

# DEGRADATION OF MYOFIBRILLAR PROTEINS BY EXTRACTABLE LYSOSOMAL ENZYMES AND m-CALPAIN, AND THE EFFECTS OF ZINC CHLORIDE

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## ABSTRACT

A study was conducted to examine the effects that physiological levels of m-calpain (calpain requiring millimolar concentrations of  $\text{Ca}^{2+}$ ) extract and a lysosomal extract have on myofibrillar proteins in vitro, and the effects that zinc has on inhibiting proteolysis by these extracts. During a 22-h incubation period, the lysosomal extract degraded myosin heavy chain,  $\alpha$ -actinin, desmin, troponin-I, and myosin light chains 1 and 2. The effectiveness of the lysosomal extract to degrade myofibrillar proteins was significantly affected by the presence or absence of EDTA. Zinc, which is a potent inhibitor of cysteine proteinases, prevented most, but not all, of the lysosomal extract-induced myofibrillar protein degradation. Incubation of myofibrils with m-calpain resulted in the hydrolysis of troponin-T, desmin, and a 58-kDa molecular weight protein, possibly vimentin, and 5 mM  $\text{ZnCl}_2$  completely blocked these changes. Results from this study indicate that the degradation by the lysosomal extract is far more extensive than the degradation that occurs with normal postmortem storage and that possibly a non-cysteine protease is present that is capable of hydrolyzing some myofibrillar proteins under this in vitro condition, because  $\text{Zn}^{2+}$  did not block all proteolysis. However, similar changes were induced by m-calpain incubation and postmortem storage.

Key Words: Calpain, Myofibrillar Proteins, Proteolysis, Proteinases

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## Introduction

It is generally accepted that the tenderization of meat during postmortem aging is due largely to the proteolysis of certain myofibrillar proteins, in particular, the degradation of troponin-T and desmin (Koohmaraie et al., 1986). Neither myosin (Bandman and Zdanis, 1988) nor actin (Young et al., 1980) is degraded with extended cold storage. Much

work has been done to quantify proteinase activities, especially those of calpains and cathepsins, and to relate these activities to postmortem proteolysis, which corresponds to tenderness. Koohmaraie (1990a) infused  $\text{ZnCl}_2$  into ovine carcasses to determine its effect on postmortem proteolysis and meat tenderness. His results indicated that  $\text{ZnCl}_2$  infusion blocked postmortem proteolysis and tenderization because  $\text{Zn}^{2+}$  inhibited both calpains and cathepsins B and L activities. Therefore, no conclusive evidence was found regarding the relative roles of these two enzyme systems in postmortem proteolysis. However, when calpains are activated by infusion or injection of  $\text{CaCl}_2$ , myofibrillar proteolysis occurs more rapidly, thus eliminating the need for postmortem aging to improve tenderness (Koohmaraie et al., 1989, 1990).

Attempts, in vitro, to duplicate events in vivo can never be absolute, and often in vitro incubations are performed using enzyme:sub-

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strate ratios greater than those in muscle (Ouali et al., 1987). Therefore, the objectives of this study were to determine the extent of myofibrillar hydrolysis by partially purified m-calpain (calpain requiring millimolar concentrations of  $\text{Ca}^{2+}$ ) and lysosomal cysteine proteinases at their respective physiological concentrations (proteinase:myofibril ratio) and to determine the effectiveness of zinc in inhibiting proteolysis by these enzymes.

### Materials and Methods

#### Myofibrils

Ovine longissimus muscle samples were obtained immediately after exsanguination and 24 h and 14 d postmortem. Myofibrils were isolated according to the procedures of Goll et al. (1974). Myofibrils were stored in 50% glycerol, 100 mM NaCl, and 1 mM  $\text{NaN}_3$  at  $-20^\circ\text{C}$ . When needed, myofibrils were thawed at  $4^\circ\text{C}$  and centrifuged at  $2,000 \times g$  for 15 min, followed by three 100 mM NaCl washes. Protein concentrations then were determined using the biuret procedure (Gornall et al., 1949).

#### Proteinases

The lysosomal extract was obtained following Method D procedures of Koohmaraie and Kretchmar (1990). Briefly, samples from ovine longissimus, gluteus medius, semimembranosus, and supraspinatus muscles obtained immediately after exsanguination were extracted. Each extract was then loaded on separate S-carboxymethylated-papain-Sepharose columns to remove cystatin. The eluates were pooled and concentrated using a YM-10 ultra-filtration cell<sup>4</sup> and then dialyzed against 50 mM sodium acetate, pH 5.5. Protein concentration was determined (1.1 mg/ml) using BCA protein assay reagent<sup>5</sup>. Cathepsin B + L specific activity ( $10.8 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein) was determined fluorimetrically according to the method of Koohmaraie and Kretchmar (1990) using the substrate N-CBZ-phenyl-arginine-7-amido-4-methylcoumarin.

