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The role of Ca^{2+}-dependent proteases (calpains) in *post mortem* proteolysis and meat tenderness

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Summary — This manuscript summarizes research results from our laboratory regarding the role of endogenous proteases in *post mortem* proteolysis resulting in meat tenderization. Proteolysis of key myofibrillar proteins is the principal reason for ultrastructural changes in skeletal muscle associated with meat tenderization. Proteases should have the following characteristics to be considered as possible candidates for bringing about *post mortem* changes: i) to be located within skeletal muscle cells; ii) to have access to the substrate *ie*, myofibrils); and iii) to be able to hydrolyze the same proteins that are degraded during *post mortem* storage. Of the proteases located within skeletal muscle cells and thus far characterized, only calpains have all of the above characteristics. Numerous experiments conducted in our laboratory have indicated that the calcium-dependent proteolytic system (calpains) is responsible for *post mortem* proteolysis. Some of this evidence includes: 1) incubation of muscle slices with buffer containing Ca^{2+} accelerates *post mortem* proteolysis; 2) incubation of muscle slices with Ca^{2+} chelators inhibits *post mortem* proteolysis; 3) infusion or injection of carcasses with a solution of calcium chloride accelerates *post mortem* proteolysis and the tenderization process such that *post mortem* storage beyond 24 h to ensure meat tenderness is no longer necessary; 4) infusion of carcasses with zinc chloride, a potent inhibitor of calpains, blocks *post mortem* proteolysis and the tenderization process; and 5) feeding a β-adrenergic agonist to lambs results in a reduction of the proteolytic capacity of the calpain system, which leads to a decreased rate of *post mortem* proteolysis and produces tough meat. Based on these results, we have concluded that calpains are the main proteolytic system responsible for *post mortem* proteolysis, and that one of the main regulators of calpains is their endogenous inhibitor, calpastatin.

calpains / calpastatin / proteolysis / *post mortem* / calcium

Introduction

It is now well documented that *post mortem* storage of carcasses at refrigerated conditions results in a significant improvement in meat tenderness. The first scientific report regarding *post mortem* tenderization of meat was that of Lehman [1], in 1907, who reported that there was a 30% increase in meat tenderness during an eight-day *post mortem* storage period. Although the improvement in meat tenderness is an accepted phenomenon, the mechanism through which these changes are brought about has remained a controversial issue.

Tenderness is probably the most important organoleptic characteristic of red meat. Therefore, it is important that the mechanism(s) of *post mortem* tenderization be identified, so that methodology can be developed to manipulate the process advantageously. Development of such methodology will have at least two major effects on the animal industry as a whole and specifically on the meat industry: 1) *post mortem* storage of up to 21 days to ensure meat tenderness may no longer be necessary; and 2) perhaps would eliminate the toughness problems that occur when *post mortem* storage fails to produce tender meat (such as with meat from *Bos indicus* breeds of cattle and from animals fed β-adrenergic agonists). The purpose of this manuscript is to review and summarize the results of experiments conducted in our laboratory regarding the mechanism of *post mortem* tenderization. Whenever possible, I will compare and discuss our results with those reported by other laboratories. Throughout this manuscript *post mortem* storage is defined as holding of carcasses at refrigerated temperatures (2–4°C) and should be distinguished from other methods of storage, such as storage at higher temperatures. Also, research efforts from this laboratory have been directed towards explaining the observed variation in meat tenderness from slaughter-age animals and should not be extrapolated to explain tenderness variation in meat from younger or older animals.

Role of proteolysis in meat tenderization

During *post mortem* storage of carcasses, numerous changes occur in skeletal muscle which result in loss of structural integrity of this tissue. This loss of structural integrity is responsible for meat tenderization (table I).

Table I. Summary of key changes that occur in skeletal muscle during *post mortem* storage at 2–4°C (adapted from [2–4]).

1. Z-disk weakening and/or degradation which leads to fragmentation of myofibrils.
2. Disappearance of troponin-T and simultaneous appearance of polypeptide with molecular weight of 28–32 kda This is perhaps the most publicized *post mortem* change in skeletal muscle. However, because of its location in the myofibrils, the exact relationship between meat tenderness and troponin-T is not yet understood.
3. Degradation of desmin which leads to fragmentation of myofibrils, probably through disruption of transverse cross-linking between myofibrils.
4. Degradation of titin. Effects of titin degradation on meat tenderness are not yet understood.
5. Degradation of nebulin. Effects of nebulin degradation on meat tenderness are not yet understood.
6. Appearance of a 95 000 kDa polypeptide, probably from degradation of myofibrillar proteins with molecular weights of greater than 95 000 kDa. Neither its origin or significance to meat tenderness is known.
7. Perhaps the most significant observation is that the major contractile proteins, myosin and actin, are not affected even after 56 days of *post mortem* storage.

