MEAT TENDERNESS - THE μ-CALPAIN HYPOTHESIS

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ABSTRACT

Interest in calpains in the tenderisation of meat post-mortem has been increasing since their discovery and characterisation over 25 years ago when it was proposed that calpain could be responsible for at least some of the tenderness of meat. The involvement of proteases, such as calpains which degrade structural proteins, is an intuitively attractive concept which is receiving increasing experimental support from assays, inhibitor studies, modelling and structural changes in muscle related to tenderisation and tenderness.

The hypothesis is that μ-calpain becomes increasingly active with the release of calcium ions from the sarcoplasmic reticulum during rigor mortis development. Activated calpain is unstable and its activity decreases with storage time. The decreased activity reduces the rate of tenderisation over time, so producing an exponential decay in the rate of proteolysis and tenderisation with storage time. Eventually the activity and tenderising come to a virtual stop which in beef takes several weeks in chill storage. The ultimate level of tenderness is largely determined by the total amount of calpain activity which is controlled by the amount of it and its inhibitor (calpastatin) in the muscle and also by the pH and the temperature after slaughter. This hypothesis gives a good basis for understanding the variations in tenderness due to variations in animal production and in post-mortem conditions.

INTRODUCTION

Calpains (EC 3.4.22.17) are intracellular calcium-activated neutral proteinases and classified in the group of cysteine endopeptidases (EC 3.4.22) including cathepsins B, L, H, papain and bromelain. They are found in all vertebrate cells and have an optimum activity between pH 7.2 and 8.2 and in muscle are found in the cytosol (non-lysosomal). Two calpain isoforms have been studied mainly although an mRNA codes for a third calpain (skm-calpain) specific for skeletal muscle but no protein form of this molecule has been purified and its role cannot be defined. Chicken muscle is unusual in expressing 4 distinct calpains (Sorimachi *et al.*, 1995). The 2 isoforms most studied are named μ- and m-calpains (calpain-I and -II respectively) because of their requirement of μM and mM concentrations of calcium ions respectively for activation. The μ-calpains require between 3 and 50μM and m-calpain between 400 and 800μM for half maximal activity. Both μ- and m-calpains have been purified and sequenced from a variety of sources although none have been crystallised. They are heterodimers composed of a large subunit (80kDa) and a small subunit (28kDa). The 80kDa subunit of μ-calpain is about 50% homologous with that of m-calpain and the 28kDa subunits of μ- and m-calpains are identical. Bovine skeletal muscle contains about 25μg of μ-calpain/g of wet muscle. Calpastatin (Murachi, 1989) is a collective name for a family of stable proteins which specifically inhibit calpains. Calpastatin is capable of inhibiting 4 molecules of calpain, binding at both the active site and calcium binding site of calpain. In muscle there is sufficient quantity of calpastatin to inhibit all the μ- and m-calpain and in heart muscle sufficient calpastatin to inhibit 10 times the amount of calpain present.

Calcium ions bind at the C-terminal parts (domains IV and VI) of both sub-units and the molecule undergoes autolysis to produce an active enzyme which then undergoes further autolysis to produce finally inactive fragments. This is represented schematically in Figure 1. Activating by calcium ions (Figure 1, grey ovals) causes an autolysis of both subunits reducing the size of the larger to 76kDa and that of the smaller subunit to 18kDa. Storage causes further autolysis at sites within the larger sub-unit but the enzyme remains intact and without loss of enzyme activity. Further autolysis and eventual disintegration of the molecule causes complete

loss of activity. Both the active isoforms can be permanently inactivated by the active-site directed inhibitor, E-64 and the later active form can be inactivated permanently with EDTA or EGTA (Dransfield, 1998). In muscle, the level of available calcium ions may be sufficient to activate skm-calpain which requires physiological concentrations of calcium for activation. If it were activated, it would be particularly difficult to isolate. Its inactivation by EDTA during the extraction and purification procedures may be the reason why a protein corresponding to skm-calpain has not been isolated.

