

Effect of Calpastatin on Degradation of Myofibrillar Proteins by μ -Calpain Under Postmortem Conditions^{1,2}

Geert H. Geesink and Mohammad Koohmaraie³

Roman L. Hruska U.S. Meat Animal Research Center, ARS, USDA, Clay Center, NE 68933-0166

ABSTRACT: To improve our understanding of the regulation of μ -calpain activity in situ during postmortem storage of muscle, the effect of different calpastatin levels on proteolysis of myofibrillar proteins by μ -calpain in a system closely mimicking postmortem conditions was studied. Increasing the amount of calpastatin in the incubations limited both the rate and extent of proteolysis of myofibrillar proteins and autolysis of μ -calpain. Excess calpastatin (i.e., a μ -calpain:calpastatin ratio of 1:4) did not inhibit proteolysis completely. West-

ern blot analysis revealed that proteolysis of myofibrillar proteins virtually ceased after 7 d of incubation, despite the presence of partly autolyzed, therefore seemingly active, μ -calpain. A series of incubations of autolyzed μ -calpain revealed that the autolyzed form of this enzyme is unstable at an ionic strength observed in postmortem muscle. The possible significance of these results in terms of the regulation of μ -calpain activity in postmortem muscle is discussed.

Key Words: μ -Calpain, Calpastatin, Proteolysis, Tenderness

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Introduction

The calcium-dependent protease μ -calpain has been implicated as a major cause of postmortem tenderization of skeletal muscle through degradation of key myofibrillar and associated proteins (for review see Koohmaraie, 1996). Incubation of myofibrils with μ -calpain under conditions mimicking the storage of postmortem muscle (i.e., pH 5.5 to 5.8 and 4°C) results in degradation of myofibrillar proteins similar to that observed in postmortem muscle (Koohmaraie et al., 1986; Huff-Lonergan et al., 1996). Calpastatin, the specific inhibitor of the calpains, was, however, not incorporated into these incubations. The activity ratio of μ -calpain:calpastatin is approximately 1:4, 1:2.5, and 1:1.5 in beef,

lamb, and pork longissimus, respectively (Ouali and Talmant, 1990; Koohmaraie et al., 1991b). Thus, the question arises of how μ -calpain can be active in postmortem muscle when muscle contains an excess of calpastatin (Boehm et al., 1998). To the best of our knowledge, no studies have been performed in which all elements that may influence μ -calpain activity in postmortem muscle (i.e., pH, ionic strength, refrigeration temperatures, calpastatin, and storage for up to 14 d postmortem) have been studied simultaneously. Such information would further our understanding of the involvement of μ -calpain and calpastatin in postmortem proteolysis and postmortem tenderization. Therefore, the objective of this study was to examine the effect of postmortem conditions on μ -calpain and myofibrillar proteins in a system that closely mimics postmortem muscle.

Materials and Methods

Purification of μ -Calpain

μ -Calpain was purified from bovine sternomandibularis (1 kg) according to methods described by Edmunds et al. (1991) with minor modifications. Briefly, muscle was trimmed of visible fat and connective tissue within 1 h postmortem and homogenized in three volumes of extraction buffer (100 mM Tris/HCl, pH 8.3, 10 mM EDTA, .05% [vol/vol] 2-mercaptoethanol [MCE], 100 mg/L ovomucoid, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 6 mg/L leupeptin). After centrifugation,

¹Mention of a trade name, proprietary product or specific equipment is necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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³To whom correspondence should be addressed: P.O. Box 166 (phone: (402) 762-4221; fax: (402) 762-4149; E-mail: koohmaraie@email.marc.usda.gov).

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the supernatant was salted out between 0 and 45% ammonium sulfate saturation, and, after dialysis, μ -calpain was purified using successive chromatography over DEAE-Sephacel (Pharmacia LKB Biotechnology, Piscataway, NJ), Phenyl-Sepharose (Pharmacia LKB Biotechnology), Butyl-Sepharose (Pharmacia LKB Biotechnology), Mono Q (Pharmacia LKB Biotechnology), and Sephacryl S-300 (Pharmacia LKB Biotechnology). μ -Calpain (13.4 units/mL, 46 units/mg) was stored at 4°C in elution buffer (40 mM Tris/HCl, pH 7.35, .5 mM EDTA, and 1 mM NaN₃). Calpain activity was determined as described by Koohmaraie (1990).

