Effect of pH and Ionic Strength on Bovine m-Calpain and Calpastatin Activity[1,2]

T. L. Kendall*,[3], M. Koohmaraie†, J. R. Arbona†, S. E. Williams*, and L. L. Young‡

*Department of Animal Science, University of Georgia, Athens 30602;
†Roman L. Hruska U.S. Meat Animal Research Center, ARS, USDA, Clay Center, NE 68933-0166;
and ‡Poultry Processing and Meat Quality Research Unit,
Richard B. Russell Agricultural Research Center, ARS, USDA, Athens, GA 30613

ABSTRACT: The effects of bovine skeletal muscle m-calpain and calpastatin on the degradation of casein and isolated bovine myofibrils were characterized under various pH values (7.0, 6.2, 5.7) and ionic strengths (32 to 400 m*M* KCl) at 25°C. Caseinolytic assays indicated that m-calpain activity increased with increasing pH ($P < .01$) but decreased with increasing ionic strength ($P < .01$). Regardless of the presence of m-calpain, SDS-PAGE of myofibrils showed increased solubilization of myofibrillar proteins as pH and ionic strength increased. However, only in the presence of m-calpain were changes normally observed during postmortem storage reproduced. Protein release attributed to m-calpain activity increased with pH, but the effects of elevated ionic strength on the ability of m-calpain to hydrolyze myofibrillar proteins were not evident from SDS-PAGE, except for the decreased troponin-T degradation by m-calpain at the higher ionic strengths. A pH × ionic strength interaction was observed for calpastatin activity determined by caseinolytic assays ($P < .01$). No changes in m-calpain inhibition were detected at pH 7.0 and 6.2 at different ionic strengths. However, at pH 5.7 the ability of calpastatin to inhibit m-calpain decreased with increasing ionic strength. No changes in m-calpain inhibition could be detected with SDS-PAGE. Based on these results, it can be concluded that although m-calpain and calpastatin activities decrease with increasing ionic strength, their activities in the presence of myofibrils were not affected by ionic strengths typically found in postmortem muscle.

Key Words: Bovine, Calpain, Protein Degradation, Myofibrils, Calpastatin

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Introduction

Postmortem tenderization is widely hypothesized to be the result of degradation and(or) weakening of the myofibrillar proteins near the Z-disks. One possible mechanism is weakening of sarcomere structure due to endogenous muscle proteinase hydrolysis (for reviews see Goll et al., 1983; Koohmaraie, 1988, 1992; Ouali, 1990). Calpain has been identified within the skeletal muscle cell and exists as two isoenzymes with different calcium sensitivities; μ-calpain requires micromolar order of Ca^{2+} and m-calpain millimolar order of Ca^{2+} for activation. Koohmaraie et al. (1986, 1988) demonstrated that changes in postmortem muscle ultrastructure parallel changes observed in calpain-treated myofibrils. To sort out calpain involvement in postmortem proteolysis, calpain activity at different pH and incubation temperatures has been investigated to evaluate proteolysis under conditions similar to those observed postmortem. Proteolysis of myofibrillar proteins diminished as pH and temperature decreased; however, calpain activity still remained sufficient to mimic the changes observed during postmortem storage (Koohmaraie et al., 1986; Zeece et al., 1986). In addition, research suggests that calpain activity can be altered by ionic strength (Tan et al., 1988; Wang and Jiang, 1991). Therefore, because intramuscular ionic strength increases as muscle undergoes rigor mortis (Winger and Pope, 1980-81), calpain activity may be affected under postmortem conditions.

[1]The technical assistance of Sue Hauver of the Roman L. Hruska U.S. Meat Animal Research Center is gratefully acknowledged.

[2]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

[3]Present address: Dept. of Anim. Sci., Univ. of Arizona, Tucson 85721.

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Few reports have dealt with examining the combined effects of ionic strength and pH on calpain and calpastatin activity (Tan et al., 1988), and, to the best of our knowledge, nobody has used myofibrils as the substrate to study this relationship. Such information would further our understanding of the involvement of calpains in postmortem proteolysis and probably also their role in tenderization. Therefore, the objective of this study was to characterize the effects of pH and ionic strength on bovine m-calpain activity and calpastatin inhibition of m-calpain.