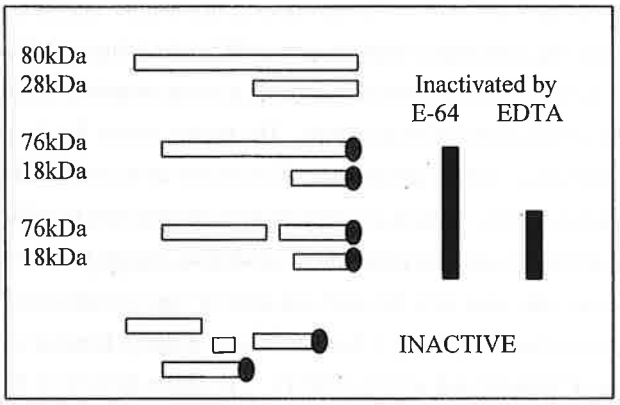


Figure 1 Schematic of calpain autolysis

ORIGIN OF THE INTEREST IN CALPAINS IN MEAT TENDERNESS

The interest in calpains in meat tenderness originates from investigations into the structural basis of tenderness changes during storage. During storage, mild homogenisation produces increased fragmentation of the muscle fibre close to the Z-disc (Takahashi *et al.*, 1967). This structural change was thought to be related to the weakening of raw meat post-mortem and could be prevented by the addition of EDTA (Davey and Gilbert, 1969) suggesting the involvement of divalent cations. This was later confirmed when it was shown that the tensile strength of chicken glycerinated muscle fibres decreased when soaked in 5 to 10 mM calcium chloride (Nakamura, 1972). At the same time, it was observed that incubating muscle strips in a calcium containing solution caused degradation of the Z-disc (Busch *et al.*, 1972). These later observations led to the isolation of m-calpain (then called CAF or calcium activated factor) and the demonstration that muscles with more CAF underwent more post mortem tenderisation (Goll *et al.*, 1974). At the same time, incubating raw muscle slices with a solution containing CAF caused tenderisation in meat after cooking (Penny *et al.*, 1974). Studies continued leading to the isolation of the more labile μ-calpain (Mellgren, 1980).

THE μ-CALPAIN HYPOTHESIS

The hypothesis is that, in meat, the *in situ* activity of μ-calpain causes proteolytic breakdown which in turn weakens the structure producing tender meat after cooking. Variations in ultimate tenderness are the result of differences in activity of μ-calpain.

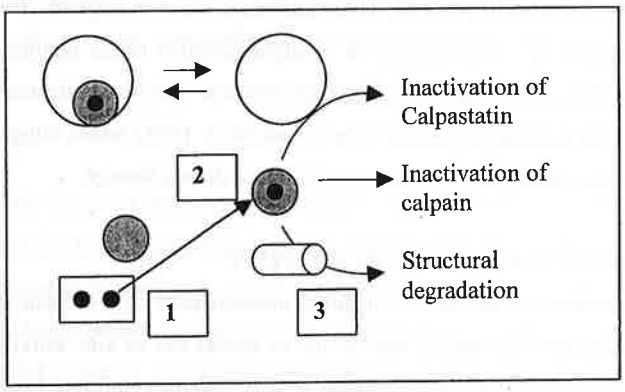
For this to happen three processes are essential. These are:

1. Increase in concentration of 'free' calcium ions
2. Calpain activation by calcium ions
3. Proteolysis by calpain activity capable of weakening the muscle structure sufficiently to be more tender in the cooked meat.

Without these processes calpain cannot be active in post-mortem muscle and could not be responsible for tenderisation nor the tenderness of meat.

The main processes and the involvement of calpastatin are shown schematically in Figure 2.

