Session 4 Muscle biology and biochemistry

L 2 PATTERNS OF VARIATION IN ENZYME ACTIVITY AND CYTOSKELETAL PROTEOLYSIS IN MUSCLE

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Introduction

Unexplained variations in tenderness are consistently a problem in the meat industry world-wide and an ongoing source of consumer dissatisfaction. Reduction or elimination of unacceptably high toughness in prime table cuts would bring large economic benefits in terms of increased repeat sales and brand/mark trust and loyalty. A basic understanding of the causes of variability in toughness is therefore important to the meat industry, because an understanding of the mechanisms controlling this aspect of eating quality is desirable if we are to ensure high acceptability. Despite considerable research on this subject, we have still only a partial explanation of such variations. This warns us that the complete picture is multivariate and complex.

Structures involved in meat tenderisation

Current models of meat texture concentrate on the proteolytic degradation of myofibrils as the greatest cause of the development of tenderness during post-mortem storage (Dransfield, 1993; Koohmaraie, 1996). There is considerable evidence that degradation of the collagenous component of the extracellular matrix does occur during conditioning of meat (Nishimura et al., 1995; Nishimura et al., 1998) and that degradation also occurs in the proteoglycan component of the extracellular matrix (Eggen et al., 1998), but textural measurements on whole meat (Bouton & Harris,1972) and on isolated perimyial connective tissue (Lewis and Purslow, 1989) show that this has a negligible effect on texture after the meat is cooked to 60°C or above. There is a consensus view that the majority of myofibrillar degradation during ageing is due to μ-calpain (Dransfield, 1999; Koohmaraie, 1996) although a question has been raised (Boehm et al., 1998) about the potential role of m-calpain. Today there is some experimental evidence that lysosomal enzymes are released into the sarcoplasm during storage of beef (Ertbjerg et al., 1999). The principal reason for rejecting the possiblility that lysosomal enzymes (cathepsins) are involved in tenderness development is that they have the potential to degrade myosin and actin (Whipple and Koohmaraie, 1991), whereas little myosin and actin degradation takes place in normal post-mortem conditioning. It has been shown that incubation of myofibrils with μ-calpain results in a pattern of degradation similar to that occurring during conditioning of meat (Huff-Lonergan et al., 1996; Koohmaraie et al., 1986). In fact, most work on proteolysis relies on the correlation between myofibrillar protein degradation, or proteolytic activity in ageing meat (i.e. the net balance between calpain activity and calpastatin inhibition measured in vitro, often using non-meat substrates) and some physical measurement of texture. To date there seems to have been little work that actually demonstrates that the degradation seen is causally linked to a change in the strength or toughness of muscle fibres.

Whilst there is no broad agreement of which proteins within the myofibrils are the principle substrates of the major proteolytic enzymes, recent attention has been directed towards the cytoskeletal proteins (Taylor et al, 1995; Wheeler et al., 2000). These studies show that cytoskeletal proteins are degraded during conditioning of meat. The approach generally taken has been to identify specific, individual proteins as "good candidate" substrates and to correlate variations in their degradation with variations in measured toughness.

Our current programme of research on proteolysis contains several related areas of work that aim to further our understanding of the structural mechanisms of tenderness development and to ask basic questions about the sources of variations in tenderness. The aim of this paper is to review some developments in answering the following important questions:

- Do calpains actually reduce the strength of muscle fibres?
- If cytoskeletal proteins are degraded in conditioning, what is the precise nature of cleavage due to μ-calpain and m-calpain? How different is this to cathepsin degradation?
- What is the basis of variations in proteolysis between different muscles? Is it related to fibre type composition?

Direct measurements of a reduction in muscle fibre strength due to calpain incubation

Mechanical measurements of the breaking strength of single muscle fibres isolated from meat have been developed over the last 6 years (Mutungi et al, 1995, 1996; Willems and Purslow, 1996; Willems and Purslow, 1997; Christensen et al., 2000a) and clearly demonstrate that muscle fibres from conditioned meat are indeed weakened (Mutungi et al, 1996). Fibres from 11-day aged porcine longissimus muscle showed reduced strength at all temperatures studied between 20°C and 80°C in comparison with fibres from muscle aged 3 days. The structural sequence of events within aged fibres as they are stretched to breaking point is similar to the events within unaged fibres, but just occur at lower loads. This indicates that the exact sites of breakdown within the fibres do not significantly change, i.e. ageing does not induce fresh points of weakness, but just increases their fragility.