Materials and Methods

Purification of m-Calpain and Calpastatin. Calpastatin and m-calpain were purified from bovine skeletal muscle using the procedures of Edmunds et al. (1991) and D. E. Goll (University of Arizona, personal communication) with some modification. Briefly, 6 kg of longissimus muscle from one steer at < 1 h postmortem was homogenized in a buffer (pH 7.8) that contained 20 m*M* Tris, 10 m*M* EDTA, 10 m*M* β-mercaptoethanol (**MCE**), 2 m*M* phenylmethanesulfonyl fluoride (Boehringer Mannheim, Indianapolis, IN), 100 mg/mL ovomucoid (Sigma Chemical, St. Louis, MO), and 5 μ*M* Epoxysuccinyl derivative (**E-64**) (Peptides International, Louisville, KY). After centrifugation and dialysis, the dialysate was loaded onto a DEAE-Sephacel (Pharmacia LKB Biotechnology, Piscataway, NJ) column to separate μ-calpain, m-calpain, and calpastatin (Koohmaraie, 1990). Fractions having m-calpain activity were pooled and purified using successive chromatography on phenyl sepharose (Pharmacia LKB Biotechnology), red reactive agarose (Sigma Chemical), and butyl Sepharose (Pharmacia LKB Biotechnology). Fractions from DEAE-Sephacel having calpastatin activity were pooled and purified using successive chromatography on phenyl Sepharose, Sephacryl S-300 HR (Pharmacia LKB Biotechnology) (active fractions were pooled and heated at 90°C for 10 min), DEAE-Sephacel, and phenyl Sepharose. Immediately before use, both m-calpain and calpastatin were dialyzed for 18 h against 100 m*M* Tris (pH 7.0), .5 m*M* EDTA, 1 m*M* NaN_3, and 10 m*M* MCE. The m-calpain and calpastatin preparations used in this study were examined for purity by SDS-PAGE. On a 12.5% polyacrylamide gel, m-calpain showed two bands of approximately 80,000 and 30,000 Da (Figure 1a) and calpastatin (Figure 1b) showed a single major band at 66,000 Da.

Myofibril Isolation and Preparation. Myofibrils were isolated from bovine skeletal muscle by a modification of the procedure of Goll et al. (1974). Eight grams of longissimus muscle was homogenized in 80 mL of standard salt solution (**SSS**; 20 m*M* potassium phosphate, 2 m*M* $MgCl_2$, 2 m*M* ethylene glycol tetraacetic acid (**EGTA**), 1 m*M* NaN_3, 100 m*M* KCl, pH 6.8) and centrifuged for 10 min at 1,000 × *g* at 4°C. The pellet was washed with 48 mL of SSS and centrifuged as before. The pellet was homogenized in 64 mL of SSS and strained through a household strainer before centrifugation. This step was performed twice to facilitate removal of connective tissue. The pellet was washed successively with 64 mL of SSS plus 1% Triton X-100 (twice), 64 mL of 100 m*M* KCl (six times), and 64 mL of 100 m*M* NaCl (three times). Isolated myofibrils were stored in a solution of 100 m*M* NaCl and 1 m*M* NaN_3 at 4°C until they were used ($\leq$ 24 h). The myofibrils were washed three times with 100 m*M* Tris (pH 7.0) and 1 m*M* NaN_3 to remove NaCl. Biuret protein assay was performed on the preparation to determine myofibrillar protein concentration (Gornall et al., 1949). Protein concentration was adjusted with 100 m*M* Tris (pH 7.0) and 1 m*M* NaN_3 to 8 mg/mL for the m-calpain assay and to 10 mg/mL for the calpastatin assay. Immediately before use, 500-μL aliquots were pelleted for 3 min at 2,500 × *g* and pellets were resuspended in 1 mL of incubation buffer.