Figure 2. Schematic of the proposed stages involved in the tenderisation by the calpain system (Dransfield, 1993)



FREE CALCIUM CONCENTRATION

The total concentration of calcium ions is about 1.5mmol/kg of wet muscle (Lawrie, 1991; Dransfield, unpublished). However concentrations as low as 0.6mmol/kg (Doornenbal and Murray, 1981) and as high as 8mmol/kg (Dumont and Huzdik, 1990) have been reported for beef. Concentrations of calcium ions are similar in pork (1.5mmol/kg), beef (1.6 mmol/kg) and in avian (0.9mmol/kg) muscles (Zarkadas *et al.*, 1987). In living muscle, the 'free' calcium is about 0.2μM (Kurebayashi *et al.*, 1993) and is much lower than that required to activate μ- or m-calpains. The majority of the calcium is maintained in the sarcoplasmic reticulum by the action of the calcium pumps. The energy source for the pumps is ATP but during development of rigor mortis, the level of ATP decreases and the pumps fail. This causes an increase in 'free' calcium concentration which rises to about 100μM at full rigor (Jeacocke, 1993). A level of 100μM free calcium ions is sufficient to activate μ-calpain, but not m-calpain, *in vitro*. A similar value (150μM) was also predicted by modelling calpain loss during storage (Dransfield, 1993). The concentration of free calcium ions may vary also with the type and state of rigor mortis development. In lamb longissimus muscle held at 15°C, free calcium levels rose from 0.1μM at 5 hours to 25μM at rigor (Jaime *et al.*, 1992) and slightly higher concentrations were found in muscles held at 4° (30μM) and at 0°C (37μM). The major increase in free calcium ions occurs during the development of rigor mortis, but it is not known if this rise continues with further storage. An early study had indicated that, in a 183,000g supernatant, the level of calcium may rise to nearly 1mmol/kg after 10 days storage of meat and in a TCA supernatant from beef, Ca ions were between 0.64 and 0.97 mmol/kg at 2 weeks storage (Parrish *et al.*, 1981). However, recent studies showed that, in goat meat, free Mg (4mmol/kg) and K (70mmol/kg) ions showed little change from 1 to 2 days (Feidt and Brun-Bellut, 1999) so it seems unlikely that free Ca ion concentration would rise substantially after full rigor mortis. The overall osmotic pressure (due mainly to inorganic ions) increases little after full rigor mortis (Zamora *et al.*, 1996). Further work is warranted to reconcile these diverse results and to establish the levels of free calcium during development of different types of rigor mortis and during prolonged chilled storage.

The activation of calpains by liberated calcium ions led to the conclusion that tenderisation would begin prior to full rigor which is when the pH falls to about 6.2 (Dransfield *et al.*, 1992) in normal rigor mortis development. The activity would then accelerate due to rapid release of more calcium ions as rigor mortis development accelerates within the cell reaching more than 90% of its final value at full rigor.

CALPAIN ACTIVATION BY CALCIUM

There is no direct evidence that calpains are active in post-mortem muscle, indeed there is no evidence that they are active in living muscle. However, we believe that μ-calpain can be active by looking at the circumstantial evidence. If calpains were active the autolytic products (Figure 1) should be present in the muscle. One such fragment, the 76kDa fragment has been detected by antibodies in post mortem muscle (Geesink and Goll, 1995). However, this fragment was not active *in vitro* and its origin is uncertain. Another line of evidence is that the detected calpain level decreases with increasing time of storage, which should be the case if the enzyme had been activated (Figure 1). This has been shown in rabbit, lamb and beef (see Dransfield, 1993; Figure 3). The activity of μ-calpain relative to the number of active sites (determined by back-titration with E-64) decreased with increase in storage time (Dransfield, 1998) which suggests some autolysis of μ-calpain takes place during storage.

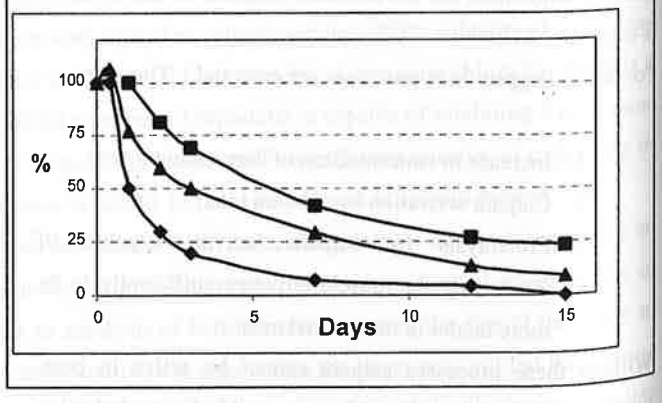


Figure 3 Changes in toughness (squares), μ-calpain (diamonds) and calpastatin (triangles) during chill storage of beef longissimus
Data adapted from Zamora *et al.*, 1996