Purification of Calpastatin

Calpastatin was purified from bovine sternomandibularis (.5 kg) as described by Geesink et al. (1998). Briefly, muscle was extracted as described for purification of μ -calpain, and 15% (wt/vol) trichloroacetic acid (TCA) was added to the extract. After centrifugation, the pellet was suspended in .1 M Tris base, and the pH was adjusted to 7.5 with 1 M NaOH. This suspension was dialyzed against three changes of TEMA (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, .1% MCE, and 1 mM NaN₃). The suspension was clarified by centrifuging at 30,000 $\times g_{\max}$. The supernatant was heated in a microwave oven to 85°C, kept in a waterbath at 90°C for 10 min, and cooled on ice. Precipitated material was pelleted by centrifuging at 30,000 $\times g_{\max}$. Calpastatin was purified from the supernatant by successive chromatography over Affigel blue (Bio-Rad Laboratories, Hercules, CA), Mono Q (Pharmacia LKB Biotechnology), and Sephacryl S-300 (Pharmacia LKB Biotechnology). Calpastatin (29.2 units/mL, 906 units/mg) was stored at 4°C in elution buffer. Calpastatin activity was determined as described by Koohmaraie (1990).

Myofibril Isolation

Myofibrils were prepared from bovine sternomandibularis within 1 h postmortem according to the procedures of Goll et al. (1974). Myofibrils were stored in 50% glycerol, 100 mM NaCl, and 1 mM NaN₃ at -30°C.

Incubations

Myofibrils were washed three times in a mixed salt solution (MSS: 12 mM NaCl, 12.6 mM MgSO₄·7H₂O, 70 mM KH₂PO₄, 3.4 mM NaOH, 64.2 mM KOH, 11.1 mM H₂SO₄, 132 mM lactic acid, 100 mM Tris, and 1 M Tris was added to pH 5.8, 4°C) formulated on the basis of the postrigor sternomandibularis muscle composition as described by Winger and Pope (1980–81). As pointed out by Winger and Pope (1980–81), the strong buffering effects of the organic constituents of muscle dictate that a strong base should be used to neutralize the lactic acid in the salt solution. To satisfy the requirement that the inorganic ion composition of the solution closely resemble that of muscle, Tris was chosen to neutralize the acid. A reducing environment is required for expres-

sion of calpain activity. Obviously, the conditions in postmortem muscle are reducing enough to allow expression of calpain activity. We do not know which compounds contribute to a reducing environment in postmortem muscle, but to meet the requirement of a reducing environment 1% MCE was added to the salt solution. After the third wash, the myofibrils were re-suspended in MSS with 1.3 mM CaCO₃. The protein concentration of the myofibril suspension was determined using the biuret assay (Gornall et al., 1949). Aliquots of 11.2 mg myofibrils (.5 mL) were incubated with .12 units of μ -calpain and 0, .25, or .50 units of calpastatin. Control incubations contained no calpain or calpastatin. Samples were incubated for 0, 1, 2, 7, and 14 d at 5°C. The pH of the suspensions was not affected by the addition of μ -calpain and calpastatin and was stable throughout the incubations. After incubation, myofibrils were centrifuged at 6,000 $\times g_{\max}$ for 3 min at 5°C, and the pellet and supernatant were prepared for SDS-PAGE by addition of protein denaturing buffer (PDB; 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% MCE, .02% bromophenol blue, and 10% glycerol).

Effect of pH on Inhibition of μ -Calpain by Calpastatin

To ensure that any change in calpastatin activity would be detected, a ratio of calpastatin to μ -calpain was selected so that μ -calpain activity (.45 units) in the presence of calpastatin was approximately 30% of μ -calpain in the absence of calpastatin in our standard assay (Koohmaraie, 1990). To account for a pH effect on substrate and calpain activity, each activity assay consisted of three separate 2-mL reactions: μ -calpain + calpastatin + media, μ -calpain + media, and media alone. The media consisted of 3.5 mg/mL casein in MSS with 1.3 mM CaCO₃, pH 5.7, 6.2, or 7.0. All reactions were incubated for 60 min at 25°C and were stopped by addition of 2 mL of 5% (wt/vol) TCA. Activity was determined by measuring the A₂₇₈ of the supernatant (Koohmaraie, 1990). Assays were performed in quadruplicate.

Effect of Salt Concentration on μ -Calpain Stability

μ -Calpain (480 μ L, 37 units/mL, in elution buffer) was autolyzed for 1 min at room temperature by the addition of 12 μ L of 100 mM CaCl₂. Autolysis was stopped by the addition of 48 μ L of 200 mM EDTA/NaOH (pH 7). Aliquots (30 μ L) of the autolyzed μ -calpain were mixed with an equal volume of 0, 200, 600, or 1,000 mM NaCl and incubated at room temperature for 5, 15, 30, or 45 min. Calpain activity after these incubations was determined on 20- μ L samples in duplicate in a 2-mL reaction volume according to Koohmaraie (1990). The remaining 20 μ L of sample was mixed with an equal volume of PDB. The effect of salt concentration on native μ -calpain was determined in a similar fashion, with the difference that EDTA was added to the sample before CaCl₂.