The m-calpain was partially purified from ovine longissimus muscle obtained within 30 min after exsanguination. The initial steps were conducted according to those described by Koohmaraie (1990b). After DEAE-Sepharcel chromatography, the fractions containing active m-calpain were pooled and dialyzed against Buffer A (20 mM 3-(N-morpholino) propanesulfonic acid [MOPS], .5 M NaCl, 1 mM EDTA, 5 mM  $\beta$ -mercaptoethanol [MCE], .02%  $\text{NaN}_3$ , pH 7.5). It was then loaded on a Reactive Red 120-Agarose<sup>6</sup> column that had been equilibrated with Buffer A. The column was washed with eight column volumes of Buffer A. The bound proteins were then eluted with Buffer A without NaCl at 30 ml/h. Active fractions were pooled and salted out between 0 and 65% ammonium sulfate. The pellet was dissolved in 50 mM Tris-HCl, pH 7.5, .5 mM EDTA, and 10 mM MCE and dialyzed against the same buffer. All these procedures were conducted at  $4^\circ\text{C}$ . Protein concentration (.79 mg/ml) was determined using the Bio-Rad dye-binding protein assay method<sup>7</sup>. Activity was determined using casein as the substrate according to the method of Koohmaraie (1990b). Specific activity was 40.5 units/mg of protein.

#### Incubation Conditions

For the lysosomal extract-myofibril incubation, 1 ml of buffer (340 mM sodium acetate, 10 mM MCE, 60 mM glacial acetic acid, pH 5.5) with and without 4 mM EDTA was used with 6.2 mg of sedimented myofibrils (centrifuged at  $2,000 \times g$  for 15 min). Based on preliminary data,  $177 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  of muscle was used as cathepsin B + L extractable (physiological) activity after the removal of cystatin. Therefore, 273  $\mu\text{l}$  of the lysosomal extract was added to the incubation buffer. The myofibrils were incubated with and without EDTA for .25, .5, 1, 3, 5, 9, 12, and 22 h at  $37^\circ\text{C}$ . Incubations were also performed with buffer containing either 5 mM  $\text{ZnCl}_2$ , 5 mM  $\text{CaCl}_2$ , or both, in the absence of EDTA.

For the calpain-myofibril incubation, 1 ml of 50 mM Tris-HCl, pH 7.5, 10 mM MCE buffer that contained either 10 mM EDTA, 5 mM  $\text{CaCl}_2$ , 5 mM  $\text{ZnCl}_2$ , or 5 mM  $\text{CaCl}_2$  + 5 mM  $\text{ZnCl}_2$ , and 16.6  $\mu\text{l}$  of partially purified m-calpain was used with 6.2 mg of sedimented myofibrils. The extractable level of m-calpain activity measurable by the substrate casein is

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1.2 activity units/g of muscle (Koochmarai et al., 1991), in the absence of its specific inhibitor. Myofibrils were incubated at 25°C for .25, .5, 1, 3, 6, 9, 12, and 22 h.

A combination incubation also was performed to determine whether the lysosomal extract's effect on the myofibrils would change if first hydrolyzed by m-calpain. Myofibrils were incubated first with m-calpain for 1 h, following the same procedures previously outlined for the m-calpain incubations using the 50 mM Tris-HCl, pH 7.5, 10 mM MCE, 5 mM CaCl<sub>2</sub> buffer. The myofibrils were washed twice with 3 ml of 100 mM NaCl. A sample then was removed for SDS-PAGE analysis. The remainder was incubated with lysosomal extract in the presence of 4 mM EDTA, as earlier discussed.

Controls, which consisted of all the assay components except the enzymes, were incubated. Incubations were terminated by centrifugation at 2,000 × g for 15 min at 4°C with two subsequent 100-mM NaCl washes. After the last wash, 1 ml of 100 mM NaCl was added to the myofibrillar pellet and protein concentration was determined using the biuret procedure. Samples were then prepared for SDS-PAGE by dissolving myofibrils in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% MCE, .02% bromophenol blue, and 10% glycerol and boiling for 5 min. Electrophoretic procedures of Laemmli (1970) were followed. Myofibrillar proteins were separated using a discontinuous 7.5 to 15% acrylamide gradient slab gel with a 75:1 acrylamide to bisacrylamide ratio. The acrylamide solution (30%) contained 50% glycerol. The amount of protein loaded was either 60 or 80 µg, depending on the well size. The myofibrillar proteins were identified based on SDS-PAGE reports of Porzio and Pearson (1977) and Greaser et al., (1983); in addition, reference was made to known molecular weight standards.

## Results

### *Myofibrillar Proteins Hydrolyzed During Postmortem Aging*

Samples obtained either within 30 min after exsanguination or 14 d postmortem are used to show proteolysis resulting from normal postmortem storage, and they appear in all figures (1 to 7) as a reference. The changes occurring in the myofibrillar proteins by 14 d include the

hydrolysis of desmin and troponin-T and the appearance of a 32-kDa component. This 32-kDa component is probably the 30-kDa polypeptide that is often associated with postmortem aging. But by using a higher ratio of acrylamide to bisacrylamide (75:1) along with a 7.5 to 15% gradient gel, the lower molecular weight proteins are more accurately separated. There was no visual evidence of myosin, actin, or  $\alpha$ -actinin degradation with postmortem aging.

### *Incubation with Lysosomal Extract*

In this study, the effects of EDTA on the myofibrillar proteins hydrolyzed by the lysosomal extract were assessed, because others (Okitani et al., 1977; Penny and Ferguson-Pryce, 1979) have reported the presence of an EDTA-activated enzyme system. In the presence of EDTA and the lysosomal extract (Figure 1), myosin degradation was evident after 1 h and its degradation continued up to 22 h of incubation, but actin degradation was minimal. The band corresponding to  $\alpha$ -actinin became less intense and appeared as a triplet with longer incubations. Desmin was faintly apparent after 9 h of incubation and was not visible by 22 h. The lysosomal extract also hydrolyzed troponin-I and myosin light chains 1 and 2, but not myosin light chain 3. In addition, the proteins at 30 and 17 kDa were degraded.