Media Preparation. Media included 100 m*M* Tris (adjusted to desired pH value with 1 *N* acetic acid), 1 m*M* NaN_3, 6.25 m*M* $CaCl_2$, and 12.5 m*M* MCE. The conductivities of the media were measured using a conductivity meter (Radiometer America, Cleveland, OH) and compared to a standard curve of 0 to 400 m*M* KCl to determine molar ionic strength. Average molarity for the media was 32 m*M* KCl. Forty-milliliter aliquots of media were adjusted to treatment ionic strengths with 3 *M* KCl as determined by conductivity measurements. A final volume of 50 mL was achieved by the addition of KCl equimolar to the target ionic strength. Final conductivity and pH were adjusted if necessary. Final assay conditions were as follows: 4 mg/mL of casein (Hammerstein) or purified myofibrils (4 mg/mL in m-calpain assay; 5 mg/mL in calpastatin assay), 80 m*M* Tris, .8 m*M* NaN_3, 5 m*M* $CaCl_2$, 10 m*M* MCE, and KCl to desired ionic strength. Treatment pH values were 5.7, 6.2, and 7.0; treatment ionic strengths were equivalent to 32, 100, 150, 200, 250, 300, 350, and 400 m*M* KCl when casein was used, or equivalent to 50, 100, 200, 300, and 400 m*M* KCl when myofibrils were used. Three replicates of media were prepared. Winger and Pope (1980–81) reported that antemortem muscle has an ionic strength equivalent to 165 m*M* NaCl and postmortem muscle 275 to 295 m*M* NaCl. These values corresponded to 130 m*M* and 210 to 235 m*M* KCl, respectively, when NaCl solutions are compared with the m*M* KCl standard curve. Thus, the actual postmortem ranges of ionic strength are typified by our 100 to 300 m*M* KCl treatments.

m-Calpain Assay. Proteolytic activity of m-calpain was assayed using either casein (Hammerstein) or purified myofibrils as the substrate. The reaction was initiated by addition of .45 activity units of m-calpain

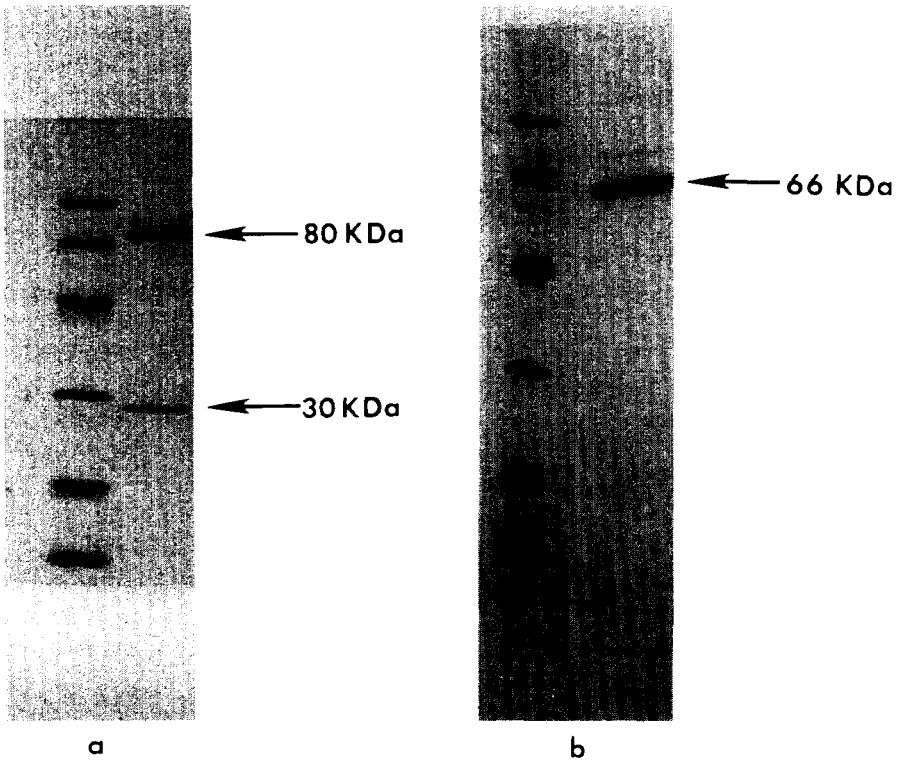

Figure 1. The SDS-PAGE of m-calpain (a) and calpastatin (b) on 12.5% polyacrylamide gels.