SDS-PAGE and Immunoblotting

The SDS-PAGE was performed according to Laemmli (1970) on .75-mM-thick 12.5 or 8% (37.5:1) separating gels with 4% (37.5:1) stacking gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) using a Semi-Phor TE70 semidry transfer unit (Hoefer Scientific Instruments, San Francisco, CA) at 150 mA for 1 h. Lanes containing molecular weight markers were stained with amido black. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (pH 7.4) containing .05% Tween-20 (TTBS) for 1 h. Primary antibodies used in these experiments included mouse anti- μ -calpain monoclonal antibody (B2F9, 1:10, Geesink and Koohmaraie, 1999), mouse anti-titin (9D10, 1:250, Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), mouse anti-myosin heavy chain (MF20, 1:25, DHSB), and mouse anti-myosin heavy chain (NA4, 1:1000, a generous gift from E. Bandman, Univ. of California, Davis). The secondary antibody was alkaline phosphatase conjugated anti-mouse IgG (1:1,000, Sigma Chemical Company, St Louis, MO). Antibodies were diluted in blocking buffer and incubated for 1 h at room temperature with gentle rocking. Membranes were washed three times with TTBS after each incubation. Antibody binding was visualized by exposure to BCIP/NBT (Bio-Rad).

Results and Discussion

Effect of Calpastatin on Degradation of Myofibrillar Proteins by μ -Calpain

In accordance with the results of Taylor and Etherington (1991), incubation of myofibrils in a buffer containing Ca resulted in an immediate solubilization of C-protein and small amounts of myosin, actin, tropomyosin, troponin-T, and troponin-I. Prolonged incubation did not result in additional solubilization of protein (Figure 1A). No degradation of myofibrillar proteins was observed in the control incubations (i.e., desmin remained intact throughout the incubation period), and no increase in protein degradation products in the 28- to 32-kDa region was observed (Figure 1A). Incubation of myofibrils with μ -calpain resulted in the appearance of a large number of bands in the soluble muscle fraction after 1 d of incubation (Figure 1B). Longer incubation resulted in an accumulation of low-molecular-weight (< 20 kDa) fragments in the soluble fraction up to 7 d of incubation. No major changes were detectable between 7 and 14 d of incubation. Typical effects of the action of calpains on myofibrils such as degradation of desmin and the appearance of protein degradation products in the 28- to 32-kDa region were complete after 1 d of incubation, whereas the degradation of troponin-I was almost completed after 2 d of incubation (Figure 1B). Incubation of myofibrils with μ -calpain and calpastatin in a 1:2 or 1:4 ratio (activity basis, calpain:calpastatin;

Figures 1C and D) resulted in changes similar to those observed for incubation without calpastatin, but at a slower rate up to 7 d of incubation. Between 7 and 14 d of incubation, little additional proteolysis was noted (Figures 1C and D).

The appearance of a large number of bands in the soluble extract could not be explained by degradation or solubilization of myofibrillar proteins visible on 12.5% acrylamide gels. Two antibodies against myosin heavy chain failed to react with any of these bands on Western blots (data not shown). These bands were, therefore, likely the result of degradation of high-molecular-weight proteins, titin being a likely candidate. Western blots against titin with antibody 9D10 confirmed that incubation of myofibrils with μ -calpain results in a large number of titin degradation products (Figure 2). Degradation products of titin were not detected in the control incubations (data not shown). The epitope recognized by this antibody is obviously resistant to proteolysis by μ -calpain. These results also show that, even at a μ -calpain:calpastatin ratio of 1:4 (activity basis), proteolysis of titin is not completely inhibited.

Based on the results presented in Figures 1 and 2, one may conclude that calpastatin not only decreased the rate, but also limited the extent of proteolysis. This effect is most evident from the results presented in Figure 2. During the early stages of the incubation without calpastatin (Figure 2A), a large number of titin fragments were generated with a molecular weight above 45 kDa. These fragments were degraded to three major lower-molecular-weight products between 14 and 31 kDa during further incubation. In the presence of calpastatin (Figure 2B and C), proteolysis clearly proceeded up to 7 d of incubation, albeit slower than in the absence of calpastatin. Beyond 7 d of incubation, little additional proteolysis was observed, and the extent of proteolysis was less than that observed in the absence of calpastatin. A similar situation was observed in postmortem muscle. Elevated levels of calpastatin in muscle, as observed in callipyge lamb, or, as a result of dietary administration β -agonists, resulted in both a decreased rate and extent of postmortem proteolysis of myofibrillar and associated proteins (Koohmaraie et al., 1991a, 1995).

