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Involvement of calpains in postmortem tenderisation A review of recent research

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INTRODUCTION

Tenderness is an important determinant of meat quality. Inconsistency and variability in tenderness have been identified as major problems for the meat industry (Morgan *et al.*, 1991). It is well documented that tenderness improves during postmortem storage of meat but the mechanisms involved remain controversial. The general consensus is that proteolysis of structural muscle proteins causes tenderisation but alternative theories suggest that changes in the actin-myosin interaction (Goll *et al.*, 1997) or a non-enzymatic effect of calcium on muscle proteins (Takahashi, 1999) or a rise in ionic strength (Ouali, 1990) may be involved. However, discussion of these theories is not within the scope of this review. With regard to postmortem proteolysis, the calpain system is thought to play a major role. Calpains are calcium-activated proteases with an optimum activity at neutral pH. In skeletal muscle, the calpain system consists three proteases, μ -calpain, m-calpain and skeletal muscle-specific calpain, p94, and an inhibitor of μ - and m-calpain, calpastatin. The involvement of calpains in postmortem tenderisation has been the subject of several reviews (Goll *et al.*, 1991; Koohmaraie, 1988, 1992a, 1994, 1996; Ouali, 1990). Since these reviews, new information has become available which has provided answers to some of the questions on the role of calpains in postmortem tenderisation. The purpose of this review is to discuss some of these issues in the context of recent results. For further information on postmortem proteolysis and meat tenderness, or characteristics of the calpain system, the reader is referred to a number of excellent reviews (Penny, 1980; Greaser, 1986; Croall and DeMartino, 1991; Ouali, 1992; Sorimachi *et al.*, 1994; Ono *et al.*, 1998).

DISCUSSION

Is μ -calpain active under postmortem muscle conditions?

The question whether μ -calpain is active at the pH and temperature of postmortem muscle appeared to be answered by a study by Koohmaraie *et al.* (1986). They observed that μ -calpain retained 24 to 28% of its activity at pH 5.5 to 5.8 at a temperature of 5°C. This level of activity was sufficient to reproduce changes in the myofibrils associated with postmortem storage of muscle. In contrast, Takahashi (1999) failed to reproduce these results and could not detect any μ -calpain activity on myofibrils below 15°C at pH 5.7 to 5.9. Results of other studies on this subject, however, support the conclusion of Koohmaraie *et al.* (1986). Huff-Lonergann *et al.* (1996), incubated myofibrils in the presence of μ -calpain at pH 5.6 and 4°C and concluded that proteolysis of muscle proteins under these conditions does occur and is similar to that occurring in postmortem muscle. More recently, Geesink and Koohmaraie (1999a)

reported that even in the presence of excess calpastatin, μ -calpain degrades myofibrillar proteins under postmortem muscle conditions (pH 5.8 and 5°C). Taken together, the available evidence suggest that μ -calpain is active under postmortem muscle conditions *in vitro*. Apart from the observation that μ -calpain can reproduce the protein degradation pattern observed in postmortem muscle, there is also more direct evidence that μ -calpain is active in postmortem muscle. One of the characteristics of the calpains is that they autolyse when activated. Western blots against μ -calpain have shown that autolysis of μ -calpain occurs during postmortem storage of muscle and that the autolysis progresses up to 21 days postmortem in ovine muscle (Geesink and Koohmaraie, 1999b).

How can μ -calpain be active in the presence of excess calpastatin?

In the presence of calcium concentrations sufficient to activate calpains, calpastatin inhibits calpain activity. The activity of calpastatin in muscle exceeds the activity of μ -calpain, which raises the question how μ -calpain can be active in postmortem muscle (Goll *et al.*, 1983, 1995). Based upon results of Cottin *et al.* (1981) on m-calpain, Dransfield (1992) suggested that calpastatin is unable to inhibit the calpains at the pH of postmortem muscle. However, other studies using m-calpain (Otsuka and Goll, 1987; Kendall *et al.*, 1993) or μ -calpain (Geesink and Koohmaraie, 1999a) found there was little or no effect of pH on inhibition of calpain by calpastatin. Other possibilities that have been suggested for living tissue include (i) translocation of calpastatin and calpain away from each other or (ii) modification of calpastatin's ability to bind to calpain either through phosphorylation or dephosphorylation of calpastatin, or through interaction with a calpain "activator" (Goll *et al.*, 1995).