to 1 mL of media. One unit of enzyme is defined as the amount of m-calpain required to increase the A_{278} of the reaction supernatant by one absorbance unit in 60 min at 25°C and pH 7.5. Reactions were incubated at 25°C for 60 min and terminated by adding 1 mL of 5% trichloroacetic acid (**TCA**) when casein was used, or 100 μL of 200 m*M* EDTA when myofibrils were used. The assay tubes were then centrifuged at 2,500 × *g* for 30 min (or at 8,800 × *g* for 60 min when myofibrils were used). To determine the quantity of proteins degraded and(or) solubilized, the A_{278} of the supernatant was measured. Reactions were performed in triplicate when casein was used, or in duplicate when myofibrils were used. To assess the effects of pH and ionic strength on the substrates alone, each treatment (pH × ionic strength) was accompanied by its own control. This control tube was used as a blank for the spectrophotometric readings to measure the activity of the corresponding treatment.

Calpastatin Assay. To ensure that any change in calpastatin activity would be detected, a ratio of calpastatin to m-calpain was selected so that m-calpain activity in the presence of calpastatin was approximately 50% of m-calpain activity in the absence of calpastatin. To account for pH × ionic strength effects on substrates, each activity assay consisted of three separate 1-mL reactions in either casein or myofibrils media: m-calpain + calpastatin, m-calpain, and media alone. The m-calpain and calpastatin were preincubated together for 4 min before adding the media. All reactions were incubated for 60 min at 25°C and were stopped and centrifuged as for the m-calpain assay. The 150, 250, and 350 m*M* buffers were omitted from the calpastatin assay when casein was used. Assays were performed in triplicate when casein was used, or in duplicate when myofibrils were used. To compensate for decreases in m-calpain activity at pH 5.7 and 6.2, values are expressed as a percentage of inhibition instead of units of activity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Samples from myofibril incubations were resolved by SDS-PAGE using a vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA) according to the method of Laemmli (1970). All supernatants from the myofibril incubations were prepared for SDS-PAGE by adding 300 μL of protein denaturing buffer (62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 5% MCE, .02% bromophenol blue, and 10% glycerol) to 300 μL of supernatant, boiling for 5 min, and storing at −20°C until they were used. Pellets from the myofibril incubations were prepared similarly for SDS-PAGE using 1 mL of protein denaturing buffer. Gel electrophoresis chemicals were purchased from Bio-Rad (Chemical Division, Richmond, CA). Myofibril incubation supernatants (100 μL) and pellets (20 μL) were resolved on 7.5 to 15% gradient separating gels (acrylamide:bisacrylamide = 75:1) with 4% stacking gels (75:1). Current was applied at 10 mA per gel. Staining was performed in a solution of .1% Coomassie Blue G250, 7% acetic acid, and 50%

methanol. Gels were destained in a solution of 7% acetic acid and 20% methanol. Molecular weight standards were myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Protein Determination. The protein contents of supernatants from both the m-calpain and calpastatin myofibril incubations were determined using the Bio-Rad Protein Assay (Bio-Rad Chemical Div., Richmond, CA). Bovine serum albumin was used to prepare a standard curve for both the standard assay and the microassay procedure. The microassay procedure was used when the protein content of the sample was assessed to be < .2 mg/mL by the standard assay procedure.

Statistical Analysis. Data were analyzed by least squares fixed variable procedures using the GLM procedure of SAS (1985), followed by Tukey's test when a significant *F*-value was obtained (Steel and Torrie, 1980). The statistical model included the effects of pH, ionic strength, and pH × ionic strength interaction. A pH × ionic strength interaction was significant for all traits measured ($P < .001$).