Along with the hydrolyzed myofibrillar proteins, various polypeptides appeared during the lysosomal extract's incubation. A group of polypeptides of 120 to 160 kDa was evident with 3 h of incubation but was extensive by 22 h. Also, 95-, 40-, 33-, 32-, and 28-kDa components became visible. However, the 95-kDa component was subsequently hydrolyzed by 9 h of incubation and an 87-kDa polypeptide became apparent. Many of these polypeptides (i.e., 120 to 160, 40, and 32 kDa) appeared in the 22-h incubation control, which did not contain any of the lysosomal extract. Also in this control, troponin-I was partially hydrolyzed. However, no changes were observed in the 12-h incubation control (data not shown), but due to the changes in the 22-h control, the ability to assess the effects that the lysosomal extract had on the myofibrillar proteins by 22 h is weakened.

Without EDTA in the incubation (Figure 2), the hydrolysis of  $\alpha$ -actinin, actin, and troponin-

nin-I by the lysosomal extract was enhanced. All three myosin light chains were degraded, as well as troponin-C, whereas in the presence of EDTA, myosin light chain 3 and troponin-C were resistant to hydrolysis. No differences in the polypeptide fragments were observed in the incubations with and without EDTA. However, the fragments appeared sooner in the incubations without EDTA. As for the 22-h incubation control without EDTA, it seemed that less degradation of myosin heavy chain and troponin-I occurred compared with the incubation control containing EDTA (Figure 1). However, myosin light chain 1, troponin-C, and the 30-kDa band seemed to be slightly affected in the 22-h control without EDTA. Also, the polypeptide fragments appearing were less intense than those in the 22-h control with EDTA, except for the 40-kDa fragment.

The presence of  $\text{Ca}^{2+}$  had no effect on the lysosomal extract's ability to hydrolyze the

myofibrillar proteins (Figure 3) compared with proteins hydrolyzed in the absence of EDTA. However, in the incubations containing  $\text{Zn}^{2+}$  and the lysosomal extract, the hydrolysis of myosin,  $\alpha$ -actinin, actin, and desmin was decreased. Also, a group of polypeptides of 120 to 195 kDa was evident, as well as 40-, 33-, 32-, and 28-kDa components. Therefore, it seems that the cysteine proteinases in the lysosomal extract were not totally inhibited by  $\text{Zn}^{2+}$ , or that a noncysteine proteinase(s) was capable of hydrolyzing the majority of the same myofibrillar proteins but to a lesser extent at this enzyme concentration. Of the incubations containing ions but no lysosomal extract, only the one containing  $\text{Ca}^{2+}$  showed evidence of hydrolysis. However, it did not differ from the 22-h incubation control without EDTA, in which troponins -I and -C, the 30-kDa band, and myosin light chain 1 were affected. However, the myofibrils incubated with  $\text{ZnCl}_2$  alone were resistant to degradation,