The inhibition of μ -calpain by calpastatin may be pH-dependent, which would allow expression of μ -calpain activity in the presence of excess calpastatin at the pH of postmortem muscle (Dransfield 1993, 1994). The inhibition of m-calpain by calpastatin was hardly or not at all affected by pH (Otsuka and Goll, 1987; Kendall et al., 1993), but the effect of pH on inhibition of μ -calpain by calpastatin has not been reported. In accordance with the results on inhibition of m-calpain by calpastatin, the inhibition of μ -calpain by calpastatin was hardly affected by pH (Figure 3). Proteolysis of myofibrillar proteins by μ -calpain under postmortem conditions in the presence of excess calpastatin, therefore, cannot be explained by an effect of pH on calpastatin.

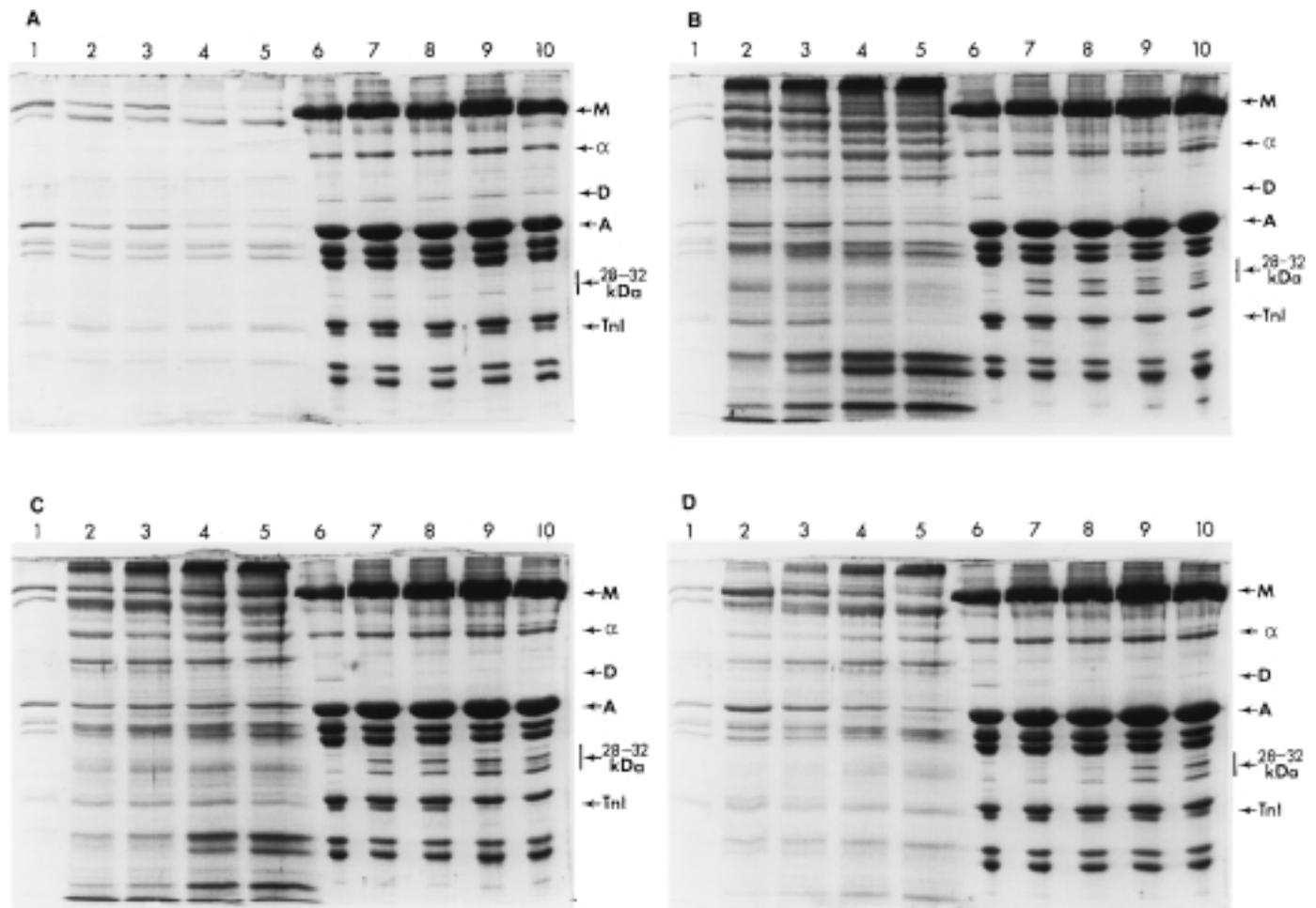


Figure 1. SDS-PAGE (12.5%) from myofibril incubations with and without μ -calpain and calpastatin at pH 5.8. (A) Incubation without μ -calpain and calpastatin. (B) Incubation with μ -calpain, but without calpastatin. (C) Incubation with μ -calpain and calpastatin in a 1:2 ratio (calpain:calpastatin). (D) Incubation with μ -calpain and calpastatin in a 1:4 ratio (calpain:calpastatin). Lanes 1 to 5 correspond to 20 μ L of the supernatant fraction after 0, 1, 2, 7, and 14 d of incubation, respectively. Lanes 6 to 10 correspond to the pellet fraction (20 μ g) after 0, 1, 2, 7, and 14 d of incubation, respectively. Abbreviations: M, myosin heavy chain; α , α -actinin; D, desmin; A, actin; Tnl, troponin-I.