Results

Effect of pH and Ionic Strength on m-Calpain Caseinolytic Activity and Calpastatin Inhibition

Figure 2 demonstrates that as pH declined from pH 7.0 to 5.7, m-calpain activity decreased markedly regardless of ionic strength ($P < .01$). At pH 6.2, activities decreased to 55 to 66% of the pH 7.0 values, whereas at pH 5.7, activities ranged from 10 to 17% of the pH 7.0 activities. The effect of ionic strength on m-calpain was pH-dependent. At pH 7.0, enzyme activity increased, reaching a maximum at 150 to 200 m*M* KCl (Figure 2), then activity declined at the higher ionic strengths. At pH 6.2, m-calpain activity was not significantly affected until at 250 m*M* KCl activity began to decline ($P < .01$). At pH 5.7, m-calpain activity increased ($P < .01$) to a maximum at 100 to 150 m*M* KCl, then decreased ($P < .01$) thereafter. When the ionic strength was equivalent to 400 m*M* KCl, m-calpain activity was reduced to 66, 71, and 84% of the base activity (32 m*M*) at pH 5.7, 6.2, and 7.0, respectively.

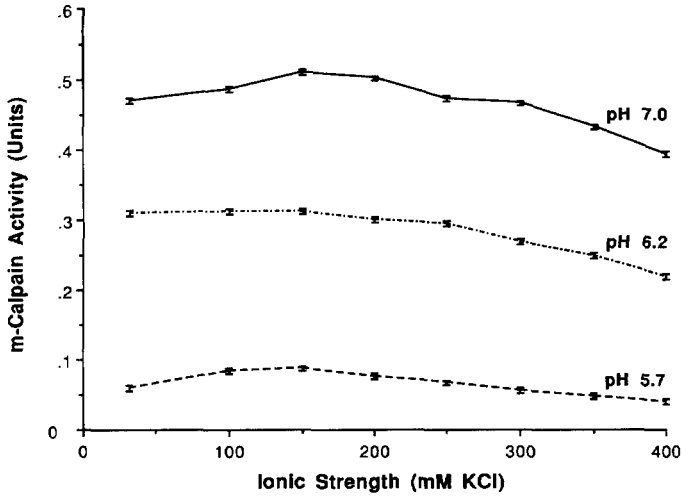

Figure 2. Effect of pH and ionic strength on m-calpain caseinolytic activity.

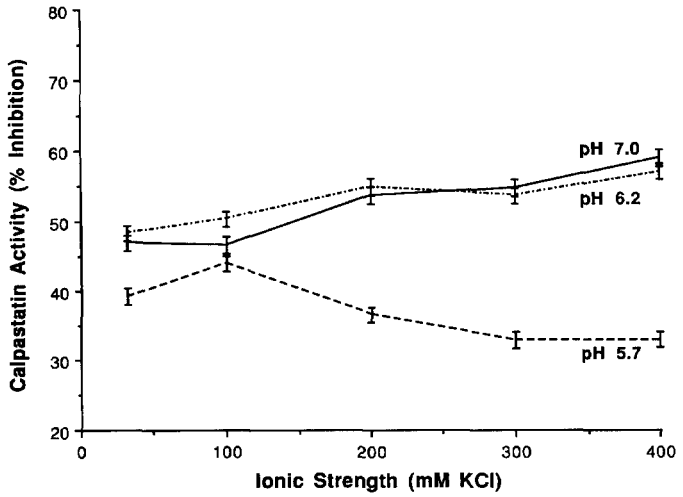

Figure 3. Effect of pH and ionic strength on calpastatin activity against m-calpain caseinolytic activity.

A pH × ionic strength interaction was observed for calpastatin activity determined by caseinolytic assays ($P < .01$). No changes in m-calpain inhibition were detected at pH 7.0 and 6.2, regardless of ionic strength (Figure 3). However, at pH 5.7 the ability of calpastatin to inhibit m-calpain activity decreased with increasing ionic strength after ionic strength equivalent to 100 m*M* KCl and reached a minimum at 300 m*M* KCl. At 200 to 400 m*M* KCl, calpastatin activity was lower at pH 5.7 than at either pH 7.0 or 6.2 ($P < .01$).