Clearly, the majority of the changes that occur in skeletal muscle (table I) which lead to the disruption of the muscle cell and meat tenderization are the result of proteolysis. In fact, as early as 1917, Hoagland *et al* [5] reported that proteolysis is an important factor contributing to *post mortem* changes in skeletal muscle, including meat tenderness. Penny [6] reported that: « There is no doubt that proteolytic enzymes are responsible for the changes during conditioning (*post mortem* storage) ».

To further substantiate the argument that proteolysis is the principal reason for the observed meat tenderization during *post mortem* storage, the results of several experiments will be summarized (table II). In addition to those mentioned in table II, we have also found that the major reason for the observed differences in meat tenderness between *Bos taurus* (tender) and *Bos indicus* (tough) breeds of cattle is the reduced rate of myofibrillar protein degradation during *post mortem* storage [16, 17]. Also, differences in the rate of *post mortem* tenderization and proteolysis in skeletal muscle from pigs, sheep and cattle were apparently due to the differences in the rate of myofibrillar protein degradation. The results clearly indicate that proteolysis of key myofibrillar proteins is the principal reason for the ultrastructural changes in skeletal muscle resulting in the loss of muscle cell integrity (*ie*, tenderization).

Table II. Experimental evidence demonstrating the role of proteolysis in *post mortem* meat tenderization.

1. Incubation of muscle slices with calcium chloride induces proteolysis of myofibrillar proteins and fragmentation of myofibrils. However, incubation of muscle slices with calcium chelators (EDTA and EGTA) prevents both degradation of myofibrillar proteins and myofibril fragmentation [7].
2. Infusion of carcasses with calcium chloride accelerates *post mortem* changes (degradation of myofibrillar proteins, tenderness) in skeletal muscle such that *post mortem* storage to ensure meat tenderness is no longer necessary [8–10].
3. Infusion of carcasses with zinc chloride inhibits all *post mortem* changes measured (degradation of myofibrillar proteins, myofibril fragmentation, tenderization) [11].
4. Muscle from β-adrenergic agonist (BAA)-fed lambs, which does not undergo *post mortem* proteolysis (no detectable degradation of myofibrillar proteins and myofibril fragmentation during *post mortem* storage), is tougher than muscle from untreated lambs [12–14]. However, calcium chloride infusion of carcasses from BAA-fed lambs induces degradation of myofibrillar proteins and eliminates their meat toughness [15].

Which proteolytic system causes tenderization

Skeletal muscle is composed of three classes of proteins: sarcoplasmic, connective tissue and myofibrillar. Although some proteolytic degradation of sarcoplasmic proteins may occur during *post mortem* storage, their degradation probably does not contribute directly to increased tenderness (for review see [2]). Also, proteolytic changes in collagen (the principal protein of the connective tissue fraction) during *post mortem* storage comparable to those of myofibrillar proteins have not been observed [18]. Therefore, the principal mechanism of *post mortem* tenderization is limited to the proteolysis of myofibrillar proteins. It is important to bear in mind that throughout this manuscript and for the most part – all experiments conducted in our laboratory – we have studied the cause of tenderization in animals of similar age. Therefore, we agree with the conclusion of Tarrant [18] as long as animal age is kept constant. Indeed, we accept the argument that the connective tissue fraction could play a significant role in meat tenderness from animals of different ages.

Proteases should have the following characteristics to be considered as possible candidates for bringing about *post mortem* changes that result in meat tender-

ization: i) they should be located within the skeletal muscle cell (for details see [2]); ii) have access to the substrate (*ie*, myofibrils); and iii) have the ability to hydrolyze the same proteins in an *in vitro* system that are degraded during *post mortem* storage. If a proteolytic system had these characteristics, it would be impossible to exclude its potential involvement in *post mortem* proteolysis and the tenderization process.

There are many proteases in skeletal muscle; however, thus far only calpains and certain lysosomal enzymes have been shown to degrade myofibrillar proteins. For this reason, over the last decade we have focused our attention on these two proteolytic systems to sort out their involvement in *post mortem* proteolysis. This by no means excludes the possible direct or indirect involvement of other proteolytic systems (such as the multicatalytic proteolytic system [19]) in this process.