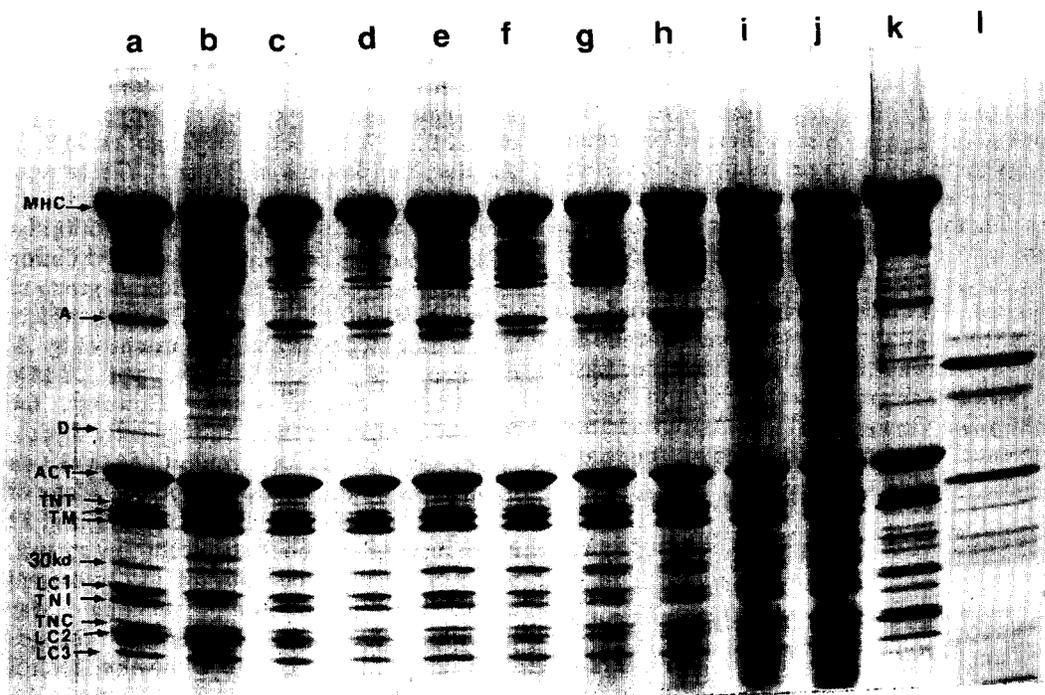


Figure 1. The SDS-PAGE of myofibrillar proteins incubated with the lysosomal extract in the presence of EDTA. Lane a, reference, longissimus sample obtained within 30 min after slaughter; lane b, 22-h control without extract present; lanes c to j, 25-, 5-, 1-, 3-, 6-, 9-, 12-, and 22-h incubations, respectively; lane k, reference 14-d aged longissimus sample; and lane l, extract alone. Abbreviations: MHC = myosin heavy chain, A =  $\alpha$ -actinin, D = desmin, ACT = actin, TN = troponins, TM = tropomyosins, and LC = myosin light chains.

and no differences were observed between this sample and the d-0 sample.

#### Incubation with *m*-Calpain

The number of myofibrillar proteins hydrolyzed by *m*-calpain was minimal, and no detectable differences appeared between the d-0 sample and the incubation control (Figure 4). The proteins hydrolyzed include troponin-T, desmin, and a 58-kDa band, which could possibly be vimentin, whereas  $\alpha$ -actinin seemed to be only partially hydrolyzed regardless of incubation time. Correspondingly, there was the appearance of a 32-kDa polypeptide product.

Because only a slight decrease in the intensity of the band corresponding to  $\alpha$ -actinin occurred among the incubation times greater than 1 h, the question had to be

answered whether or not *m*-calpain itself was being hydrolyzed or whether there was a lack of substrate. Therefore, another incubation was conducted, in which an additional 16  $\mu$ l of partially purified *m*-calpain was added to each incubation at intervals of .25, .5, 1, and 3 h; the 6-h incubation received a total of 80  $\mu$ l of *m*-calpain.

The results indicate that by increasing *m*-calpain concentrations, troponin-I is hydrolyzed to a greater extent and the 32-kDa band intensity peaks after 30 min of incubation, and it also is subsequently degraded through 6 h (Figure 5). These changes occurred with 3 h of incubation and 64  $\mu$ l of *m*-calpain, whereas only slight changes were observed with 6 h of incubation and 80  $\mu$ l of *m*-calpain. Therefore, it was concluded that there was a lack of substrate by 3 h. Also, it seems that desmin, the 58-kDa band, and troponin-T are more



Figure 2. The SDS-PAGE of myofibrillar proteins incubated with the lysosomal extract in the absence of EDTA. Lane a, reference, longissimus sample obtained within 30 min after slaughter; lane b, 22-h control without extract present; lanes c to j, .25-, .5-, 1-, 3-, 6-, 9-, 12-, and 22-h incubations, respectively; lane k, reference 14-d aged longissimus sample; and lane l, extract alone. Abbreviations: MHC = myosin heavy chain, A =  $\alpha$ -actinin, D = desmin, ACT = actin, TN = troponins, TM = tropomyosins, and LC = myosin light chains.

susceptible to m-calpain degradation than are  $\alpha$ -actinin and troponin-I.

The proteolytic effects of m-calpain were completely inhibited by  $Zn^{2+}$  (even in the presence of  $Ca^{2+}$ ) and EDTA (Figure 6). No differences were observed among these and the d-0 sample or the incubation control. The only treatment in which extensive proteolysis occurred was m-calpain +  $Ca^{2+}$ , in which troponin-T, desmin, and the 58-kDa band were degraded. With this proteolysis, a 32-kDa fragment became visible, which corresponds with the d-14 sample. The addition of  $Ca^{2+}$  alone decreased the intensity of desmin and a faint 32-kDa band was apparent.

In the combined incubation, in which the myofibrils were first hydrolyzed by m-calpain then later subjected to the lysosomal extract (Figure 7), no differences from proteolysis by the lysosomal extract alone were observed.

Therefore, it does not seem that m-calpain altered any myofibrillar protein to be later hydrolyzed by the acidic proteinases. However, the band representing troponin-T was removed by m-calpain but became apparent in the lysosomal extract's incubations. Consequently, this fragment must be from a higher molecular weight protein and the lysosomal extract's ability to hydrolyze troponin-T cannot be assessed.

### Discussion

One of the major contributors to postmortem tenderization is the proteolysis of certain myofibrillar proteins, including desmin and troponin-T (Koochmaraie et al., 1986). For years, researchers have tried to determine which proteases are responsible for this proteolysis (for review, see Goll et al., 1983;

