, the Ca^{2+} concentrations that exist in muscle post-mortem are less than that required of m-calpain for activation (Boehm, Kendall, Thompson, & Goll, 1998). Recently the evidence for a significant role of μ-calpain in post-mortem proteolysis has further been strengthened from observations made in μ-calpain knockout mice (Geesink, Kuchay, Chishti, & Koohmaraie, 2006). In these animals post-mortem proteolysis was significantly inhibited, although some proteolysis was observed in the knockout mice.

The authors attributed this protein degradation to autolysis of m-calpain, however, it does not rule out the possibility that another protease is responsible for the proteolysis observed. Therefore the experimental evidence available suggests that μ-calpain is the enzyme component of the calpain system that has the most significant role in post-mortem proteolysis and meat tenderisation. In cattle the identification of single nucleotide polymorphisms (SNP) in the CAPN1 gene, encoding μ-calpain, which is associated with tender meat, has further strengthened the importance of μ-calpain in tenderisation. Two bovine CAPN1 gene SNPs, which introduce amino acid substitutions (non-synonymous SNPs) in μ-calpain, are associated with tenderness (Page et al., 2002). In pigs or sheep, however, no such relationships between polymorphisms in CAPN1 and meat quality have yet been reported.

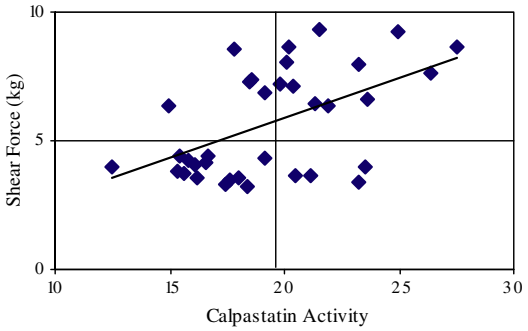

Fig. 2. Correlation between slaughter (2 h) calpastatin activity ($\times 10^7$ fluorescence units/kg) and 8 day shear force in porcine *longissmus dorsi* (LD) (unpublished observations).

6. Relationship between calpastatin and meat toughness

The single calpastatin gene contains multiple promoters that generate several different transcripts that are also alternatively spliced into multiple mRNAs, resulting in multiple protein isoforms being derived from a single gene (Meyers & Beever, 2008; Parr, Sensky, Arnold, Bardsley, & Buttery, 2000; Parr, Sensky, Bardsley, & Buttery, 2001; Parr et al., 2004; Raynaud, Jayat-Vignoles, Laforêt, Levéziel, & Amarger, 2005a; Raynaud et al., 2005b). The micro-heterogeneity of calpastatin in different cells and tissues may determine its intracellular localisation and its physiological role, being a potential mechanism by which the single calpastatin gene can regulate the activity of the products from multiple calpain genes. Calpastatin inhibits both μ- and m-calpain and this process requires calcium concentrations that are reported to be close to or below those that are required to activate calpain (Goll et al., 2003) (Table 1). However, calpastatin is itself susceptible to proteolysis but the resulting fragments retain inhibitory activity. More recent crystallography-based observations have identified the nature of the interaction of calpastatin with calpain, which has unique aspects for a protein inhibitor interacting with a proteolytic enzyme (Hanna, Campbell, & Davies, 2008; Moldoveanu, Gehring, & Green, 2008). Calpastatin is an unstructured protein but when it binds calpain it adopts a structure which allows inhibition to take place. Calpastatin contains 4 inhibitory domains, each of which can inhibit calpain activity. Within these domains there are three regions A, B and C, predicted to interact with calpain. The binding of calcium to calpain causes changes in the calpain molecule enabling it to become active but also allowing calpastatin to interact with the enzyme. The peptide chain helices found in regions A and C, within a inhibitory domain, interact with calpain at two separate sites causing the inhibitory domain to wrap around calpain. The region between A and C, region B, then blocks the active site of calpain. The crystallography studies suggest that region B does not directly bind to the active site thereby preventing it becoming a substrate for the enzyme. These experiments go some way to explain the observation that calpastatin requires calcium in order to interact with calpains (Goll et al., 2003). However, if region B does not interact with the active site what is the mechanism by which calpains are reported to cleave calpastatin, as the inhibitor is degraded post-mortem (Doumit & Koohmaraie, 1999)? Part of this cleavage may be caused by other proteinases such as caspases (see below), which presumably will modify calpastatins activity. However, Moldoveanu et al. (2008) reported that that the inhibitory domains are not equivalent at inhibiting calpains which might, as suggested by Mellgren (2008), be the mechanism by which calpains degrade calpastatin via the cleavage of the weaker inhibitory domains, creating specific peptide fragments that retain inhibitory activity.