MODELLING CALPAIN ACTIVITY

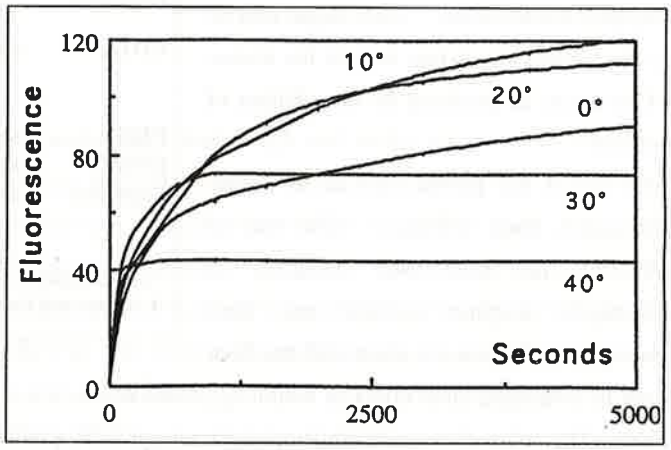
Because of the lack of a direct measurement of μ-calpain *in situ,* one alternative was to try to model the *in situ* activity from knowledge of the activity *in vitro* and the conditions prevailing in muscle during its conversion to meat.

The factors such as calcium ion concentration, pH, instability and temperature have been reported in the literature and sufficient was known of the changes in pH and temperature to combine the two sets of data to model the activity. Conditions could then be set to predict the activity under a variety of conditions observed in different meats. Unfortunately, this modelling could only be done for a 'typical muscle' as little was known of the variability in levels of calpains and calpastatin between muscles. One of the main assumptions in the model (Dransfield, 1993) is that calpastatin also serves as a substrate for calpain which has yet to be shown *in situ* but which was needed to account for the observed decrease in calpastatin in post-mortem muscle (Figure 3).

CALPAIN AND CALPASTATIN INSTABILITY

One of the main characteristics is that, once activated, calpains are unstable and this has led to the idea that tenderisation is enzyme limiting. This would account for the exponential nature of the decay in toughness with time which is a reflection of the exponential decay of calpain activity with time (Figure 4). When activated therefore we expect a decrease in amount of calpain activity in the muscle extract. Typical results for the decrease in μ-calpain (Figure 3) shows that the μ-calpain in chilled beef decreases to about 50% after 24 hours. The calpastatin usually decreases more slowly, reaching 70% after 24 hours. Similar results have been observed for other meats in chill (Vidalenc *et al.*, 1983; Ducastaing *et al.*, 1985; Koohmaraie *et al.*, 1986; Boehm *et al.*, 1998). It is clear that for calpain to operate in the tenderisation, the rate of decrease in calpain and calpastatin activities should be directly related to the decrease in toughness (Figure 2). However whilst the decrease in calpastatin is usually found to be closely related to that of the toughness, the rate of decrease of μ-calpain is usually faster than that in toughness (Figure 3). The observed decrease in μ-calpain activity from muscle is also faster than that calculated for the conditions which exist in post-mortem muscle (Dransfield, 1993). The discrepancy could be due the underestimation of μ-calpain in muscle by using EDTA in the extraction procedures, since the use of EDTA with extracts containing activate calpain will destroy some of the calpain activity (Figure 1; Dransfield, 1998). In extreme cases, where the level of μ-calpain at 24 hours has been reported (Boehm *et al.*, 1998) to be as low as 20% of its value at death, this could be due to the use of E-64 during the extraction procedure which would permanently inactivate all the activated calpain. If calpastatin were a substrate for calpain, it would be expected to relate directly to the activity of μ-calpain and therefore to tenderisation. This probably explains why measures of calpastatin are usually better correlated to toughness than are those of μ-calpain.