We have recently used this single fibre technique to directly show, for the first time, a causal link between the calpain activity and reduction in muscle fibre strength (Christensen et al, 2000a,b). Individual muscle fibres incubated in Ca^{2+}solution without μ-calpain or in EGTA solution (calcium-activated protease control) required 137 kPa ± 14 kPa and 140 kPa ± 10 kPa, respectively, to fracture. Addition of μ-calpain to the calcium solution decreased the forces at which the fibres fractured to 46 kPa ± 8 kPa after just 10 minutes incubation. It is perhaps comforting that these studies do directly confirm that μ-calpain does have a direct effect in reducing fibre strength, and also interesting to note that the effect of presence/absence of Ca^{2+} in the control incubation medium has a negligible effect on fibre strength by comparison, so arguing against the hypothesis (Takahashi, 1992; Takahashi, 1999; Tatsumi and Takahashi, 1992) that tenderisation is due to the direct action of the Ca^{2+} ion. However, the *initial stiffness* of the fibres is affected by the presence or absence of calcium, as shown in figure 1. Single fibres incubated in

EGTA were initially much stiffer than fibres incubated in Ca^{2+} solution alone or a solution containing both Ca^{2+} and μ-calpain. This indicates that the presence or absence of calcium may cause configurational changes in some of the load-bearing proteins, altering their resistance to extension. However, because the breaking stress and strain values of Ca^{2+}-incubated fibres (without calpain) are similar to the EGTA-incubated fibres, Ca^{2+} does not weaken these structures.

The precise site of action of μ-calpain at the sarcomeric level is unclear. We have previously speculated (Christensen et al., 2000) that nebulin plays a role in maintaining the tensile strength of the myofibrils. Nebulin is located in the I-band region running longitudinally to the muscle fibre direction in close proximity to the actin filaments. Structural studies using electron microscopy have reported that fragmentation of the myofibril often takes place adjacent to Z-lines (Gann and Merkel, 1978). This fragmentation could result from degradation of nebulin, or titin. Current work underway in our laboratory seeks to identify the interaction of calpains with nebulin. Degradation of the Z-line region of titin is also known to occur (Boyer-Berri & Greaser, 1997). However, the mechanical contribution of these proteins in post-mortem myofibrils still needs to be investigated. The specific role of desmin for the tensile strength and integrity of myofibrils has been studied using desmin knockout mice (Li et al., 1997). Although results from knockout models often are obscured by cross-compensation mechanisms, it was found that absence of desmin resulted in mice with weaker muscles that fatigued more easily. This protein therefore seems to play a major role in the mechanical properties of the muscle fibres and has been the point of focus for several aspects of our research programme on proteolysis. We have also examined its role in post-mortem variations in water-holding (Kristensen & Purslow, 2000b)

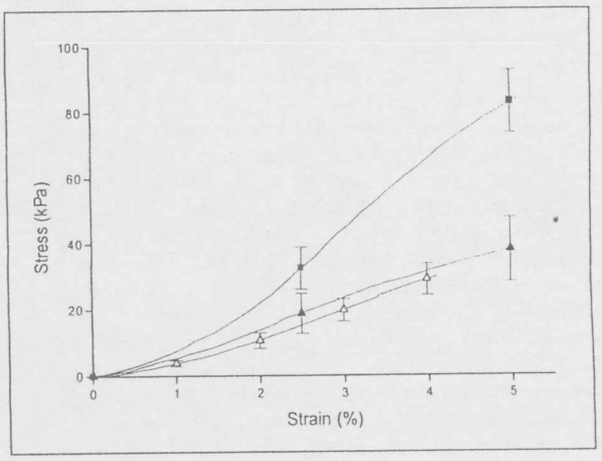

Figure 1. The effect of μ-calpain on the initial (low strain region) mean stress-strain curves of singl · fibres. Fibres were isolated 24 hours post-mortem and either incubated for 10 minutes in a buffer solution containing 0.2 mM EGTA (n=6) (■), 0.1 mM $CaCl_2$ (n=8) (▲) or 0.1 mM $CaCl_2$ with added μ-calpain (n=13) (△). Values are expressed as means ± standard error. From Christensen et al. (2001a).