A consistent observation of the calpain system's involvement in tenderness is that high levels of calpastatin are associated with poor quality meat; the model being that high levels of calpastatin reduce the activity of calpain thereby reducing the proteolysis required for tender meat. In ruminant species there is relationship between calpastatin activity in the muscle 24 h after slaughter and the degree of tenderisation achieved after conditioning, with differences in calpastatin accounting for 40% of the variation in tenderness (Shackelford et al., 1994). Our studies on a random selection of commercially slaughtered pigs have shown that high levels of calpastatin (both activity and protein levels) in the first few hours after slaughter are associated with an increased incidence of toughness (Parr et al., 1999b; Sensky et al., 1998), (Fig. 2). Therefore by monitoring calpastatin at these times it would be potentially possible to predict whether or not any given carcass will tenderise to an acceptable degree. Although several calpastatin-based approaches of predicting meat quality have been described (Geesink, van der Palen, et al., 2005; Grant, Stringer, Studer, Lichlyter, & Lorenzen, 2005) the use of antibody-based methods of calpastatin detection is complicated by the fact that there is considerable micro-heterogeneity in calpastatin forms that are generated by alternative splicing and phosphorylation (Parr et al., 2000, 2001). The function of such variability may be to control the inhibitory activity of calpastatin, and therefore the identification of these isoforms and the mRNA species from which they originate may lead to an even closer predictor of toughness/tenderness. Given the potential this has for marker-assisted breeding programmes, a number of groups have now identified calpastatin gene polymorphisms and have shown that some of these are predictive of carcass quality in cattle and in pigs (Barendse, 2002; Ciobanu et al., 2004). Currently there are markers within the calpastatin and μ-calpain genes that are able to identifying beef cattle with the genetic potential to produce tender meat (Casas et al., 2006) and these are commercially available as a genetic test (GeneSTAR Molecular Value Predictions, Pfizer Genetics Ltd.). In pigs, five genetic polymorphisms have been identified that are mis-sense mutations leading to amino acid substitutions within subdomains of the calpastatin protein (Ciobanu et al., 2004).

Variability in calpastatin and its inhibition of calpain mediated-proteolysis is likely to be affected by environmental effects. For example elevated plasma adrenaline increases calpastatin activity and expression in pigs, implying that the link between stress and meat toughness may indeed be partially mediated via the calpain system (Parr et al., 2000; Sensky et al., 1996). The hypothesis that environmental factors influence calpastatin function and subsequently that this affects meat quality is further strengthened by the observation that the gene's sequence variation, that specifies the haplotype linked to pig meat quality (Ciobanu et al., 2004), alters peptide consensus sequences predicted to be phosphorylated by cyclic 3′, 5′-monophosphate-dependent protein kinase (PKA).