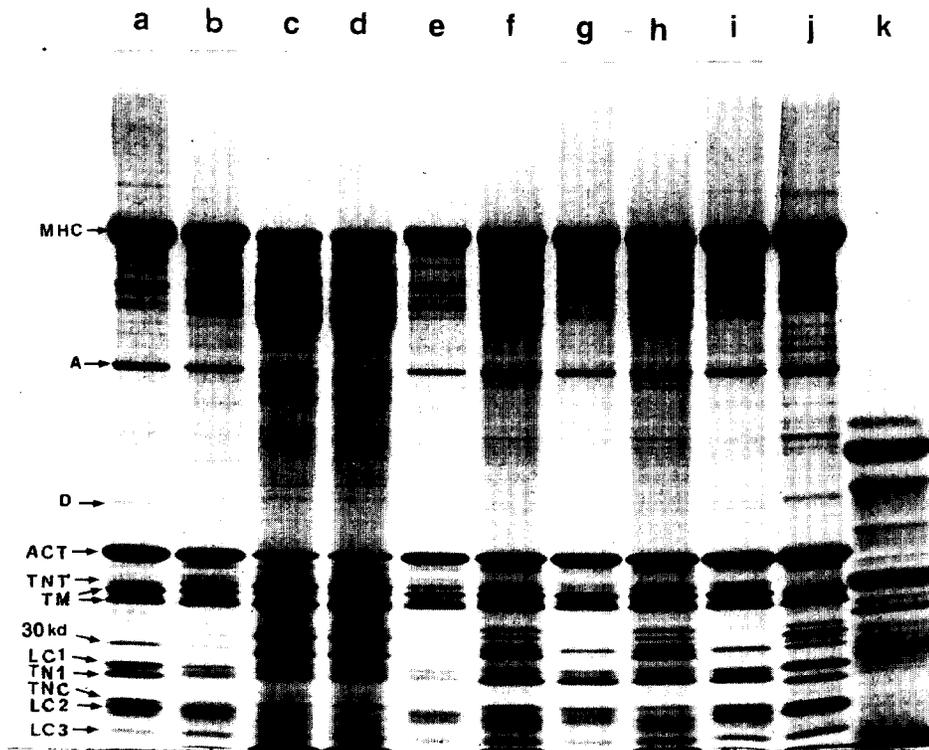


Figure 3. The SDS-PAGE of myofibrillar proteins incubated with and without the lysosomal extract in the presence and absence of calcium and zinc. Lane a, reference, longissimus sample obtained within 30 min after slaughter; lanes b to i are 22-h incubations; lane b, control without EDTA and extract; lane c, extract - EDTA; lane d, extract +  $CaCl_2$ ; lane e,  $CaCl_2$  - extract; lane f, extract +  $ZnCl_2$ ; lane g,  $ZnCl_2$  - extract; lane h, extract +  $CaCl_2$  +  $ZnCl_2$ ; lane i,  $CaCl_2$  +  $ZnCl_2$  - extract; lane j, reference 14-d aged longissimus sample, and lane k, extract alone. Abbreviations: MHC = myosin heavy chain, A =  $\alpha$ -actinin, D = desmin, ACT = actin, TN = troponins, TM = tropomyosins, and LC = myosin light chains.

Koohmaraie, 1988). Of these enzyme systems, calpains and lysosomal proteinases have received the greatest attention because to date they are the only characterized proteases known to degrade myofibrillar proteins.

The calpains are both found intracellularly; however,  $\mu$ -calpain requires micromolar and m-calpain millimolar concentrations of  $\text{Ca}^{2+}$  for activation (Dayton et al., 1981). Physiological  $\text{Ca}^{2+}$  levels after death are sufficient to activate  $\mu$ -calpain, but not m-calpain. Research has shown that both  $\mu$ -calpain and m-calpain hydrolyze the same myofibrillar proteins (for review, see Goll et al., 1989). Therefore, m-calpain was used in this study because its specific activity is higher with fewer chromatographic steps. The optimum conditions for the calpains are 25°C and pH 7.5 (Dayton et al., 1976; Koohmaraie et al., 1986). Therefore, some may believe that calpain's contribution to meat tenderness is minimal, because temperature and pH decrease under normal slaughter conditions. However, Koohmaraie et al. (1986) reported that 24 to 28% of  $\mu$ -calpain's activity

was retained at pH 5.5 to 5.8 and 5°C. Also, Penny and Ferguson-Pryce (1979) found calpain to remain active at pH 5.5.

Cathepsins and other acidic proteinases are found within the lysosome. Thus, their ability to hydrolyze myofibrillar proteins is dependent on their release, which may not occur even after 3 wk of aging (LaCourt et al., 1986). For some cathepsins, the optimum pH is in the pH range of postrigor meat (Goll et al., 1983). Therefore, some may believe that their contribution to meat tenderness occurs later post-mortem, although their maximum activity is expressed at 37°C (unpublished data).

Two myofibrillar proteins that are hydrolyzed under normal postmortem conditions (2 to 4°C and 5.5 to 5.7 pH) are desmin (Young et al., 1980) and troponin-T (Penny and Dransfield, 1979). Also, Hwan and Bandman (1989) reported a partial degradation of  $\alpha$ -actinin by 14 d postmortem under normal conditions and degradation was enhanced with 25°C storage. However, no evidence supports the fact that either myosin or actin is