Figure 4 Inactivation of μ-calpain at different temperatures
The graph shows proteolysis (fluorescence) by μ-calpain in relation to the temperature and over time. Curves from Dransfield *et al.*, 1998.



In vitro, temperature has a large effect on the rate of activity, as with all enzymes, but calpains are unusual in that temperature also determines the total amount of proteolysis (Figure 4). More proteolysis occurs at intermediate temperatures than at low (0°C) or high (40°C) temperatures.

EVIDENCE FROM USE OF ACTIVATORS AND INHIBITORS

Addition of calcium ions should activate and thereby cause a decrease the levels of both μ- and m-calpains and should also produce increased tenderising. The effect on ultimate tenderness is likely to be by bringing into play m-calpain which is thought not normally to be activated in meat. The tenderising effect of calcium ion addition has been shown many times in a variety of meats and as often been cited as evidence for the involvement of calpains in tenderisation. However, the concentration of calcium ions required to produce a maximum effect on tenderisation is about 30mM (Alarcon and Dransfield, 1995), an order of magnitude higher than that required to fully activate calpains. Other inorganic ions also produce tenderisation, for example Mg ions, although the effect with Mg ions is less than that produced by calcium ions (Alarcon and Dransfield, 1995). This tenderising cannot be

explained by a direct effect on calpains because calpains are not activated by Mg ions. This could be explained by an indirect effect of the added Mg ions liberating Ca ions bound to proteins (the majority of which are bound to proteins other than calpains). Unfortunately in none of the studies on the effect of added cations has the free calcium ion concentration been measured. This needs to be further investigated before the mechanism of the tenderising effect of calcium addition can be assessed.

The effect of addition of calcium ions should be to increase the amount of tenderisation but not to change its exponential rate constant, since this is determined by the stability of the enzyme and not by the calcium ion concentration. Unfortunately this has rarely been studied. Some observations have shown that the final level of toughness after ageing does not change. This is inconsistent with a calpain-alone mediated tenderisation. There might also be a direct effect of calcium ions on the texture of the meat, as produced by the addition of sodium salts and it has proved difficult to clearly distinguish these effects. The use of inhibitors has been used classically to investigate enzyme activity and their mechanism of action and some work has been done to investigate their effect in inhibiting tenderisation.

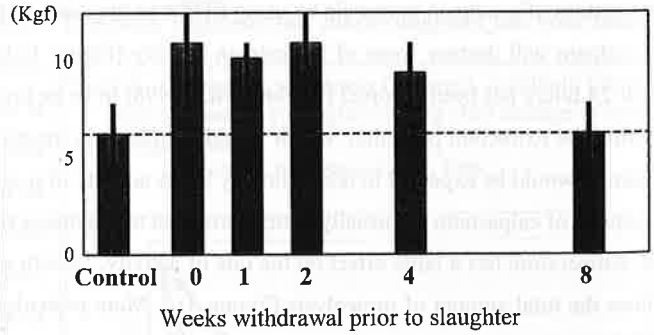
Compound	Effect	μM	Remarks*
Leupeptin	cysteine	60	Complete inhibition of ageing (2)
E-64	cysteine	1	No toughening in beef (4)
		1	No effect in chicken (1)
		140	Inhibition of ageing beef (2)
Iodoacetate	cysteine	100	Complete inhibition of ageing (4)
'Peptide'	cysteine	100	Inhibition (4)
Zinc ions	calpains+?		Prevention of ageing at all pH's (5)
			Stops ageing in sheep meat (6)
EDTA	calpains	100	No inhibition (4)
		2000	Reduced ageing (3)
		2000	Not completely blocked (2)
PMSF	serine	100	Some inhibition of ageing
DNME	aspartic	100	Some toughening at 15°C (4)
Pepstatin	aspartic	1	Some toughening at 15°C (4)
		60	No inhibition of ageing (2)

*1, Etherington *et al.*, 1990; 2, Uytterhaegen *et al.*, 1994; 3, Aalhus *et al.*, 1993; 4, Alarcon and Dransfield, 1995; 5, Watanabe *et al.*, 1996; 6 Koohmaraie, 1990.