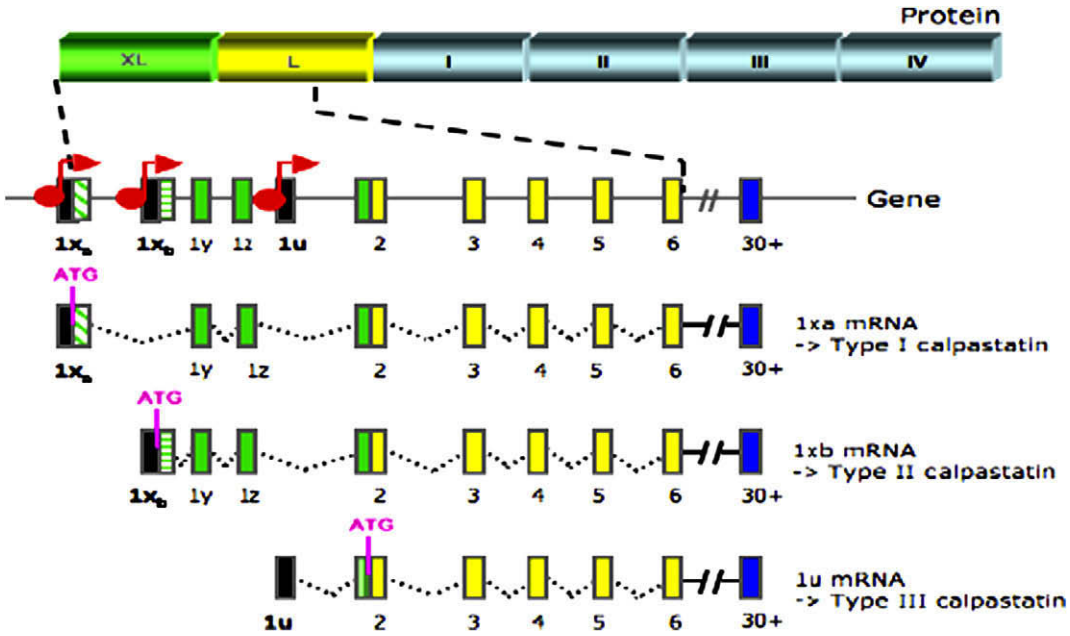

Fig. 3. The promoter structure and transcripts generated from the calpastatin gene. The diagram shows the generalised representation of the structure of the 5′ end of the calpastatin gene in pigs and cattle below the domain structure of calpastatin, boxes indicate identified exons. Indicated are the predominant transcripts that originate from the promoters and transcription start sites, represented by the circles and arrows, respectively. These transcripts have exons alternative spliced to produce mRNA that encode for the three types of calpastatin protein (Type I, II and III calpastatins). Indicated by the initiating codon ATG is the 5′ most start site of translation within each mRNA. Black boxes indicate non coding regions; hatched boxes indicate a amino acid coding region located within an exon that also contains non coding region; dark grey boxes indicate regions that code for the N terminal XL region of calpastatin; light grey boxes indicate regions that code for calpastatin (adapted from Parr et al., 2001).

This kinase is part of the signalling pathway stimulated by adrenergic agonists. Porcine calpastatin can be phosphorylated by PKA (Parr et al., 2000) and in other species PKA-mediated phosphorylation alters calpastatin's solubility and cell localisation with the phosphorylation sites being located within peptide sequences encoded by an exon within the N terminal Leader domain (L-domain), a region of the protein which does not have inhibitory activity (Averna et al., 2001; De Tullio et al., 2009). It is likely that any effect of calpastatin on meat quality is influenced by an environment interaction.

Understanding the mechanism by which such regulation can control calpastatin function is of paramount importance to enable further meat quality improvement and may yield specific genetic or protein based markers that are indicative of tender meat. Our current studies have focused on determining the factors that regulate calpastatin gene expression. Calpastatin gene expression is regulated via several promoters associated with the 5′ exons 1xa, and 1xb, which are in tandem and 1u, which is 3' and distal to 1xa and 1xb (Meyers & Beever, 2008; Parr et al., 2000; Raynaud et al., 2005a) (Fig. 3). Each of these promoters appears to be responsible for the expression of different calpastatin mRNAs (encoding Type I, II and III calpastatins, respectively, which have different N termini) that can be alternatively spliced to potentially give a variety of protein isoforms. The activity of the promoter regions of pigs and cattle have been examined and it appears that each species has differing activities, with the 1u promoter and 1xb promoter being most active in pig and cattle, respectively (Parr et al., 2004; Raynaud et al., 2005a). Within the calpastatin gene each promoter has a different response to secondary messenger pathways, mediating the response to factors that stimulate growth or a stress response (Parr et al., 2004; Sensky et al., 2006). The differential between transcriptional activity of the calpastatin gene promoters between species and their differing response to stimuli probably is in part responsible for the variation in calpastatin expression between species, breeds and individuals that contributes to variations in meat tenderness.