Identifying the location of cleavage sites in the desmin molecule caused by specific enzymes

We have recently investigated the way in which the principal cytoskeletal protein in the intermediate filaments of skeletal muscle (desmin) is cleaved by the two major enzyme systems in muscle, calpains and cathepsins (Baron et al, 2000, 2001). Both μ- and m-calpain rapidly degrade the isolated protein, as shown in figure 2. The 55 kDa native molecule is cleaved into a similar series of well-defined fragments with molecular weights in the range 39 – 50 kDa by both m- and μ-calpain. N-terminal sequencing of the principal fragments in the 39 – 50 kDa range have been carried out. The results reveal that the primary mode of attack by both m- and μ- calpains is cleavage of the non-helical head and tail regions of the molecules, which are responsible for the interactions building the individual molecules into the polymeric structure of intermediate filaments. Sequencing also reveals that the major degradation products from both m- and μ-calpain have exactly matching new N-terminal amino acid sequences, showing that the sites of cleavage in the protein due to the two enzymes are identical. Calpains therefore seem to rapidly depolymerise the intermediate filaments, but have only limited ability to cleave within the alpha-helical rod domain of the molecule. In contrast, degradation products from cathepsin B (Baron et al., 2001) show an almost continuous molecular weight distribution on SDS-PAGE gels, reflecting the enzyme's ability to sequentially break the desmin molecule down into very small fragments and amino acids. It therefore seems that the breakdown of intermediate filaments post-mortem is a two-step cascade process; calpain activity depolymerises filamentous structure and cleaves desmin into major fragments, which are then further degraded by other enzyme systems, notably cathepsins. These results confirm and expand the ideas of Goll et al (1992) who presented detailed arguments to suggest that calpains are responsible for large-scale disassembly of myofilaments in the sarcomere as an integral part of muscle growth and turnover, but not the release of amino acids during protein turnover.

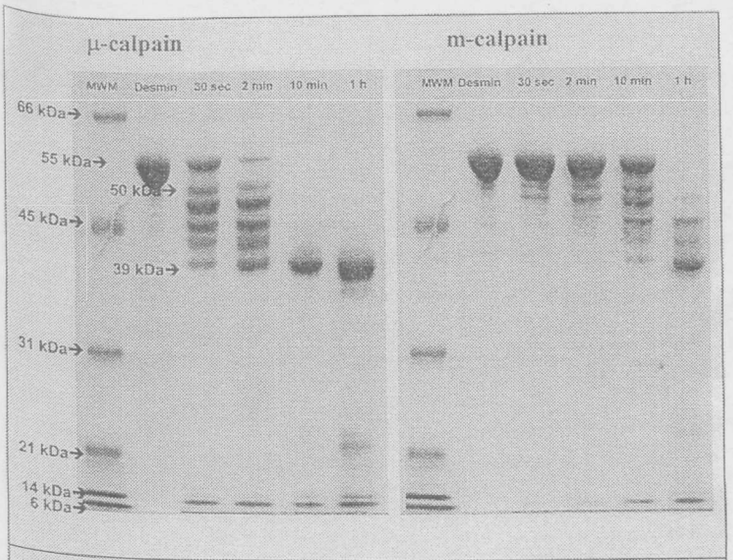

Fig 2: Kinetics of the degradation of desmin by μ-calpain and m-calpain followed using SDS-PAGE electrophoresis after 30 sec, 2 min, 10 min and 1 h at 25 °C. Arrows indicate the molecular weight of the different fragments. MWM: molecular weight marker.