Calpain and calpastatin activity is almost always assayed with casein as a substrate. However, casein is a poor substrate compared to physiological substrates of the calpains such as microtubule-associated protein 2 (Tompa et al., 1995). The effectiveness of calpastatin to inhibit degradation of casein is, therefore, likely greater than its effectiveness in inhibiting the proteolysis of physiological calpain substrates as evidenced by the degradation of titin in the presence of excess calpastatin (Figure 2). In addition, the sensitivity of the commonly used assays is not sufficient to detect the low levels of proteolytic activity that were evident after days of incubation in the present study.

Effect of Calpastatin on Autolysis of μ -Calpain

The effect of calpastatin on autolysis of μ -calpain is shown in Figure 4. The gel system did not allow for complete resolution between the 78- and 76-kDa autoly-

sis products, but conversion of the native 80-kDa subunit to the 78- to 76-kDa autolysis products is clearly visible. After 1 d of incubation, conversion of the 80-kDa subunit to the 78- to 76-kDa autolysis products was virtually complete under all conditions. Further autolysis to a 61-kDa autolysis product and lower-molecular-weight autolysis products (data not shown) were inhibited by calpastatin in a dose-dependent manner.

Stability of Autolyzed μ -Calpain

The experiments discussed above yielded some puzzling results. Incubation of μ -calpain and myofibrils in the presence of calpastatin resulted in proteolysis of myofibrillar proteins up to 7 d of incubation with little additional proteolysis between 7 and 14 d of incubation (Figures 1 and 2). Yet, the amount of μ -calpain that represents an active protease in vitro (Inomata et al.,