The results of several trials are given in summary in the Table. The range of cysteine inhibitors used, except E-64, produces some form of inhibition of tenderisation, the most effective being leupeptin and iodoacetate. Surprisingly, there is little effect of EDTA in preventing ageing.

The serine (PMSF, phenylmethanesulfonyl fluoride) and aspartic (DNME, diazoethyl norleucine methyl ester) active site inhibitors have generally less affect than cysteine inhibitors. In general therefore the use of inhibitors has not provided convincing evidence for the involvement of any particular group of enzymes. This is perhaps not surprising, bearing in mind the difficulties of getting an inhibitor to the site of the intracellular calpains and the complexity of the muscle system. The evidence does suggest a more important role of the cysteine proteinases.

Figure 5 Toughness of Longissimus from ß-agonist treated lamb
Values are the means and SD from 4 animals



ANIMAL PRODUCTION

The introduction of ß-adrenergic agonists showed an improvement in efficiency of meat production but it soon became evident that they resulted in tougher meat when administered to cattle, pigs, sheep and poultry. Following ß-agonist treatment, the levels of calpains and calpastatin change. The μ-calpain is usually depressed whilst calpastatin is increased which is consistent with a reduced proteolysis and net increase in protein accretion. The activity of μ-calpain would therefore be expected to decrease and the rate of tenderisation would consequently be reduced. The observed effects of ß- agonists on rate of tenderisation would therefore be

consistent with a calpain-mediated reduced proteolysis. The effect of ß-agonists on carcass characteristics and on tenderness are reversible. For example, after stopping feeding of 4ppm ß-agonist, the induced toughness gradually returns to normal (Figure 5). Little change occurs up to 4 weeks after removal but after 8 weeks the toughness has fully returned to that of the control. (Figure 5, Dransfield and Ross, 1988).

This shows a relatively rapid turnover of the drug and complete removal of the effect as far as tenderness is concerned. The rapid return to normal is probably faster than could be accounted for by turnover of connective tissue proteins.

More recent and equally interesting observations have been shown for callipyge sheep. Callipyge phenotype in sheep has a muscle mass about one third greater than in half-siblings not expressing the trait. Calpastatin levels in the affected muscles are twice as high (Koohmaraie *et al.*, 1995) suggesting that the increased growth resulted from a decreased protein degradation. Shear force values of longissimus muscle can be 3 times higher in callipyge sheep and a delayed and the frequency of breaks at the I-band increased in normal but showed no change in callipyge sheep (Taylor and Koohmaraie,1998).

These results show the link between animal growth and tenderness of meat which could be linked through calpain-mediated myofibrillar protein turnover.

IMPORTANCE OF RIGOR MORTIS TO TENDERNESS

Many studies has shown the importance of rigor development to tenderness. Both tough and tender meats can be produced depending on the conditions. The calpain system is also strongly affected by rigor mortis development and rigor mortis development critical in determining the proteolysis by calpain. Rapid rigor development would be expected to produce an earlier activation of calpains. Tenderisation would then start earlier. The proteolysis would then depend on the temperature at that time. If the temperature is reduced in proportion to the increase in pH decline, the total proteolysis would be unaffected. In this case the tenderisation would start earlier but reach the same level. This is the case with electrical stimulation when used with faster cooling than with non-electrically stimulated controls. However if rigor is allowed to develop at higher temperatures would also cause an early decrease in pH and early activation of μ-calpain. But subsequently, the higher temperature would give more rapid inactivation (Figure 4) and lead to tougher meat. This may be the origin of the toughness and lack of ageing found in extreme cases of PSE meats and when electrical stimulation is applied and followed by slow cooling.