The answer to the above question for postmortem muscle is probably more mundane: calpastatin does not completely inhibit calpain activity. Calpastatin is degraded by calpain (Mellgren *et al.*, 1986; Nakamura *et al.*, 1987) even in the presence of excess calpastatin (Doumit and Koohmaraie, 1999). This indicates that calpastatin does not completely inhibit calpain activity. In accordance with this, Koohmaraie (1992b) observed slow autolysis of μ -calpain in the presence of calpastatin. However, this does not prove that calpain is able to degrade other substrates in the presence of excess calpastatin under postmortem muscle conditions. To resolve this issue, Geesink and Koohmaraie (1999a) incubated myofibrils in the presence of μ -calpain and excess calpastatin at the pH, temperature, ionic strength, and ageing period of postmortem muscle. The results showed slow, but clear degradation of myofibrillar proteins similar to that observed during postmortem storage of

muscle. The inability to detect calpain activity in the presence of excess calpastatin using standard assays may be due to the low sensitivity of the method. Standard assays for calpain activity typically use casein as a substrate and, after an incubation period of 30 to 60 minutes, the increase in TCA-soluble casein fragments is a measure of calpain activity. The relatively short incubation period limits the detection of low levels of proteolysis. Furthermore, large casein fragments, although evidently the result of proteolysis, are not TCA-soluble (Doumit and Koohmaraie, 1999) and are not detectable in standard calpain assays.

What causes the decrease in μ -calpain activity postmortem?

A characteristic of both μ - and m-calpain is that binding of Ca^{2+} induces autolysis in a series of defined steps. The initial autolysis steps decrease the Ca^{2+} -requirements of the calpains but do not affect their activity. Further autolysis eventually results in loss of activity (Suzuki *et al.*, 1981 a, b; Mellgren *et al.*, 1982). During postmortem storage of muscle, μ -calpain activity decreases, but m-calpain activity remains nearly constant (Vidalenc *et al.*, 1983; Ducastaing *et al.*, 1985; Koohmaraie *et al.*, 1987). Based on these observations Koohmaraie *et al.* (1987) suggested that only μ -calpain is activated in postmortem muscle and, as a result of autolysis, eventually loses its activity. Results from recent studies have led to a modification of this hypothesis. In vitro, the initial autolysis steps reduce the 80 kDa subunit of μ -calpain to 76 kDa through a 78 kDa intermediate, without loss of activity. However, in postmortem muscle a relatively large amount of μ -calpain is present in its 78 and 76 kDa form, when little of its activity remains (Geesink and Koohmaraie, 1999b). The loss of μ -calpain activity in postmortem muscle is, therefore, not the result of extensive autolysis. In a follow up of this study it was found that briefly autolysed μ -calpain is unstable at the ionic strength and pH of postmortem muscle (Geesink and Koohmaraie, 2000). Earlier work by Thompson *et al.* (1990) has also shown that even under optimal conditions (neutral pH and low ionic strength) autolysed calpain rapidly loses its activity at 37°C due to the thermodynamic instability of the enzyme. Further work by Thompson *et al.* (unpublished) has shown that partially autolysed μ - and m-calpain aggregate to inactive trimers or tetramers at ionic strengths above 100 mM NaCl. The initial hypothesis of Koohmaraie *et al.* (1987) is thus modified to the extent that the loss of μ -calpain activity in postmortem muscle is initiated by autolysis and that it is instability of partially autolysed μ -calpain and not extensive autolysis which is the major cause of the loss of μ -calpain activity postmortem (Geesink and Koohmaraie, 1990). Thus, the rate and extent of postmortem proteolysis and the ensuing tenderisation, appear to be limited by (i) the instability of autolysed μ -calpain and (ii) inhibition of μ -calpain by calpastatin.

Results from a number of studies have indicated that tenderisation is negatively affected when muscles go into rigor at temperatures above 25°C (Marsh *et al.*, 1987; Hertzman *et al.*, 1993; Devine *et al.*, 1996; Simmons *et al.*, 1996). The observation that autolysed μ -calpain is unstable under postmortem muscle conditions and, furthermore, thermodynamically unstable, even under optimal

conditions, may explain these results. This suggestion is supported by the results of Geesink *et al.* (accepted) which showed that the toughening effect of rigor temperature above 25°C can be explained by a reduction in postmortem proteolysis.

Does m-calpain play a role in postmortem tenderisation?

During postmortem storage of muscle, m-calpain activity remains nearly constant (Vidalenc *et al.*, 1983; Ducastaing *et al.*, 1985; Koohmaraie *et al.*, 1987; Morton *et al.*, 1999), even after 56 days of postmortem storage (Geesink and Koohmaraie, 1999). However, when calcium levels are elevated by infusing muscles with a calcium chloride solution, postmortem proteolysis and tenderisation are accelerated and m-calpain activities decline (Koohmaraie, 1994). This suggests that m-calpain is activated when calcium levels are artificially elevated and loss of activity is a result of autolysis. On the other hand, this also implies that the stability of m-calpain in postmortem muscle is due to insufficient Ca^{2+} to activate the enzyme (Koohmaraie *et al.*, 1987). In contrast, a number of recent studies reported a significant decline in m-calpain activity postmortem in pork (Sensky *et al.*, 1996, 1999) and beef (Beltran *et al.*, 1997). A common feature of these studies is that the muscles were extracted with buffers of low buffering capacity. In a recent study, Veiseth and Koohmaraie (submitted) investigated the effect of buffering capacity on the extraction of calpain and calpastatin activity from 1-day postmortem ovine muscle. They observed that, using the extraction method of Sensky and co-workers, the pH of the extract was 5.84 and the recovery of m-calpain activity was 37% lower than using their standard extraction method (Koohmaraie 1990).