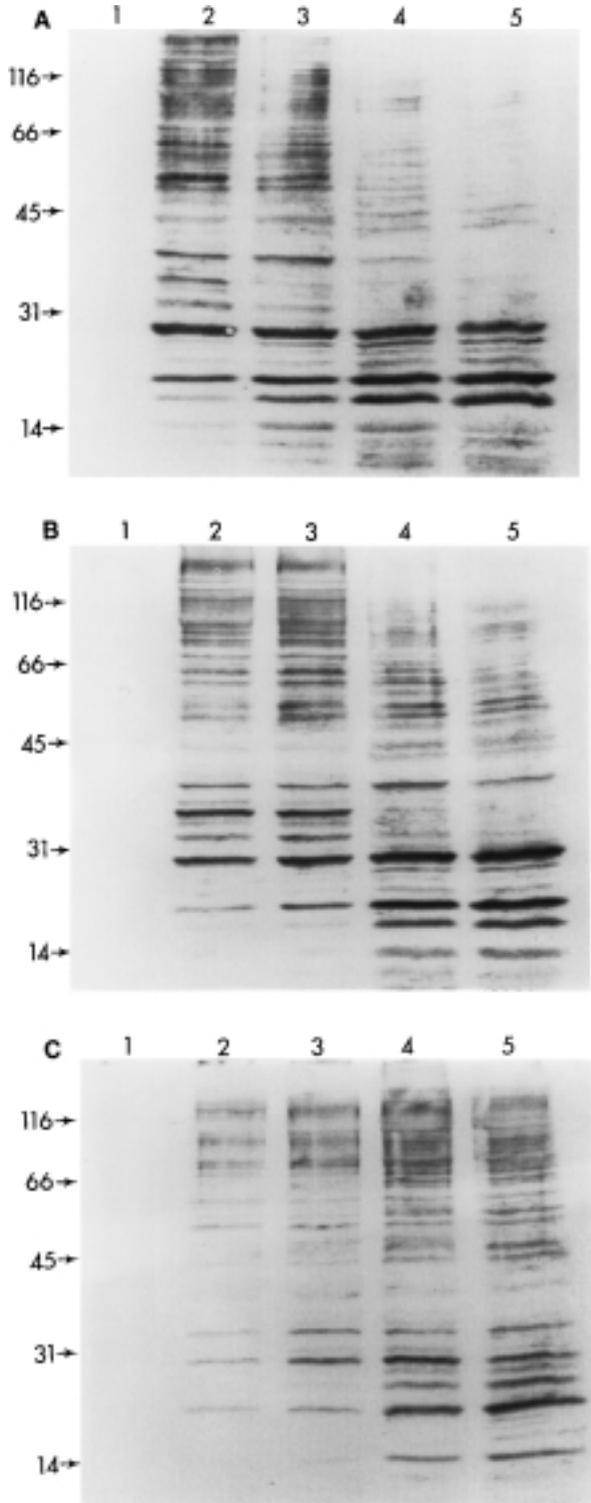


Figure 2. Western blotting (12.5% gel) of titin degradation products in 20 μ L of the supernatant fraction from incubations at pH 5.8. (A) Incubation with μ -calpain, but without calpastatin. (B) Incubation with μ -calpain and calpastatin in a 1:2 ratio (calpain:calpastatin). (C) Incubation with μ -calpain and calpastatin in a 1:4 ratio (calpain:calpastatin). Lanes 1 to 5 correspond to the supernatant fraction after 0, 1, 2, 7, and 14 d of incubation. Molecular weight markers (kDa) are indicated on the left side of the blots.

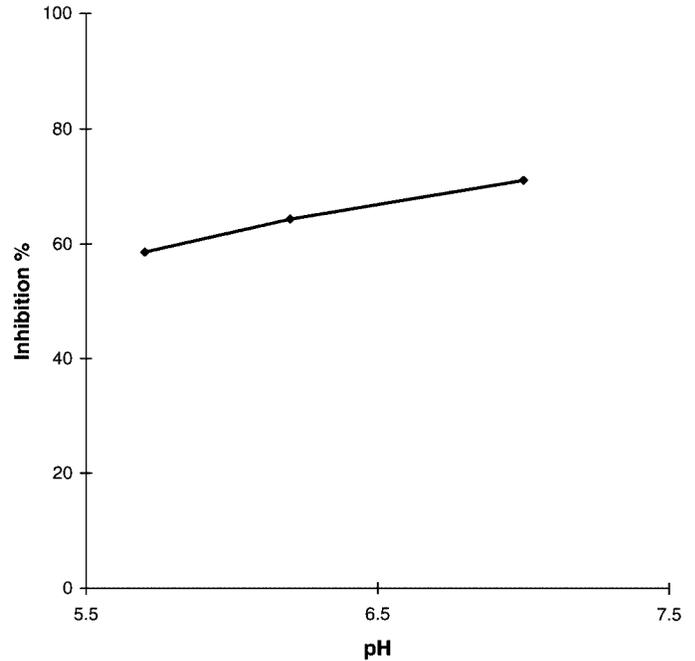


Figure 3. Effect of pH on the inhibition of μ -calpain by calpastatin. Standard deviations for pH 5.7, 6.2, and 7.0 were 1.1, .5, and 1.4, respectively ($n = 4$).

1985, 1986; i.e., a subunit of 78 to 76 kDa) remained virtually stable between 1 and 14 d of incubation (Figures 4B and 4C). This observation could not be explained by substrate limitation, because incubation in the absence of calpastatin clearly resulted in more extensive degradation of myofibrillar proteins (Figures 1 and 2). A similar phenomenon was observed in postmortem muscle. Figure 5 shows a Western blot against the large subunit of μ -calpain in the soluble muscle fraction of lamb longissimus. During 10 d of aging, little loss of immunologically detectable μ -calpain was observed. Yet, the amount of extractable μ -calpain activity decreased to approximately 10% of its at-death activity during 7 d of aging in lamb longissimus (Koochmarai et al., 1991a). The possibility remains, however, that the immunologically detectable μ -calpain is active in situ, but that the loss of activity occurs during extraction and chromatography. The available evidence on degradation of calpain substrates in situ argues against this possibility. Degradation of calpain substrates in lamb longissimus occurs relatively rapidly during the first 7 d of aging, and there is little additional degradation between 7 and 21 d of aging (Koochmarai et al., 1995). The extractable μ -calpain activity, therefore, properly reflects the activity in postmortem muscle. A previous report indicated that autolyzed m-calpain was unstable at elevated ionic strength (Brown and Crawford, 1993; Elce et al., 1997). Based on this observation, Elce et al. (1997) suggested that the main physiological importance of autolysis is to generate an active but unstable enzyme, thereby limiting the in vivo duration of calpain activity. We hypothesized that autolyzed μ -