Effect of pH and Ionic Strength on m-Calpain Activity and Calpastatin Inhibition in the Presence of Isolated Myofibrils

Hydrolysis of myofibrillar proteins by m-calpain was monitored by measuring the A_{278} of the assay supernatant (Figure 4). Absorbances were used to detect protein solubilized from the myofibrils as a result of the different treatments. There were no differences in absorbances at the three pH values from 50 to 200 m*M* KCl. However, at 300 m*M* KCl, pH 7.0

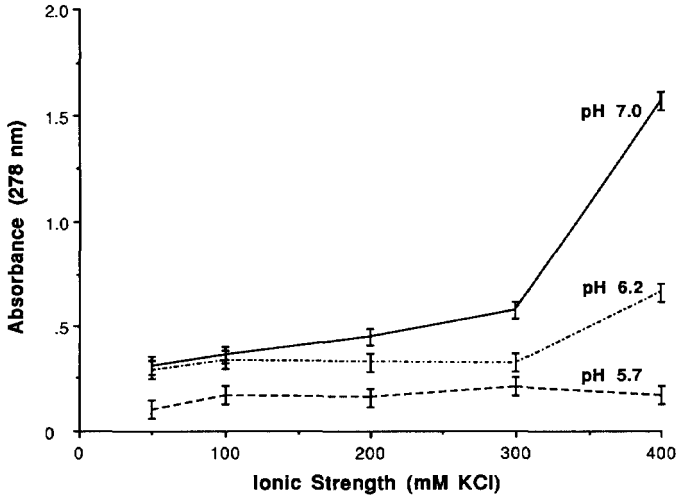

Figure 4. Least squares means and standard errors for absorbance (278 nm) of supernatant due to m-calpain activity from myofibril incubation.

exhibited a higher absorbance than either pH 5.7 or 6.2, and at 400 m*M* KCl, higher pH resulted in an increased A_{278}. Thus, as with the casein assay, there was a pH × ionic strength interaction affecting overall protein released ($P < .001$).

The amount of protein solubilized by treatment in the presence of m-calpain and by buffer alone was used to calculate protein solubilization (Figure 5). Although the pattern of protein solubilization in the presence of m-calpain seemed to increase with increasing ionic strength, the only significant increase was found at pH 5.7 and 7.0, where the amount of protein solubilized at 400 m*M* KCl vs 50 m*M* KCl increased ($P < .05$). In comparing the percentage of total protein in the supernatant due to m-calpain vs buffer effects, it becomes evident that as ionic strength increases the proportion of protein released due to m-calpain proteolysis declines. Protein solubilized in the presence of m-calpain was constant from 50 to 200 m*M*, averaging 76% of total protein solubilized at pH 5.7, 84% at pH 6.2, and 83% at pH 7.0. However, at 300 and 400 m*M* KCl, values decreased to 55, 55, and 62% of the total protein being released due to m-calpain effects at pH 5.7, 6.2, and 7.0, respectively. Thus, the direct effect of ionic strength on myofibrillar solubilization becomes more prominent at the higher ionic strengths.

A general solubilization of the myofibrillar proteins was noted in all treatments, which increased as KCl concentration increased (Figures 6a–f). Myosin, α-actinin, actin, troponin-T, tropomyosin, troponin-I, and titin appeared in the supernatant as a result of general salting-in effects due to combined pH and ionic strength effects. Protein solubilization was more pronounced as ph increased from 5.7 to 7.0. In addition, an increase in general protein solubilization, especially of actin and myosin, was observed in treatments with and without m-calpain due to increasing ionic strength. Contrary to the casein data, the decrease in m-calpain activity caused by increasing ionic strength was not easily detectable. Desmin was degraded in all calpain reactions except for pH 5.7 at 50 m*M* KCl (Figures 6d–f). The most noticeable result of increasing ionic strength was the decreased troponin-T degradation by m-calpain at the higher ionic strengths. This effect was especially apparent in the pH 7.0 and 6.2 reactions at 300 and 400 m*M* KCl (Figures 6a–f). The troponin-T doublet was absent in the supernatants at the lower ionic strengths but became increasingly visible at the higher ionic strengths.