7. The caspase system

Although there is considerable evidence to suggest that the activity of the calpain system early in the post-mortem conditioning period influences the ultimate tenderness, it has been suggested that it is not the sole proteolytic determinant of meat quality. Recently a model has been proposed which suggests that the protease family of caspases could be active post-mortem and contribute to tenderisation (Ouali et al., 2006; Sentandreu et al., 2002). Caspases are a family of cysteine aspartate-specific proteases and to date 14 members of the caspase family have been identified and can be divided according to their roles in either apoptosis or inflammation (Earnshaw, Martins, & Kaufmann, 1999). Apoptosis is the organised dismantling of the cell, characterised by cell shrinkage, DNA fragmentation, chromatin condensation, membrane blebbing and the formation of apoptotic bodies without inducing an inflammatory response (Wyllie, Kerr, & Currie, 1980). Caspases involved in apoptosis can be further subdivided into initiator caspases, such as caspases 8, 9, 10 and 12 or effector caspases, such as caspases 3, 6 and 7, depending on their location on the cell death pathway (Earnshaw et al., 1999).

Caspases are activated via three main pathways, outlined in Fig. 4. The cell death pathway or extrinsic pathway is triggered by cell surface receptors and initiator caspases 8 and 10 are activated via this pathway (Boatright & Salvesen, 2003). The intrinsic pathway involves caspase 9 and is activated in response to environmental stress such as hypoxia and ischaemia (Earnshaw et al., 1999). The ER mediated pathway is activated via stress directly upon the ER, for example disruption in Ca^{2+} homeostasis, which in turn activates initiator caspase 12. Effector caspases are activated by initiator caspases upstream and once activated target and cleave specific substrates, resulting in cell disassembly (Fuentes-Prior & Salvesen, 2004). To date more than 280 caspase targets have been identified including myofibrillar and cytoskeletal proteins (Fischer, Janicke, & Schulze-Osthoff, 2003). There are a number of mechanisms which are involved in the regulation of

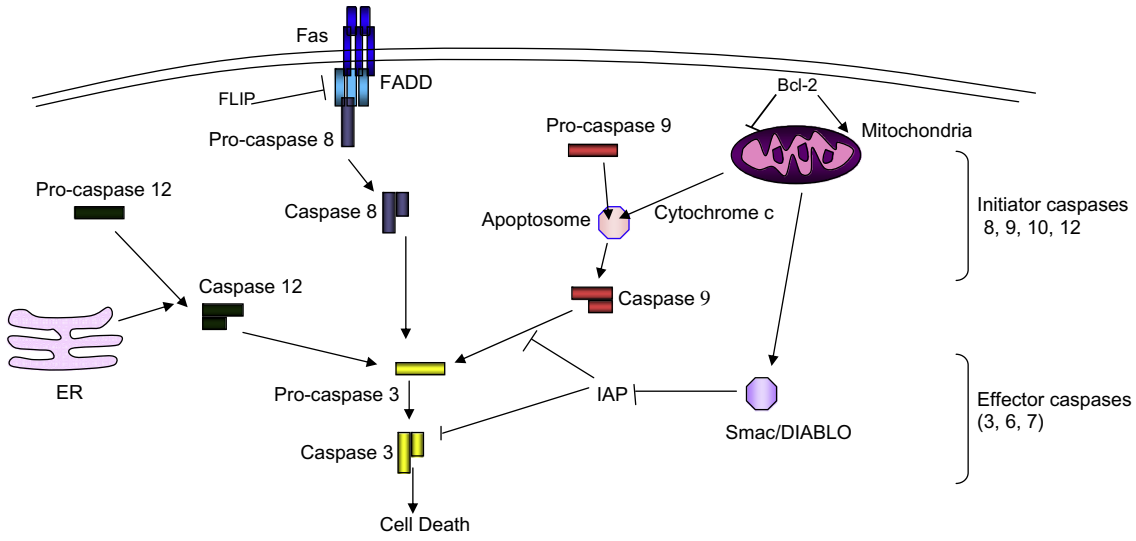

Fig. 4. Schematic diagram of the intrinsic, extrinsic and ER-mediated apoptosis pathways showing the caspases involved in each pathway. FADD- fas associated death domain, IAP- inhibitor of apoptosis, Smac- secondary mitochondrial activator of caspases, DIABLO- direct IAP-binding protein with low pI (adapted from Holcik, 2002).