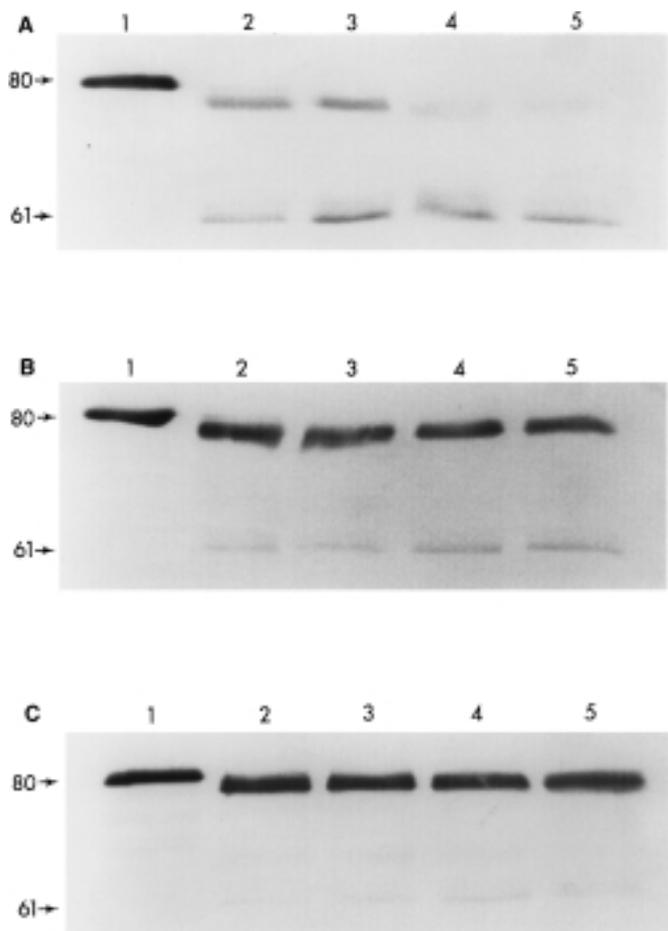


Figure 4. Western blotting (8%) gel of μ -calpain in the supernatant fraction from myofibril incubations with μ -calpain (A), μ -calpain and calpastatin in a 1:2 ratio (calpain:calpastatin) (B), and μ -calpain and calpastatin in a 1:4 ratio (calpain:calpastatin) (C) at pH 5.8. Lanes 1 to 5 correspond to 0, 1, 2, 7, and 14 d of incubation. Molecular weight markers (kDa) are indicated on the left side of the blots.

calpain also loses its activity at high ionic strength and that this loss of activity is the reason for minimal proteolysis beyond 7 d of incubation. To test this hypothesis, we incubated autolyzed μ -calpain for up to 45 min in buffers with different NaCl concentrations, after

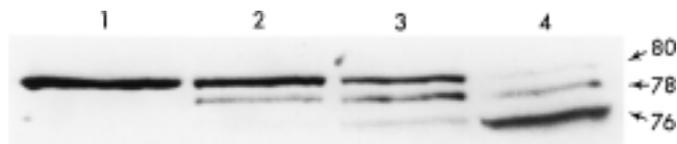


Figure 5. Western blotting (8% gel) of the large subunit of μ -calpain in the soluble muscle fraction of ovine longissimus (Dorset ewe, 3 yr of age). Lanes 1 to 4 correspond to 0, 1, 3, and 10 d of aging at 4°C. Samples were extracted according to Koohmaraie (1990), and 40 μ g of protein was loaded per lane.

which they were assayed for activity (Figure 6A). Evidently, the stability of autolyzed μ -calpain was also affected by ionic strength. At 300 mM NaCl, an ionic strength comparable to that attained in postmortem muscle (Winger and Pope, 1980–81), autolyzed μ -calpain lost 50% of its activity within 30 min of incubation, even though the amount of immunologically detectable μ -calpain remained constant during the incubations (Figure 6B). The activity of unautolyzed μ -calpain was not affected by ionic strength (data not shown). The present results, therefore, suggest that most of the loss of activity during incubation of μ -calpain with myofibrils and calpastatin occurs because of instability of autolyzed calpain, not through extensive autolysis. Loss of activity through autolysis in the presence of substrate and Ca^{2+} , but in the absence of calpastatin, seems to occur faster than loss of activity through ionic strength-induced instability of autolyzed calpain, because little of the 76- to 78-kDa subunit could be detected after 7 and 14 d of incubation (Figure 4A).