The decrease in m-calpain activity caused by pH decline was somewhat evident. However, released titin appeared in all calpain supernatants, but in increasing amounts as pH increased; titin was essentially absent in the pH 7.0 pellet preparation (Figure 6f). Similarly, calpain-mediated troponin-T degradation increased as pH increased. However, Figures 6a–f show that much of the expected degradation was present in all calpain reactions to various degrees, suggesting that the calpain was sufficiently active to act on the most susceptible proteins despite pH. Released α-actinin is evident in supernatants at all three pH values (Figures 6a–c). Likewise, troponin-I present in pellet preparations from m-calpain-treated samples decreased at all three pH values (Figures 6d–f).

Calpastatin inhibition of m-calpain activity demonstrated no significant response to pH. Protein released as measured by absorbances was similar at all three pH values (data not shown). The SDS-PAGE of m-calpain and m-calpain plus calpastatin reactions showed no apparent effect of pH on calpastatin

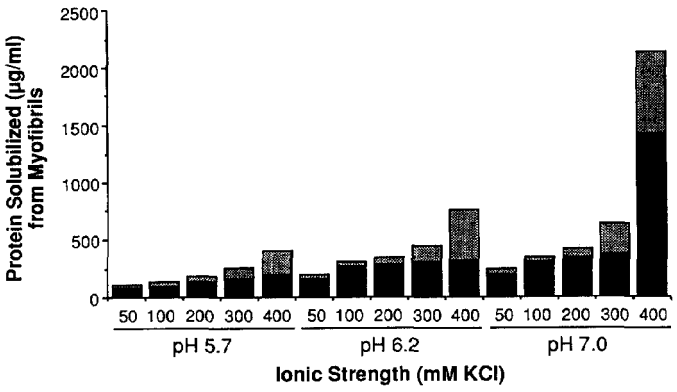

Figure 5. Least squares means of protein solubilized (μg) from myofibrils incubated with m-calpain; m-calpain activity plus buffer effect (total height); buffer effects (shaded); m-calpain effect (black).

inhibition (data not shown). Reactions at each pH exhibited similar degradation patterns with or without calpastatin. In addition, there was no detectable effect of ionic strength on calpastatin inhibitory capabilities at any of the three pH values. There were no apparent differences in SDS-PAGE of calpain reactions with and without calpastatin due to changes in the ionic strength of reaction buffers other than increased general protein solubilization due to salting-in effects.