Degradation of cytoskeletal proteins varies between muscles

We have extended our previous study (Morrison et al, 1998) of degradation of cytoskeletal proteins during post-mortem conditioning by quantitating the immunohistochemical labelling of transverse cryosections from five porcine muscles; *longissimus, iliocostalis, semitendinosus, semimembranosus* and *psoas major* using monoclonal antibody D33, which labels in a non-helical telopeptide region of the desmin molecule. It is precisely these end regions that are initially cleaved off by calpains, explaining why labelling intensity is gradually lost from the tissue sections

with time post-mortem; the cleaved telopeptide regions easily wash away. In contrast, the intensity of labelling on such sections with antibodies against the central rod region of the molecule (e.g. DER 11) does not diminish significantly over the same timescale (Morrison et al, 2000)

In agreement with previous findings, the intensity of immunolabelling for desmin decreased overall during 7 days conditioning, but in *longissimus* muscle the spatial distribution of this decrease was not uniform; labelling intensity was more quickly lost from type IIB fibres than types I+IIA muscle fibres. The ratio of the intensity of desmin labelling in I+IIA to IIB fibres is shown as a function of time post-mortem in fig 3b (Purslow et al., 2001) for the five muscles studied. An increase in this ratio with time reflects proportionately more loss of labelling in the IIB fibres with time. In contrast to the *longissimus*, however, the gradual decrease in labelling intensity for desmin was more uniform across all fibre types in the other four muscles. Only in *semimembranosus* was there a suggestion of fibre type-specific reduction in labelling, although this trend was minimal compared to that seen in *longissimus*. The results suggest that cytoskeletal protein degradation in *longissimus* is atypical of the process in porcine muscles generally. As well as a fibre type-specific variation in desmin degradation seen in *longissimus*, there also appears to be a muscle-specific effect which is not solely due to muscle fibre type distribution.

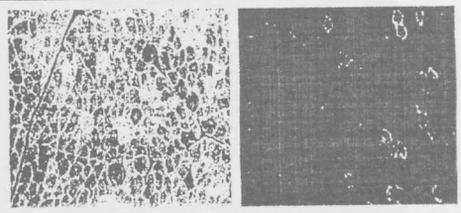

Fig 3a. Immunolabelling for desmin in porcine longissimus (D33 antibody) at 1day (left) and 7 days (right) post mortem. The brightly labelled fibres at 7 days correspond to fibre types I+IIa (Morrison et al, 1998)

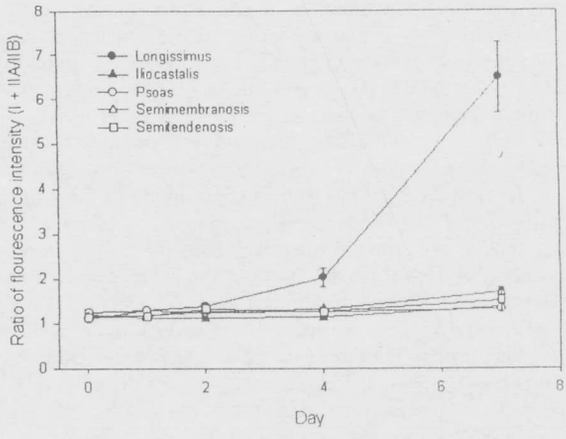

Fig.3b. The ratio of fluorescence intensity for desmin colocalising with the type I+IIA vs. type IIB fibres plotted at days 0, 1, 2, 4, and 7 postmortem for each of the five muscles studied. (Purslow et al, 2001)

Are variations in cytoskeletal degradation between muscles simply due to fibre type composition?

To investigate this muscle-specific effect further, we have studied (Christensen et al., 2000c, 2001b) the variations in proteolysis of desmin and troponin-T in porcine *longissimus* (LD) *semimembranosus* (SM), *semitendinosus* (ST), *vastus intermedius* (VI) and *soleus* (S) muscles. (This different range of muscles was chosen from this point on in our programme to give a good comparison of economically important white muscles [LD,SM,ST] with a mixed muscle [S] and a predominantly red muscle [VI] whose fibre type distributions were well-characterised.) Western blots of muscle homogenates reveal that degradation of desmin and troponin-T are faster in LD and SM than ST, despite the relatively similar fibre type distributions (all three are predominantly composed of type IIB fibres). In contrast, the pattern of degradation for troponin-T and desmin in ST was similar to that in S and VI, despite their markedly different fibre type compositions compared to ST. It seems that these inter-muscle differences in the rate of proteolytic degradation broadly rank with in fibre type distribution, but anomalies (such as the result for ST) indicate that degradation may also be influenced by other muscle-specific traits.