In the initial purification procedure for m-calpain from rabbit skeletal muscle, Bush *et al.* (1972) used iso-electric precipitation between pH 4.9 and 6.2, which implies that it is important to keep the pH of muscle extracts above 6.2 to avoid precipitation of m-calpain. Thus, it seems that the reported decline in m-calpain activity during postmortem storage is an artefact of the methodology. This suggestion is supported by the fact that the reported drop in m-calpain activity was less severe in high pH muscle (Beltran *et al.*, 1997; Sensky *et al.*, 1996), extraction of which would result in a relatively high pH of the extract. Nevertheless, the theoretical model for postmortem tenderisation proposed by Dransfield (1993) includes activation of m-calpain. The activity of m-calpain in the model is predicted to decline 24% during 2 weeks of postmortem storage. Continuation of this trend to 8 weeks of storage would lead to almost complete loss of m-calpain activity. Clearly, the observation that m-calpain activity remains stable during 8 weeks of storage (Geesink and Koohmaraie, 1999b) contradicts the suggestion that m-calpain is involved in postmortem tenderisation.

Is skeletal muscle-specific calpain, p94, involved in postmortem tenderisation?

Since its discovery by Sorimachi *et al.* (1989), skeletal muscle specific calpain, p94, also known as skm-calpain, calpain 3, or nCL-1 has attracted considerable attention because inactivating mutations in the p94 gene cause limb-

girdle muscular dystrophy type 2A (Richard *et al.*, 1995). Purification and characterisation of p94 has been extremely difficult for several reasons. Unlike the ubiquitous calpains, p94, can not be easily extracted from skeletal muscle due to its association with the giant elastic protein titin (Sorimachi *et al.*, 1995). Expression of p94 *in vitro* is hampered by rapid autolysis of the enzyme at physiological levels of calcium and, furthermore, the autolysis is not affected by calpain inhibitors (Sorimachi *et al.*, 1993). The interest in p94 with regard to postmortem tenderisation stems from the observation that p94 binds to titin at the N2 line, a site where proteolysis in the early postmortem period has been linked to tenderisation (Taylor *et al.*, 1995). Reports on the involvement of p94 in tenderisation are few and contradictory. Parr *et al.* (1999) monitored the quantity of p94 by immunoblotting during postmortem storage of porcine longissimus muscle and reported a mean half-life of p94 of about 13 hours. This result suggests that p94 autolyses and is active in postmortem muscle. However, the variability in p94 abundance and its half-life was not related to differences in postmortem proteolysis and tenderness of tough and tender muscles. Ilian and Bickerstaffe (1999), on the other hand, observed a significant correlation between the rate of tenderisation of different ovine muscles and steady state mRNA levels for p94. In a recent study, Ilian *et al.* (submitted) determined p94 levels in non-tenderising longissimus muscle from callipyge lamb and tenderising muscle from normal lamb. They detected significantly less p94 in the callipyge muscles at-death, but the rate of decline in p94 during postmortem storage was not different between the normal and callipyge muscles. Evaluation of the role of p94 will be facilitated by identification of substrates for p94, and comparison of their degradation pattern with the degradation pattern of these proteins in postmortem muscle. Recent progress in expression and isolation of active p94 indicates that this will be possible in the near future. Kinbara *et al.* (1998) succeeded in extracting active p94 from rabbit skeletal muscle but further purification led to inactivation of the enzyme. More recently, Branca *et al.* (1999) reported expression and partial purification of active p94.

CONCLUSIONS

The available evidence indicates that m-calpain is not involved in postmortem tenderisation, unless calcium levels in postmortem muscle are artificially increased. The role of p94 in tenderisation remains to be determined but recent research indicates that this issue may be resolved in the near future. Recent results have strengthened the hypothesis that μ -calpain plays a crucial role in postmortem tenderisation. Its activity under postmortem conditions *in vitro* and *in situ* have been confirmed. New information on the cause of μ -calpain activity decline postmortem is now available. This information can be used to optimise the processing conditions in order to benefit from the potential tenderising action of μ -calpain.

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