General Discussion

Postmortem tenderization of meat is a well-documented phenomenon. Although the mechanism of meat tenderization is not fully understood, it is generally accepted that proteolysis of key myofibrillar and associated proteins by μ -calpain plays an important role in meat tenderization (for review see Koohmaraie, 1992,

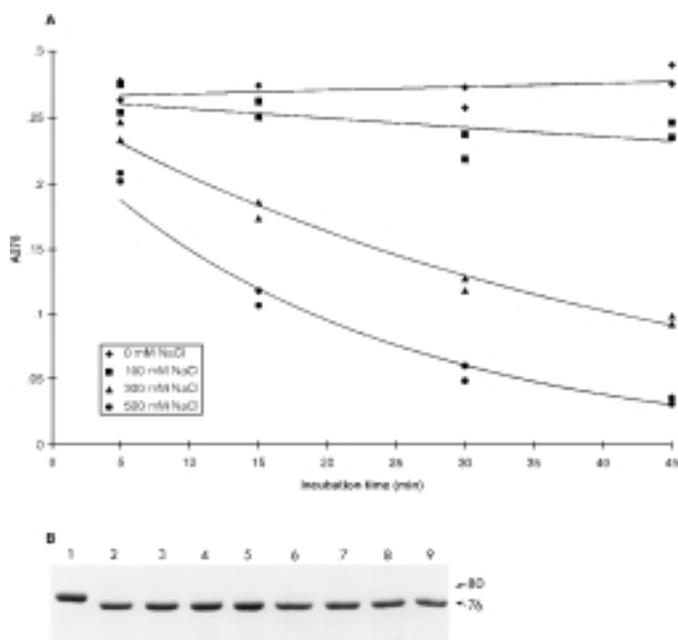


Figure 6. (A) Activity of autolyzed μ -calpain after preincubation at the indicated times and salt concentrations. (B) SDS-PAGE (8%) of the large subunit of autolyzed μ -calpain after incubation at 0 mM NaCl (lanes 2 to 5) or 500 mM NaCl (lanes 6 to 9) for 5, 15, 30, and 45 min (lanes 2 and 5, 3 and 6, 4 and 7, and 5 and 8, respectively). Lane 1 shows the large subunit of μ -calpain before autolysis.

1996). The effect of postmortem storage on the activities of the calpains and calpastatin has been extensively studied (for review see Koohmaraie, 1996). Because the activity of m-calpain remains nearly constant throughout postmortem aging, and a progressive decrease in μ -calpain is observed, Koohmaraie et al. (1987) hypothesized that μ -calpain, not m-calpain, may be involved in tenderization. This hypothesis derives from the observation that both calpains undergo autolysis in *in vitro* assays in the presence of sufficient calcium, ultimately leading to loss of activity. Furthermore, m-calpain is inactivated when sufficient calcium is present (i.e., infusion of carcasses with calcium chloride; Koohmaraie et al., 1989). Therefore, under postmortem conditions, only μ -calpain seems to be activated, and the reason for stability of m-calpain may be insufficient Ca^{2+} concentration to activate the enzyme.

Based on the available information on the involvement of the calpain system in postmortem proteolysis that results in meat tenderization, a generalized model for calpain-induced tenderization was proposed by Dransfield (1993, 1994). Two key elements in this model are that the inhibition of μ -calpain by calpastatin is pH-dependent and that the initial level of calpastatin in postmortem muscle determines the rate of tenderization, but it only marginally affects the extent of tenderization. The results of the experiments reported here indicate that calpastatin not only affects the rate but also the extent of proteolysis. Furthermore, in agreement with previous reports on m-calpain (Otsuka and Goll, 1987; Kendall et al., 1993), pH minimally affected the inhibition of μ -calpain by calpastatin.

Based on our current understanding of the available data, we propose that μ -calpain-induced degradation of key myofibrillar and associated proteins is responsible for postmortem tenderization. The extent of μ -calpain-induced proteolysis is modulated by, among other factors, calpastatin and the ionic strength-dependent instability of autolyzed μ -calpain. Apart from being affected by ionic strength, the stability of autolyzed μ -calpain is likely affected by factors such as temperature, pH, the presence of Ca^{2+} , calpastatin, and substrates. We are currently investigating the effect of these factors on the stability of autolyzed μ -calpain.

Implications

Previous results indicated that μ -calpain is probably the principal protease involved in postmortem proteolysis of key muscle proteins leading to the improvement in meat tenderness. The results of the present study indicate how postmortem conditions could influence the activity of μ -calpain and postmortem proteolysis. Inhibition of μ -calpain activity by calpastatin and instability of autolyzed μ -calpain probably limit the rate and extent of postmortem proteolysis and, as a result, meat tenderization.

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