Based on the observations reported in table III (cathepsins degrade myosin efficiently and *post mortem* storage has no effect on myosin) and because of the location of lysosomal proteases in the skeletal muscle cell, we have excluded their involvement in *post mortem* proteolysis. These enzymes are normally located in lysosomes and presumably have to be released to have access to myofibrils. It has been assumed that during *post mortem* storage lysosomes are ruptured and thereby cathepsins are released into the cytosol. However, the only experiment conducted to test the accuracy of this assumption has indicated that even after 28 days of *post mortem* storage, lysosomal rupture was not evident [20]. Some of the other reasons that have led us to believe that lysosomal proteases are not involved in this process are explained in detail elsewhere [4, 11].

Table III. Effect of *post mortem* storage, calpains and cathepsins on myofibrils (adapted from [2–4, 21]).

	Post mortem storage	*Calpains*	*Cathepsins*
Z-disk degradation	+	+	±
Titin degradation	+	+	+
Nebulin degradation	+	+	+
Myosin degradation	–	–	+
α-Actinin degradation	–	–	+
Desmin degradation	+	+	–
Actin degradation	–	–	+
Troponin-T degradation	+	+	+
Appearance of 30 K	+	+	+

In contrast to lysosomal enzymes, considerable experimental evidence supports the hypothesis that calpains are the primary enzyme system responsible for *post mortem* proteolysis and tenderization. The properties and regulation of the calpain proteolytic system have been discussed in detail [22]. Therefore, I shall focus on experimental evidence regarding the role of this proteolytic system in *post mortem* proteolysis and tenderization process.

There is considerable experimental evidence indicating that elevated calcium ion concentration is responsible for the weakening of myofibrillar structures that results in tenderization. I shall first review this experimental evidence and then attempt to explain the mechanism of action of calcium in this process.

The first report linking calcium ions to *post mortem* tenderization is perhaps that of Davey and Gilbert [23]. They reported that the weakening and disappearance of Z-disks was inhibited by EDTA. They also speculated that EDTA may exert its effect by chelating calcium ions. Their observations were later supported by others [7, 24]. Busch *et al* [24] demonstrated that myofibril fragmentation was inhibited by EDTA, but was induced by calcium ions. Koohmaraie *et al* [7] demonstrated that the disappearance of the Z-disk and myofibril fragmentation were inhibited by EDTA (a general chelator of divalent cations) and EGTA (specific for calcium in the presence of magnesium) and were accelerated in the presence of calcium chloride. The results of these experiments [7, 23, 24] and others had convinced us that elevation of calcium ions is the cause of the *post mortem* tenderization process. We, therefore, attempted to reproduce these observations *in situ*.

Immediately after slaughter and after electrical stimulation (to exhaust ATP and prevent supercontraction), the carcasses were infused with a solution of calcium chloride via the vascular system. Results indicated that the *post mortem* tenderization processes were accelerated such that ultimate tenderness values were obtained within 24 h instead of after 7–14 days of *post mortem* storage [8]. We then focused our attention on attempting to elucidate the mechanism of action of calcium ions. There are at least three possible mechanisms through which calcium ions can exert their effect on *post mortem* tenderization: i) protein solubilization due to a salting-in action by calcium chloride; ii) non-enzymatic weakening of structural proteins involved in stability of Z-disk proteins; and iii) activation of calpains. To determine whether elevated ion concentration was the mechanism of action of infused-calcium chloride (salting-in action), carcasses were infused with calcium chloride or sodium chloride at the same ionic strength [9]. Infusion of carcasses with sodium chloride did not result in acceleration of *post mortem* proteolysis or the

tenderization process. Hence, it was concluded that the observed effects with calcium chloride infusion of carcasses were due to calcium ions and not due to an elevation of ionic strength. Recently, Taylor and Etherington [25] conducted an experiment to determine the mode of action of calcium. Their results indicated that although some solubilization of myofibrillar proteins occurred in the presence of calcium chloride: 'The removal of these proteins would probably not affect the stability of the Z-disk', and therefore should not affect meat tenderness.