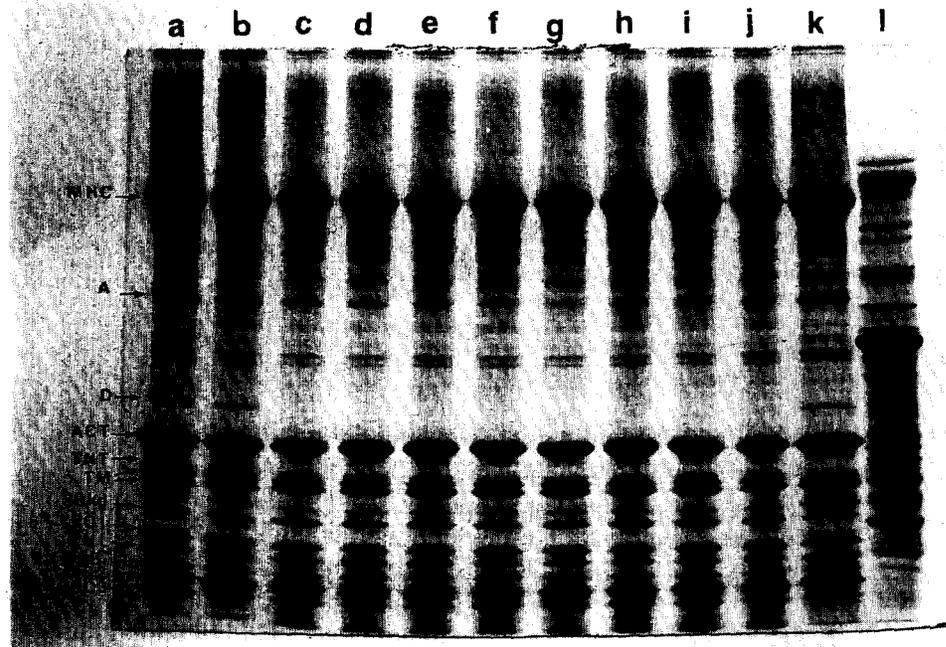


Figure 4. The SDS-PAGE of myofibrillar proteins incubated with m-calpain. Lane a, reference, longissimus sample obtained within 30 min after slaughter; lane b, 22-h control without m-calpain; lanes c to j, .25-, .5-, 1-, 3-, 6-, 9-, 12-, and 22-h incubations, respectively; lane k, reference, 14-d aged longissimus sample; and lane l, partially purified m-calpain alone. Abbreviations: MHC = myosin heavy chain, A =  $\alpha$ -actinin, D = desmin, ACT = actin, TN = troponins, TM = troponosins, and LC = myosin light chains.

hydrolyzed under normal postmortem conditions, but they may be during high-temperature storage (Penny et al., 1984; Bandman and Zdanis, 1988). In this study, the lysosomal extract degraded myosin (Figures 1 and 2), which is in agreement with the results of others who found cathepsins to degrade myosin (Schwartz and Bird, 1977; Matsukura et al., 1981; Okitani et al., 1981; Mikami et al., 1987; Ouali et al., 1987). In addition to myosin, cathepsin B (Schwartz and Bird, 1977; Ouali et al., 1987) and cathepsin L (Matsukura et al., 1981; Mikami et al., 1987) hydrolyze numerous myofibrillar proteins that are not degraded during postmortem aging, which also agrees with results from this study. However, the observed proteolysis by the lysosomal extract by 22 h is confounded with the myofibrillar breakdown occurring in the 22-h incubation control, but no breakdown occurred in the 12-h incubation control. Ouali et al. (1987) found that myofibrils incubated for long

periods with a sodium phosphate buffer, pH 5.8, at 30°C also were unstable. Currently, there is no explanation for the prerigor myofibrils' instability under these conditions. However, our study indicates that if the myofibrils are first subjected to a 50 mM Tris buffer, pH 7.5, at 25°C for 1 h, they remain stable for 22 h at 37°C in the sodium acetate buffer, pH 5.5 (Figure 7), which rules out the possibility that the degradation (increase in protein solubility) is an effect of ionic strength (Wu and Smith, 1987; Wheeler et al., 1990). In addition, when 5 mM ZnCl<sub>2</sub> was added to the sodium acetate buffer, the myofibrils remained stable for the 22-h duration (Figure 3). Therefore, it seems that an unknown proteinase(s) that is active at pH 5.5 and 37°C remains bound to the myofibrils during the extraction process, which did include two detergent washes (Goll et al., 1974). The proteinase(s) apparently is irreversibly inactivated during the 25°C and pH 7.5 incubation