Table 2
Changes in caspase 3/7 and 9 activities and protein levels of degraded poly (ADP-ribose) polymerase (PARP 89 kDa) and alpha II spectrin breakdown degradation product 120 kDa (SBDP120), the caspase-mediated proteolysis breakdown products, over time in porcine *longissmus dorsi* (LD) (adapted from Kemp, Bardsley, & Parr, 2006a, 2006b).

Time h	*n*	Caspase 3/7 activity fluorescence/μg protein	Caspase 9 activity luminescence/μg protein	PARP 89 kDa units[a]/mg protein	SBDP120 units[a]/mg protein
0	10	110.49	55.3	21.52	2.59
2	10	129.88	58.58	11.14	2.98
4	10	115.87	56.81	19.65	2.82
8	10	110.66	39.9	18.84	1.23
16	10	120.52	48.07	ND	1.01
32	10	73.16	25.71	ND	1.03
192	10	5.49	3.01	ND	1.08
s.e.d.		10.32	5.22	5.14	0.57
P value		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

ND = not detectable.
[a] Arbitrary densitometry units.

caspase activation including the skeletal muscle-specific inhibitor apoptosis repressor with caspase recruitment domain (ARC) (Koseki, Inohara, Chen, & Nunez, 1998). Interactions between these inhibitory proteins and caspases are outline in Fig. 4. The intricacies of these pathways and caspases' regulations are beyond the scope of this review and readers are referred to the relevant reviews (Boatright & Salvesen, 2003; Earnshaw et al., 1999).

Caspases can be activated early in pathological events associated with hypoxia/ischaemia (Gustafsson & Gottlieb, 2003), which is not that dissimilar to the hypoxic conditions in muscle after slaughter. In meat animals the process of exsanguination occurs after slaughter, depriving all cells and tissues of nutrients and oxygen. After death muscle continues to metabolise and therefore muscle cells will presumably engaged in the process of cell death, with apoptosis rather necrosis considered to be the most likely process of cell death (Sentandreu et al., 2002). Therefore it has been hypothesised that the process of slaughter and exsanguination could initiate the apoptotic pathways and caspase activity may contribute to early post-mortem proteolysis and meat tenderisation. Our recent work has focused on trying to determine whether caspases are active in post-mortem muscle and whether they cleave proteins found within the muscle of myofibril structures.

Our work in pigs has shown that components of the caspase system are expressed and are active in varying proportions in different skeletal muscles (Kemp, Parr, Bardsley, & Buttery, 2006b). Subsequently we examined whether caspases were active in porcine LD muscles conditioned for a period of up to 8 days (Kemp, Bardsley, & Parr, 2006a). The combined activity of effector caspases 3 and 7 and the initiator caspase 9 was found to decrease over the post-mortem conditioning period (Table 2). Caspases 3/7 and 9 activities were highest in the early stages of the post-mortem conditioning period, with less than 6% of at death activity remaining at 192 h. The ratio of activities at 0 and 32 h were taken as an approximation of the change in caspase activity during the early post-mortem period, when caspases appear most active. There was a negative relationship between shear force and the 0:32 h ratio of caspase 9 ($P < 0.05$) and caspase 3/7 activities ($P = 0.053$). Caspase substrates alpha II spectrin and poly (ADP-ribose) polymerase (PARP) were analysed in muscle samples taken across the conditioning period and their specific caspase-mediated degradation products were detected by Western blot analysis (Table 1). The caspase-specific cleavage products of PARP and alpha II spectrin are both known indicators of apoptosis and the changes observed in their protein levels corresponded with the differences detected in caspase activities. The caspase-specific degradation product of PARP (89 kDa) was detected in the muscle samples predominantly in the first 8 h after death. The *in situ* protein level of caspase-specific alpha II spectrin cleavage product across the conditioning period were found to correlate positively to caspase 3/7 activity ($P < 0.01$) and caspase 9 activity ($P < 0.05$), indicating that caspase-mediated cleavage was occurring *in situ*. In addition there