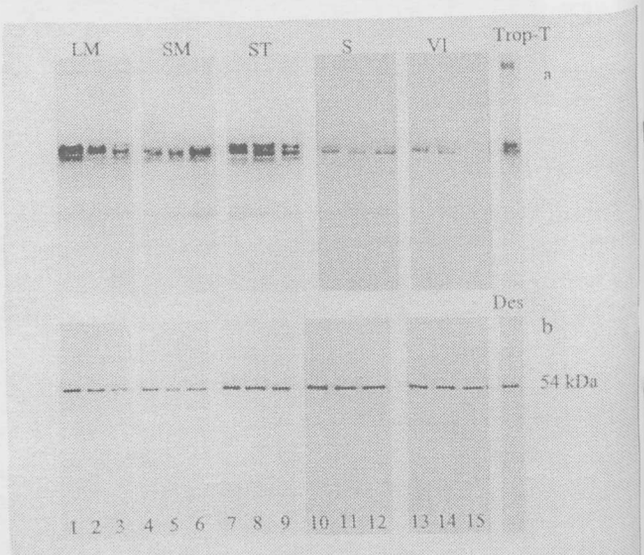

Figure 4. Western blots of type II fibres isolated from five porcine muscles, probed with (a) anti-troponin-T and (b) anti-desmin (DE-R-11) antibodies. Samples were stored for 1 day (lane 1,4,7,10,13), 3 days (lane 2,5,8,11,14) or 8 days (lane 3,6,9,12,15). Longissimus muscle (LM), Semitendinosus (ST), Semimembranosus (SM), Vastus intermedius (VI) and soleus (S). Markers of purified (a) troponin-T and (b) desmin appear in the single track on the right. From Christensen et al. (2001b)

Western blots on muscle homogenates suffer from the disadvantage that they can only show the average proteolysis occurring. The effect of fibre type is then inferred by correlating proteolysis with fibre type distribution. As described above, this correlation is not perfect, and in general correlations need not necessarily indicate direct cause-and-effect linkages between variables. One hypothesis may be that degradation rates in each type of fibre are fundamentally different (e.g. due to different amounts of enzymes, or substrates), so that the average degradation rate in the muscle is just the result of the proportion of each fibre type present. An alternative is that the local environment within the muscle as a whole affects the proteolytic behaviour of all fibre types present; this local environment may be more or less determined by the post-mortem metabolic conditions in the muscle (temperature, pH, glycolytic potential, etc) - which in turn may be heavily influenced by the proportion of different fibre types present. To decide between these alternative hypotheses, type II fibres from each of the five muscles were isolated by dissection and the rate of proteolytic degradation of desmin and troponin-T again determined from Western blots. The results (as shown in figure 4) indicate that the

rate of proteolytic degradation of desmin (calculated as the relative decrease in the intensity of labelling for the intact molecule) in type II fibres varied between muscles with broadly the same ranking as for the whole muscle homogenates (Christensen et al., 2000c, 2001). However, the degradation of troponin-T in type II fibres (again calculated as the relative decrease in the intensity of labelling for the intact molecule) was greater in VI than the other muscles. At present, we have no clear picture of why the changes in the intensity of the native troponin-T band for the isolated type II fibres do not match the findings in muscle homogenates. However, it is clear that individual fibre types do not have a fixed degradation pattern, but that the environment within the muscle as a whole modulates the proteolysis seen in all its constituent fibres.

The concept that the environment within the muscle as a whole is shared by all of the fibres present may be explained by the fact that intracellular proteolytic enzymes are free to migrate out of the cell early (e.g. within 6 hours) post-mortem, and so can affect the proteolysis in neighbouring cells – which may be of a different fibre type. (Purslow et al., 2000). However, it is likely that the overall level of proteolysis in the whole muscle is related to the metabolism and pH development post-mortem, which in turn will be related to the proportions of different fibre types present in the muscle.