The second possible mode of action of elevated calcium concentration, from endogenous (due to their release from mitochondria and the sarcoplasmic reticulum) or exogenous (infusion of carcasses with calcium chloride or incubation of muscle slices in calcium chloride solution) sources is a non-enzymatic one [26–28]. Immediately after slaughter, lamb carcasses were infused with a solution of zinc chloride. Guroff [29] reported that zinc chloride was a potent inhibitor of calpains. Therefore, if *post mortem* tenderization is brought about by non-enzymatic action of calcium ions, the process should not be affected by infusion of carcasses with zinc chloride infusion. However, results indicated that none of the *post mortem* changes (proteolysis of myofibrillar proteins, myofibril fragmentation or tenderization) occurred in carcasses infused with zinc chloride [11]. We have, therefore, concluded that the action of calcium chloride (from endogenous or exogenous sources) is mediated through the calpain proteolytic system. In support of these findings, Alarcon-Rojo and Dransfield [30] reported that the calcium chloride acceleration of *post mortem* tenderization was inhibited in the presence of synthetic inhibitor N-Acetyl-leu-leu-norleucinal), which is a substrate-like inhibitor of calpains. In addition, this inhibitor did not inhibit cathepsins B and L [30]. There is no doubt that calcium ions induce other changes in the skeletal muscle [25] and that much remains to be learned regarding its mode of action. However, the present experimental evidence suggests that its effects on tenderization are mediated by the calpain proteolytic system.

Regulation of the calpain proteolytic system in *post mortem* muscle

The process of conversion of muscle to meat is complex and involves metabolic, physical and structural changes. In a typical slaughterhouse, the animals are slaughtered by severing the carotid artery and jugular vein. After bleeding and evisceration, the carcasses are stored at about 1°C. Due to cessation of blood flow to the tissue, the oxygen supply (*ie*, source of energy) is cut off. In addition, the products of anaerobic metabolism (glycolysis) cannot be removed and accumulate in the tissue, resulting in buildup of lactic acid, which causes a gradual decline in the pH of the tissue from about 7.0 to about 5.6 over a 24-h period. At the same time, the temperature of the carcass falls from about 37°C to about 2°C over a 12-h (in cattle) period [31–33]. The combination of these complex changes results in the generation of a new environment totally different from that of living tissue. These *post mortem* conditions may change the capacity of different proteolytic systems dramatically. For example, one of the major changes is the elevation in the free calcium concentration due to its release from mitochondria and sarcoplasmic reticulum. While the concentration of free calcium in the resting muscle is less than 1 μM, in *post mortem* muscle it could reach 100 μM. These three dramatic changes alone (gradual fall in pH and temperature, and elevation of calcium concentration) would likely have a dramatic effect on the endogenous proteolytic systems. For example, serine proteases are almost totally inactive at pH values below 6.0. However, the conditions are more favorable for other proteolytic systems, such as calpains and lysosomal cathepsins. Currently, there is a debate on how calpains could possibly function in muscle tissue (for details see [2, 22]). Much of this debate occurs because the calcium requirements for proteolytic activity of the calpains (approximately 10 μM for μ-calpain and approximately 200–300 μM for m-calpain) are much higher than the free calcium concentrations found in living tissue (< 1 μM). Moreover, the calcium concentration required for binding of calpastatin to calpains is less than that required for proteolytic activity of the calpains themselves [2, 22]. However, these arguments may not be valid in *post mortem* muscle, because free calcium concentrations are sufficient to activate μ-calpain. Also, we have recently found that the drop in pH and temperature has a significant effect on the ability of calpastatin to inhibit μ-calpain (at 25°C: pH 7.5 = 87% inhibition; pH 5.7 = 55% inhibition; at 5°C: pH 7.5 = 59% inhibition; pH 5.7 = 6% inhibition) [34].

Previous studies have indicated that under normal *post mortem* conditions (*ie*, slaughter and holding at 2°C for up to 14 days) m-calpain is remarkably stable, whereas there is a gradual decline in the activities of μ-calpain, and calpastatin loses its activity rapidly [31, 35, 36]. Both μ- and m-calpain undergo autolysis in the presence of sufficient calcium with the eventual loss of activity [29, 37–45]. However, this loss of enzymatic activity is highly temperature-dependent [9] and greatly reduced in the presence of substrate [42]. We have therefore, [31] suggested that the reason for loss of μ-calpain *post mortem* is its autolysis due to the elevated calcium concentration in *post mortem* muscle. Although this seems to be a plausible

hypothesis (elevated calcium concentration activates μ-calpain which in turn hydrolyzes the few myofibrillar proteins that it can utilize as substrate, upon depletion of these proteins μ-calpain will undergo autolysis leading to its inactivation), another explanation could be its hydrolysis by another protease. We believe that both of these hypotheses are likely and that the role of a third protease *(ie*, the one that hydrolyzes μ-calpain) could be very significant. We are in the process of testing the accuracy of this hypothesis.