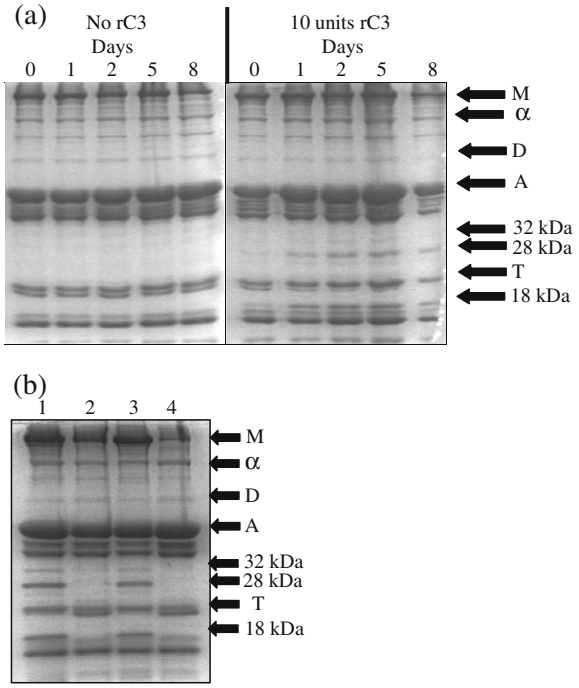

Fig. 5. The effects of incubating porcine myofibrils with recombinant caspase 3 (rC3). (a) The effect of incubating myofibrils for 0, 1, 2, 5 and 8 days at 4 °C without or with 10 units of rC3. (b) The effect of co-incubation: lane 1 10 units rC3 + 5 mM EDTA, lane 2 no rC3 + 5 mM EDTA, lane 3 10 units rC3 + 50 μl semi-purified calpastatin, lane 4 10 units rC3 + Ac-DEVD-CHO (0.1 μg/μl). In both figures the major degradation products generated by caspase-mediated proteolysis are indicated by their molecular weights. Abbreviations: M, myosin heavy chain; α, α-actinin; D, desmin; A, actin; T, troponin-I (adapted from Kemp and Parr, 2008).

was also a negative relationship between shear force and the level of the caspase generated alpha II spectrin 120 kDa degradation product ($P < 0.05$). These findings indicate that changes in caspase activity and caspase-mediated cleavage take place in muscle during the conditioning period and this could be associated with the development of tender meat. Using a similar approach to that described above Underwood, Means, and Du (2008) examined the changes in muscle caspase 3 activity during the post-mortem conditioning period in cattle. In this study they were unable to find any associations between caspase 3 activity, measured within 10 min of slaughter, in muscles that developed into beef with extremes of shear force. However, as described below, others have found a very strong association between the expression of a factor involved in apoptosis and the meat quality in beef (Bernard et al., 2007).

One of the criteria specified by Koohmaraie (1988) for a protease to be considered to be involved in post-mortem proteolysis and meat tenderisation is that it must be able to mimic the *in situ* post-mortem changes in myofibrils. Kemp and Parr (2008) demonstrated caspases ability to replicate myofibril degradation patterns observed *in situ*. Full length human recombinant caspase 3 (rC3) was expressed in *E. coli* and purified to homogeneity and incubated with myofibrils prepared from porcine LD using the procedure of Goll, Young, and Stromer (1974) in a buffer designed to simulate post-mortem muscle conditions according to Winger and Pope (1981). Incubation of myofibrils with rC3 resulted in the visible increase of myofibril degradation with the detection of proteolytic products at 32, 28 and 18 kDa (Fig. 5a). Analysis of these proteolytic products were identified using MALDI-TOF mass spectrometry and found to arise from the degradation of actin (32 kDa), troponin T (28 kDa) and myosin light chain (18 kDa). Additionally there was visible degradation of a number of myofibril proteins including desmin and troponin I, identified by MALDI-TOF mass spectrometry (Fig. 5a). The degradation of desmin is thought to significantly contribute to tenderisation (Hopkins & Taylor, 2002; Koohmaraie & Geesink, 2006) and there is a reported positive association between troponin T degradation and meat tenderness (Huff-Lonergan et al., 1996). These degradation patterns were not observed in myofibrils incubated with the caspase 3 specific inhibitor Ac-DEVD-CHO (Fig. 5b). However, co-incubation with either the endogenous calpain inhibitor calpastatin or the Ca^{2+} chelator EDTA and rC3 induced an increase in band intensities at 32, 28 and 18 kDa (Fig. 5b), suggesting that inhibition of calpain activity could somehow increase caspase activity. This study has shown that rC3 was capable of causing myofibril degradation, hydrolysing myofibrillar proteins under conditions that are similar to those found in muscle in the post-mortem conditioning period.