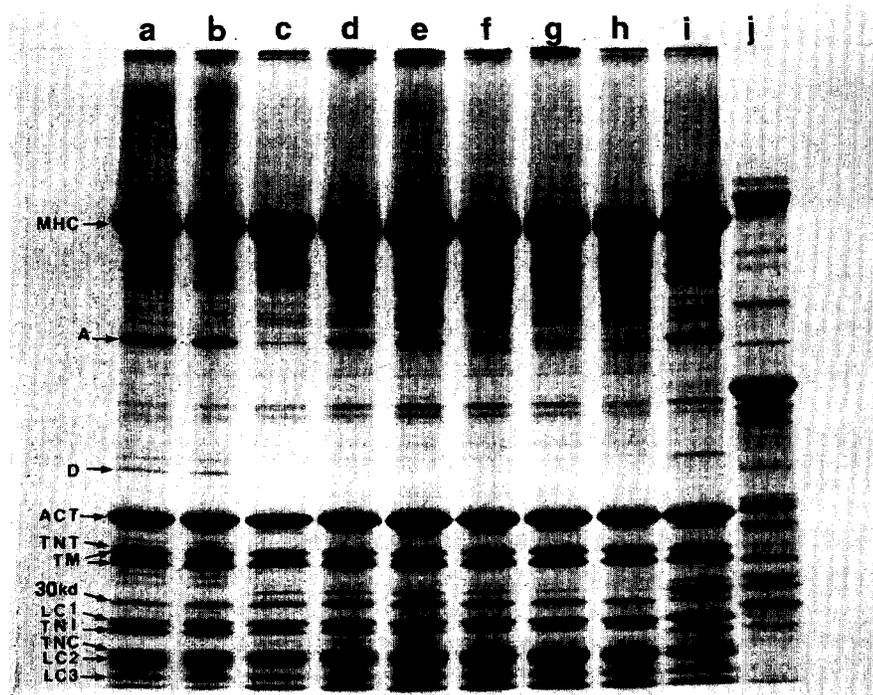


Figure 5. The SDS-PAGE of myofibrillar proteins incubated with higher m-calpain concentrations. Lane a, reference, longissimus sample obtained within 30 min after slaughter; lane b, 22-h control without m-calpain; lane c, 22 h 16.6 µl of m-calpain; lane d, 15 min, 16 µl of m-calpain; lane e, 30 min, 32 µl of m-calpain; lane f, 1 h, 48 µl of m-calpain; lane g, 3 h, 64 µl of m-calpain; lane h, 6 h, 80 µl of m-calpain; lane i, reference, 14-d aged longissimus sample, and lane j, partially purified m-calpain alone: 16 µl of m-calpain = .013 mg of protein. Abbreviations: MHC = myosin heavy chain, A =  $\alpha$ -actinin, D = desmin, ACT = actin, TN = troponins, TM = tropomyosins, and LC = myosin light chains.

and is inhibited by  $Zn^{2+}$  but remains active in the presence of EDTA (i.e., incubation 22-h control; Figure 1) and  $Ca^{2+}$  (Figure 3).

Another interesting observation was the enhanced myofibrillar degradation by the lysosomal extract in the absence of EDTA. Therefore, an unknown metalloproteinase(s) may be present that is active under these assay conditions, which would also be consistent with the decreased myofibrillar degradation in the presence of EDTA. In contrast, Matsukura et al. (1981) found that EDTA was a potent enhancer of cathepsin L activity, and others have reported the incidence of an EDTA-activated enzyme system (Okitani et al., 1977; Penny and Ferguson-Pryce, 1979). However, regardless of the presence or absence of EDTA, myosin was hydrolyzed by the lysosomal extract.

The myofibrillar proteins hydrolyzed by the extract containing m-calpain in vitro closely mimic those degraded with normal postmortem

aging (Dayton et al., 1976; Ouali et al., 1983; Elgasim et al., 1985; Koochmarai et al., 1986; Zeece et al., 1986). In this study, the myofibrils incubated with m-calpain in the presence of 5 mM  $CaCl_2$  mimicked the 14-d sample, except that  $\alpha$ -actinin seemed to be hydrolyzed to a greater extent and the 58-kDa band did not appear (Figures 4 and 6). The 58-kDa band is possibly vimentin and is a substrate for calpain (Nelson and Traub, 1983). However, it seems not to be subjected to proteolysis in vivo.

In other in vitro studies, troponin-I, tropomyosin,  $\alpha$ -actinin, and C-protein may have been degraded by calpain (Dayton and Schollmeyer, 1980; Penny et al., 1984; Elgasim et al., 1985; Zeece et al., 1986). Of these proteins,  $\alpha$ -actinin seemed to be partially degraded by m-calpain in this study. In addition, when the concentration of m-calpain was increased (Figure 5), the degradation of troponin-I was enhanced. Dayton et al. (1975)