The third component of the calpain proteolytic system is their specific endogenous inhibitor, calpastatin. Results of several experiments reported recently seem to indicate that calpastatin is one of the principal regulators of the calpains in *post mortem* muscle. Firstly, infusion of carcasses with zinc chloride which prevented the *post mortem* proteolysis and tenderization process, completely blocked the inactivation of calpastatin [11]. Secondly, infusion of carcasses with calcium chloride, which results in acceleration of *post mortem* proteolysis and tenderization process, also accelerates the process of calpastatin inactivation [8, 9]. Thirdly, the rate of inactivation of calpastatin is highly correlated with the rate of *post mortem* proteolysis and tenderness in meat from *Bos indicus* breeds of cattle [16, 17] and in meat from animals fed a β-adrenergic agonist, $L_{644,969}$ [13, 14]; and finally, the differences in the rate of *post mortem* proteolysis and tenderization of meat from different species are negatively correlated with their calpastatin activity [33, 46]. From these results, it becomes apparent that calpastatin is indeed a powerful regulator of the calpains and efforts should be made to elucidate the mechanism of its inactivation. We believe that the inactivation of calpastatin is an enzymatic process and the protease involved is activated with calcium and inhibited by zinc [11].

Finally, I would like to suggest that our present knowledge of the regulation of calpains in *post mortem* muscle is by no means complete and much remains to be learned. Although quantification of the components of the calpain proteolytic system may be very useful, the results should be interpreted with caution, because the measured activity and actual proteolytic capacity, *in situ,* may be very different. One of the best examples to illustrate this point is the activity of the calpains in slow-twitch red *vs* fast-twitch white muscles. The activity of μ-calpain, m-calpain and calpastatin has been shown to be similar or even higher in red than white muscle [46]. Also, it has been demonstrated that *post mortem* proteolysis and tenderization process do not occur in the red muscle. Therefore, it has been argued that the calpain content does not agree with the proposed role of this proteolytic system in *post mortem* proteolysis [47]. However, Cassens *et al* [48] demonstrated that the slow-twitch red muscle has a three- to four-fold higher zinc content than fast-twitch white muscle. They reported that the largest portion of muscle zinc (64 and 86% in the white and red muscle, respectively) was found in the fraction composed primarily of myofibrils and nuclei [48]. Their findings have recently been substantiated by Kondo *et al* [49] who reported that red muscle contained 4.3 times higher zinc than white muscle. Therefore, higher content of zinc could be one of the reasons for the lack of *post mortem* proteolysis and tenderization in red muscle [32, 50]. In addition to zinc, other factors could also be involved in the regulation of calpains in *post mortem* muscle, such as a calpain activator. In 1982, DeMartino and Blumenthal, while examining the role of calmodulin on the activities of calpains in brain, identified a protein which was capable of stimulating the activities of both μ-calpain and m-calpain [51]. It stimulated the activities of both calpains up to 25-fold but it did not alter their calcium requirement for activity. Recently, Pontremoli et *al* [52] reported successful isolation of an activator from rabbit skeletal muscle. Whether calpain activator exists in skeletal muscle of meat producing animals (*ie,* cattle, sheep and pigs) remains to be determined. Yet, other possible regulators of calpains are carnosine, anserine and L-1-methyl-histidine. In muscle these dipeptides are found at relatively high (mmol) concentration [53, 54]. It has been reported that these dipeptides are mild activators of calpains [55]. In addition, carnosine increased the inhibitory activity of calpastatin, whereas anserine and L-1-methyl-histidine reduced the inhibitory effect of calpastatin [55]. These are some examples that should clearly indicate that while two tissues (*eg,* red and white muscle) might have identical calpain content, they might not have the same proteolytic capacity and thus we should be careful in interpreting quantitative data.

In summary, based on information currently available, we conclude that *post mortem* changes associated with meat tenderization are the result of proteolysis of key myofibrillar proteins. Experimental evidence indicates that the calpain proteolytic system is probably responsible for this *post mortem* proteolysis. There is no doubt that factors other than proteolysis will affect meat tenderness (elevated ionic strength during *post mortem* storage, connective tissue, etc). However, we believe that the principal reasons for the observed differences in the rates of *post mortem* tenderization (*eg,* among species or muscles) are differences in the rate of degradation of key myofibrillar proteins, which is probably mediated by the calpain proteolytic system. Also: 'The ultimate test of any theory's validity must be its capacity to explain the wide tenderness variability found among carcasses that have undergone normal *post mortem* treatment: muscles that remain attached to the carcass or side,

sides that remain suspended during cooling, and cooling that remains within the usual rate limits of meat-industry practice. Without this vital extension to works' operating conditions, a laboratory-generated hypothesis will necessarily remain of only academic interest' [56].

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