Calpain proteolytic potential varies between muscles

In the preceding section we mentioned the idea that muscle-to-muscle variations in tenderisation may be due to differences in the amounts or activity of calpains and calpastatin. To investigate this we have measured m-calpain and μ-calpain proteolytic activity and calpastatin activity as a function of time post mortem in the same five muscles as used for the fibre type study; LD, SM, ST, VI and S (Ertbjerg & Purslow, 2000; Ertbjerg et al, 2001). The amounts of the catalytic subunit of the enzyme were also measured by Western blots. The amounts of catalytic subunit of the enzyme were also measured by Western blots. In all these muscles, μ-calpain activity decreased rapidly with post-mortem storage, while m-calpain activity was little affected by storage. The change in *activity* of μ-calpain mirrored changes in the *amounts* of the intact 80 kDa form of the enzyme present. A stable 76 kDa fragment of the catalytic subunit appeared, indicating that the rapid decrease of activity was due to limited degradation of the enzyme rather than extensive degradation resulting in inactivation. Calpastatin activi.y in all five muscles decreased more slowly during storage than μ-calpain activity, in agreement with reports on bovine muscles. However, calpastatin activity was less and the proteolytic potential (as estimated by the ratio of activity of μ-calpain:calpastatin early after slaughter) was greatest in the two fast-twitch white muscles (LD and SM) than in ST, S and VI, predicting that calpain-mediated proteolysis can occur faster in porcine LD and SM muscles.

This predicted pattern of calpain-mediated proteolysis is in good agreement with the rate of proteolytic degradation of desmin and troponin-T in both type II fibres from these five muscles and whole muscle homogenates (Christensen et al., 2000c, 2001) discussed above. Figure 5 shows a ranking comparison of (a) the μ-calpain:calpastatin ratio on the day of slaughter measured by Ertbjerg et al (2001) to the fraction of intact troponin-T and (b) calpastatin activity to the percentage of a 47-kDa desmin fragment generated after 8 days of post-mortem storage seen by Christensen et al (2001). The reciprocal variations between these parameters are extraordinarily clear for troponin-T (fig 5a) and (with the exception of the result for SM) almost as clear for desmin (fig. 5b). Variations in the calpastatin activity appear to dominate the μ-calpain:calpastatin ratio and focus our attention on the factors that control the expression and activity of this inhibitor.

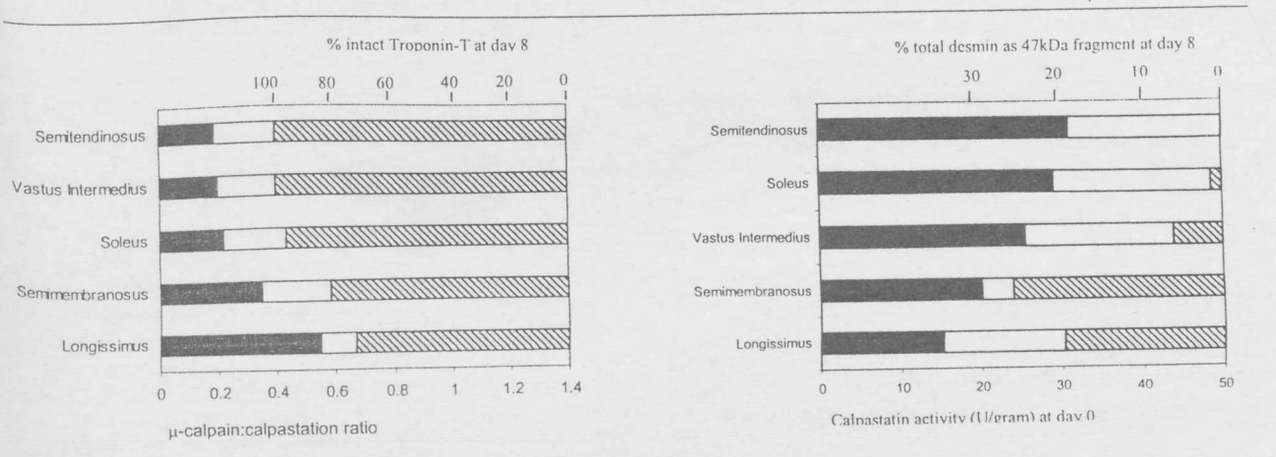

Fig.5. Comparison of (left) the μ-calpain:calpastatin ratio at day 0 (solid bars) vs. fraction of intact troponin T at day 8 (hatched bars) and (right) calpastatin activity at day 0 (solid bars) vs. the percentage of a 47-kDa desmin fragment generated at day 8 (hatched bars).