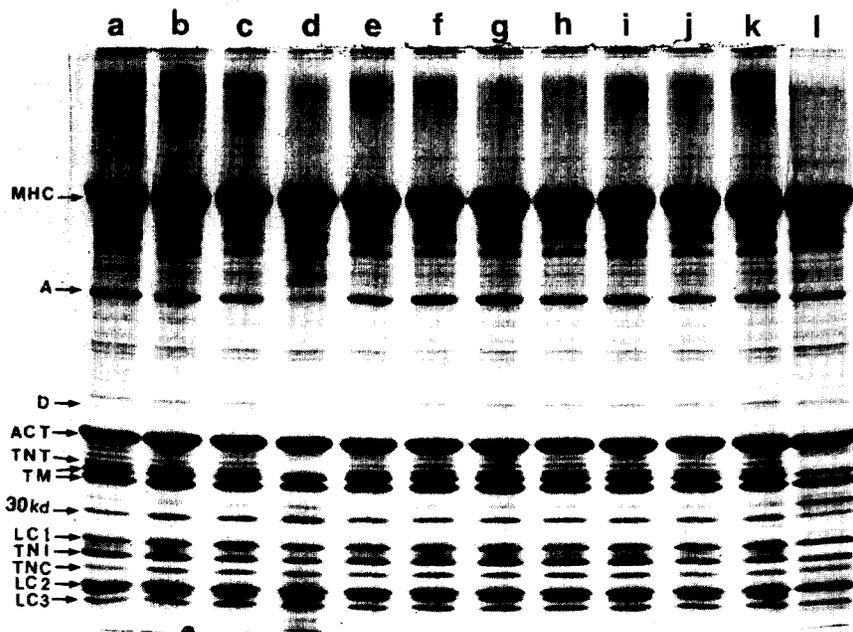


Figure 6. The SDS-PAGE of myofibrillar proteins incubated with and without m-calpain in the presence and absence of calcium, zinc, and EDTA. Lane a, reference, longissimus sample obtained within 30 min after slaughter; lanes b to k are 22-h incubations; lane b, control without m-calpain; lane c, m-calpain alone; lane d, m-calpain +  $CaCl_2$ ; lane e,  $CaCl_2$  - m-calpain; lane f, m-calpain +  $ZnCl_2$ ; lane g,  $ZnCl_2$  - m-calpain; lane h, m-calpain +  $CaCl_2$  +  $ZnCl_2$ ; lane i,  $CaCl_2$  +  $ZnCl_2$  - m-calpain; lane j, m-calpain + EDTA; lane k, EDTA - m-calpain; lane l, reference, 14-d aged longissimus sample. Abbreviations: MHC = myosin heavy chain, A =  $\alpha$ -actinin, D = desmin, ACT = actin, TN = troponins, TM = tropomyosin, and LC = myosin light chains.

found that purified  $\alpha$ -actinin was not susceptible to calpain proteolysis, but calpain releases  $\alpha$ -actinin from the myofibril. Therefore, whether or not  $\alpha$ -actinin actually was degraded or released from the myofibril was not determined in this study. Our results also agree with those of Koohmaraie et al. (1984) in which the 30-kDa band (32 kDa in this study) was subjected to further degradation with longer incubation times and higher calpain concentrations. When  $ZnCl_2$  was added to the incubation, no proteolysis took place, which supports the *in vivo* results of Koohmaraie (1990a).

From these results, it seems that the proteinases contained in the lysosomal extract extensively degraded many myofibrillar proteins, whereas m-calpain degraded a minimal number. In addition, calpain's effect is totally inhibited in the presence of  $Zn^{2+}$ . But, in neither case was the *in vitro* proteolysis

identical to that *in vivo*. So, there is still much unknown about these proteinase activities in postmortem muscle and the role their inhibitors have in their regulation. Also, *in vitro*, the proteinase is put in direct contact with its substrate (i.e., myofibrils), whereas *in vivo* substrate availability may be limited.

#### Implications

Myofibril incubations with the lysosomal extract always resulted in some type of myosin degradation, which does not occur with cold storage. The addition of  $ZnCl_2$  did not totally inhibit proteolysis in the lysosomal extract incubations, as it did in those containing m-calpain. Troponin-T and desmin were degraded by m-calpain, and the proteolysis of these proteins is associated with postmortem tenderization. Therefore, we conclude that the myofibrillar proteolysis by m-calpain closely resem-

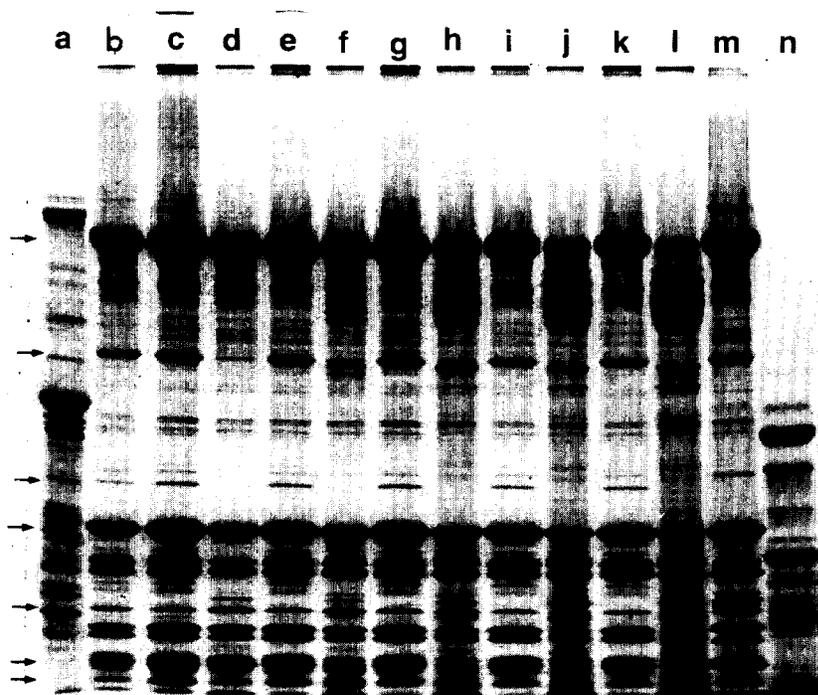


Figure 7. The SDS-PAGE of myofibrillar proteins incubated first with m-calpain followed by lysosomal extract proteolysis in the presence of EDTA. Lane a, partially-purified m-calpain alone; lane b, reference, longissimus sample obtained within 30 min after slaughter; lane c, 1 h, pH 7.5 control; lane d, 1 h, m-calpain +  $CaCl_2$ ; lanes e, g, i, and k; 1 h, pH 7.5 control followed by 1 h, pH 5.5 control (e); 6 h, pH 5.5 control (g); 12 h pH 5.5 control (i) or 22 h, pH 5.5 control (k); lanes f, h, j, l; 1 h m-calpain +  $CaCl_2$  followed by 1 h extract (f); 6 h extract (h); 12 h extract (j) or 22 h extract (l); lane m, reference, 14-d aged longissimus sample; and lane n, extract alone. See Figure 2 for myofibrillar protein identification.

bles degradation that occurs during normal postmortem storage, more so than that by the lysosomal enzymes.

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