More recently callipyge lambs were examined to determine the changes in caspases during the conditioning period in this model of tough meat (Kemp, King, Shackelford, Wheeler, & Koohmaraie, 2009). As has been reported in previous studies there was high levels of muscle calpastatin activity and shear force in the callipyge lambs compared to non-callipyge lambs. Combined caspase 3/7 activity was found to decrease over the post-mortem storage period in both groups of lambs along with caspase 9, which preceded the changes in caspase 3/7; and a positive relationship observed between the two caspase activities ($P < 0.001$). Overall caspase activities were higher across the post-mortem conditioning period in normal sheep than in callipyge lambs in the LD and semimembranosus muscles but not in the infraspinatus muscle, which is not affect by the callipyge phenotype. A negative relationship was found between calpastatin activity at 0 and 2 days post-mortem and the peak post-mortem caspase 3/7 activity in LD in the non-callipyge animals. The interpretation of these observations was that caspases may contribute to the decrease in calpastatin in normal lambs, but the level of calpastatin in callipyge lambs was such that caspases could not degrade it sufficiently to overcome the high levels of calpastatin and, thus, calpastatin activity was the over-riding factor in post-mortem proteolysis in these animals.

There is increasing evidence of interactions between the calpain and caspase protease systems, predominantly focusing on the substrates that are targeted. Nakagawa and Yuan (2000) demonstrated that a disruption in Ca^{2+} homeostasis in the ER as a result of ischemic injury induced calpain-mediated activation of caspase 12 and that the anti-apoptotic protein Bcl-X_L was cleaved by m-calpain transforming it into a pro-apoptotic protein. Calpain inhibition through over-expression of calpastatin has been shown to increase caspase 3 activity and apoptosis (Neumar, Xu, Gada, Guttmann, & Siman, 2003). Additionally the endogenous calpain inhibitor calpastatin is also cleaved by caspases 1, 3 and 7, generating distinct degradation patterns (Wang et al., 1998). Therefore if caspases are active in the muscle post-mortem they may influence meat quality by proteolysis of calpastatin. This in turn could result in the activation of calpains which are known to be involved in meat tenderness and thus reducing toughness. Recently, the gene DNAJA1 has been identified to be down-regulated in longissimus thoracis muscle from Charolais bulls with high meat quality using microarrays. Its expression was shown to inversely relate to tenderness and explained up to 63% of the variation observed (Bernard et al., 2007). DNAJA1 encodes a member of the heat shock protein family (Hsp40), which co-chaperones the 70 kDa heat shock protein (Hsp70). The DNAJA1/Hsp70 complex has been shown to directly inhibit apoptosis by preventing the pro-apoptotic protein Bax to translocate to the mitochondrial membrane, where it undergoes conformational changes triggering the release of cytochrome c, an essential component in activating the intrinsic caspase 9 pathway (Gotoh, Terada, Oyadomari, & Mori, 2004). Bernard et al. (2007) suggest that if apoptosis and caspases are involved in

post-mortem proteolysis then the reduced anti-apoptotic activity of down-regulated DNAJA1 could facilitate cell death in the post-mortem period and, consequently, increase tenderisation. However, this study was performed in cattle and the use of micro-arrays is a relatively new tool for identifying markers of meat quality, therefore, further research is needed to understand the relationships between gene expression and meat quality.

8. Conclusions

The ultimate tenderness of meat is dependent on the degree of alteration and weakening of myofibrillar structures and has been largely attribute to endogenous proteolytic enzymes (Sentandreu et al., 2002). The calpain system has been shown to influence post-mortem proteolysis and the calpain-specific inhibitor has an important role in influencing tenderisation and acts as a marker for meat quality. However, it must be remembered that other novel proteolytic systems, such as caspases may contribute to post-mortem proteolysis and meat tenderisation and the extent of which is yet to be fully examined.

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