Conclusions

To understand the development of tenderness post mortem in its fullest sense, we should ideally have a complete picture of

(a) What proteins and structures are physically involved in toughness/tenderness determination, i.e. what mechanically-competent structures/proteins determine how easily meat is broken up in the mouth.

(b) Which structures/proteins are degraded by proteolysis so as to reduce the mechanical strength of the tissue (i.e. identify key substrates). How variable they are in terms of amount, structure or isoform, which may lead to variations in resistance to proteolysis.

(c) Which enzyme systems are responsible - for precisely the structural weakening in (b).

(d) Exactly how and where do these enzymes act; what controls their efficacy, and mechanisms of inhibition; how variable are their amounts and activity.

There are hundreds of proteins within muscle tissue. A " good candidate" approach can test whether there is evidence that a chosen sub-set of proteins and structures are substrates for the proteolytic processes thought to develop tenderness during post-mortem conditioning. However, it is likely that a very wide range of proteins and structures are undergoing proteolysis. Current investigations in our laboratories are using molecular biology techniques which aim to identify as complete a list of substrates for calpains as possible. Many of these will be irrelevant to tenderisation; the proteins involved are not structural, or are not in the areas of the sarcomere, myofibril or muscle fibre that are actually involved in the fracture processes dictating the texture of the whole tissue. Many may be associated "indicators" of relevant degradation; troponin-T is a potential example here. Variations in troponin-T degradation correlate well with variations in tenderness development, but it is perhaps difficult to imagine troponin-T as being a structural, load-bearing protein. However, its degradation may be a particularly clear indicator of the weakening of thin filaments, and it is easier to think of proteolytic weakening of these structures as a causal mechanism of increasing sarcomeric fragility at the Z-disc/I-band junction that occurs in conditioning. Equally, degradation of titin at specific sites close to its insertion onto the Z-disc (Boyer-Berri & Greaser, 1997) is a possible mechanism, but we should be careful not to focus on just one simple mechanism in what is obviously a complex and multifactorial situation. It is quite likely that the whole class of cytoskeletal proteins (including titin, nebulin, desmin, vinculin and talin) are preferred substrates of the calpain enzyme system. In this regard, desmin degradation may be an easily observed "indicator" of general cytoskeletal degradation.

It may be that degradation of nebulin or titin is a key event in weakening within the sarcomere. However, just as desmin is mechanically required in the living muscle cell to integrate the myofibrils and attach them to the sarcolemma via the costameres (so as to coordinate force production), so the mechanical linkage of adjacent myofibrils and myofibrillar attachment to the sarcolemma is an important aspect of the mechanical strength and integrity of whole composite structure of a muscle cell, as has been demonstrated in earlier studies of the strength of single post-mortem muscle fibres (Mutungi et al, 1996). Studies to extend these findings are required; we need to quantify what the relative contribution of endosarcomeric versus extrasarcomeric weakening is to the development of tenderness.

The research represented in this review concentrates on developing a detailed understanding in some precise areas in this overall scheme. Calpains do demonstrably weaken muscle fibres. They rapidly cleave desmin, primarily in the non-helical part of the molecule. This is enough to weaken the polymeric structure of the intermediate filaments. Desmin degradation varies between muscles. There may be more, or less, desmin in fibres of different fibre type, and varying amounts of enzymes per individual muscle cell, but there is a clear picture emerging that the metabolic profile within a whole muscle largely determines the degradation of all cells within it, regardless of type. We can clearly look to variations in the inhibitor of calpains, calpastatin, as an easy means to manipulate tenderness development. Early indications from strategic feeding experiments (Therkildsen et al, 2001a,b; Kristensen et al., 2001a) are that manipulation of the balance of calpain/calpastatin ratio just before slaughter due to nutrition of the animal is a practical means of manipulating the tenderness of meat. A greater understanding of the control of factors regulating calpain & calpastatin expression, activity and deactivation is clearly going to be useful in the task of predictively manipulating proteolysis to achieve controlled gains in meat tenderness.

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