With the start of any activation of μ-calpain at about pH 6.3 under normal conditions of rigor development, tenderisation would start prior to full rigor development, at time when toughness measurements in both raw and cooked meat cannot be made in the same context and are not comparable with those made post-rigor, that is, they are not made at pH 5.5 without muscle shortening. Estimates have been made as to the value of the toughness at death. By calculation, this was estimated between 13 and 25 kg shear force for beef (Dransfield *et al.*, 1992; Dransfield, 1993) and measured, following the injection of Zn ions to prevent proteolysis, to be about 17kg for lamb meat (Watanabe *et al.*, 1996). This is considerably higher than the value taken at 24 hours (Figure 2) suggesting that a large part of the tenderisation occurs prior to full rigor development (24 hours in beef). This has led to the idea of tenderisation being the increase in tenderness throughout and ageing that part of the tenderisation which is measured after full rigor mortis.

Under cold-shortening conditions, calpastatin appears to decrease more slowly than under non-cold-shortening conditions ((Zamora *et al.*, 1998). If calpastatin were a substrate for calpain which is responsible for the decrease in calpastatin, the slower decline of calpastatin would indicate less calpain activity. This could account for the toughness of cold-shortened meats. The level of μ-calpain is also particularly sensitive to thaw rigor. The μ-calpain in pre-rigor frozen muscle is rapidly lost during thawing (Dransfield, 1996) and is accompanied by a severe muscle contraction and often gives rise to tender meat.

The tenderness of DFD meat has been explained by increased μ-calpain activity at high (neutral) pH. What has not been clearly explained on the basis of calpain activity is the peak of toughness often observed at about pH 6.

STRUCTURAL DEGRADADTION AND PROTEOLYSIS

The early observation that muscles become more tender during storage led to the initial conclusion that tenderisation was the result of proteolysis (Hoagland *et al.*, 1917; Bate-Smith, 1948). Pre-rigor meat contains about 4 to 5 mmol free amino nitrogen, half of which is accounted for by free amino acids and peptides. After slaughter this increases. However there is relatively small release of small peptides and amino acids during the first week of storage (Locker, 1960; Parrish *et al.*, 1969; Feidt *et al.*, 1998) when most of the tenderisation occurs. The increase in amino acids probably arises from proteolysis of the sarcoplasmic proteins (Fujimaki and Deatherage, 1964) or by mast cells within the muscle (Woodbury *et al.*, 1978). Little of this increase in amino acids and peptides is thought to come from proteolysis of the myofibrillar proteins. The increase in peptides may be caused by the action of proteases, whilst the increase in free amino acids by the action of those amino-peptidases having an optimal at neutral pH (Nishimura *et al.*, 1996). Calcium independent autolysis increases with lowering of pH and is maximal at pH between 5 and 3 and activity was least in the neutral range of pH and the liberation of peptides is only slightly stimulated by addition off calcium ions (Okitani *et al.*, 1977). It appears then that there is little release of amino acids and small peptides responsible for tenderisation and that the major tenderisation occurs with a limited beaks in proteins. This pattern of limited proteolysis is consistent with proteolysis by calpains. During storage, there is little degradation of myosin and actin which is consistent with activity of calpains which are unusual in that they do not degrade myosin or actin. More recent work has focussed on the role of the cytoskeleton in meat tenderness (see Dransfield, 1997). The proteolytic cleavages in titin (at the N-terminal end of the large polypeptide chain), in nebulin (200nm from the Z-disc entry of the nebulin and titin), in desmin and in vinculin observed in post-mortem muscle are mimicked closely by calpains (Taylor *et al.*, 1995; Huff-Lonergan *et al.*, 1996). There is also less degradation of titin, desmin, nebulin and vinculin in tough muscles than in tender muscles (Parrish *et al.*, 1981; Anderson and Parrish, 1989; Huff-Lonergan *et al.*, 1995). Histological evidence (Davey and Gilbert, 1969) shows that the region adjacent to the Z-line disintegrates progressively with chilled storage. This presents one of the puzzles in the μ-calpain theory of tenderisation which is why, when added to muscle, does μ-calpain rapidly remove the Z-disc itself when after prolonged storage and tenderisation of meat, the Z-disc remain intact?

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