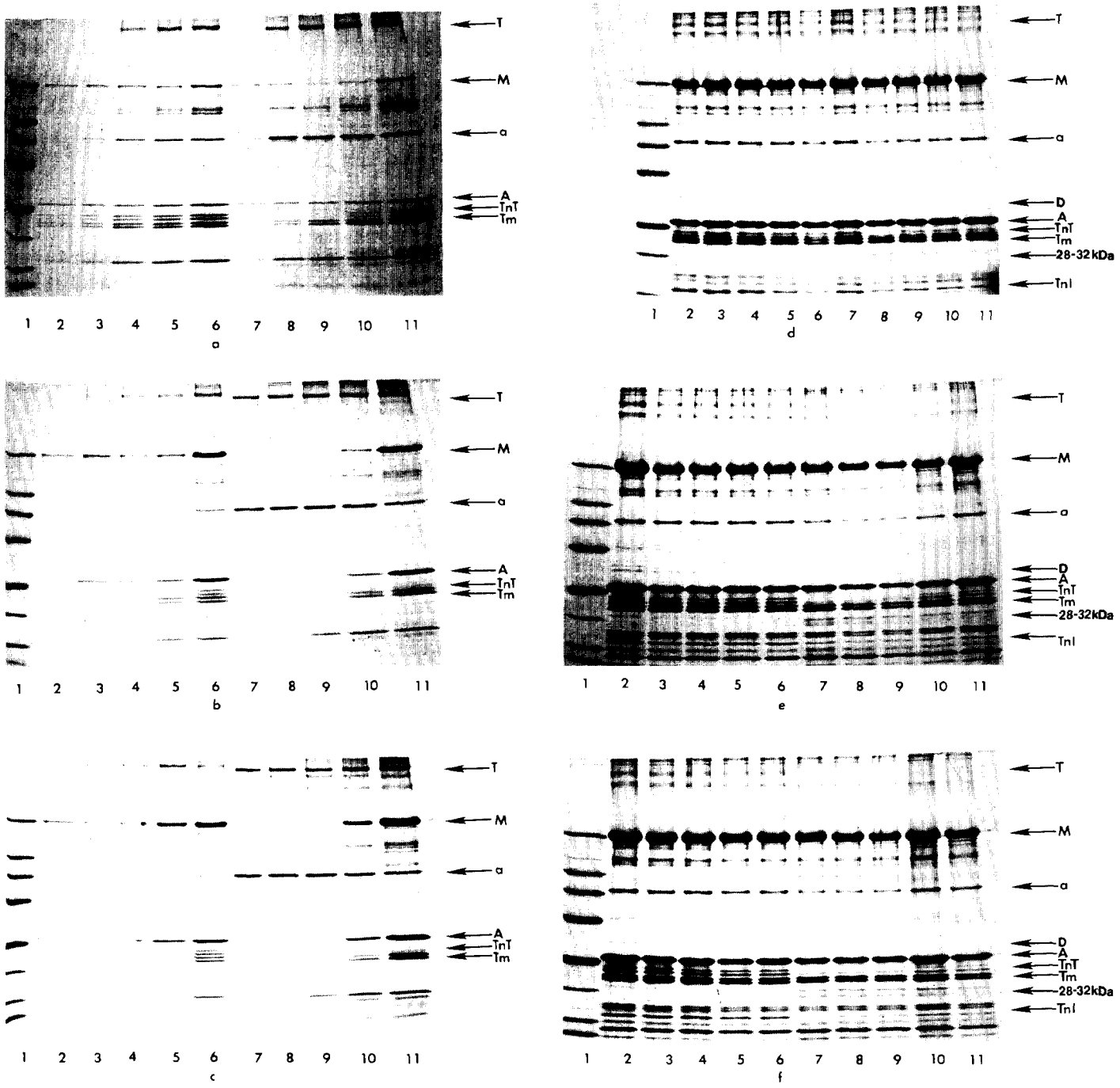

Figure 6. The SDS-PAGE from myofibril incubation with and without the presence of m-calpain. (a) Supernatant fraction at pH 5.7; (b) supernatant fraction at pH 6.2; (c) supernatant fraction at pH 7.0; (d) pellet fraction at pH 5.7; (e) pellet fraction at pH 6.2; (f) pellet fraction at pH 7.0. Lane 1 corresponds to molecular weight standards, which consisted of myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lanes 2 to 6 correspond to 50, 100, 200, 300, and 400 m*M* KCl treatments, respectively, without the presence of m-calpain; lanes 7 to 11 same treatments but with m-calpain present. Abbreviations: T, titin; M, myosin; a, α-actinin; A, actin; D, desmin; TnT, troponin T; Tm, tropomyosin; TnI, troponin I.

Discussion

It is well documented that postmortem tenderization of meat is improved significantly by storage of carcasses at refrigerated temperatures. The exact mechanism(s) of this improvement in meat tenderness remains unclear; however, it is generally accepted that proteolysis of key myofibrillar proteins plays an important role in this process (for reviews see Goll et al., 1983; Koohmaraie, 1988, 1992; Ouali, 1990). Considerable experimental evidence supports the hypothesis that the calcium-dependent proteolytic system (calpain) probably is the major proteolytic system involved in postmortem proteolysis (for reviews see Koohmaraie, 1988, 1992) and that the principal regulators of calpains in postmortem muscle include calpastatin and autolytic inactivation. In addition, infusion of whole carcasses with calcium chloride accelerates postmortem tenderization (Koohmaraie et al., 1989, 1990). The principal mechanism by which calcium chloride exerts its effect is believed to be by fully activating the calpain system (Koohmaraie, 1992). Calpains are proteinases having an absolute calcium requirement for proteolytic activity. Under normal postmortem conditions, m-calpain is remarkably stable (Vidalenc et al., 1983; Koohmaraie et al., 1987); however, it is greatly affected by $CaCl_2$ infusion (Koohmaraie et al., 1989) and injection (Koohmaraie et al., 1990). Other than $CaCl_2$ infusion and injection, there are no documented conditions that affect m-calpain so dramatically.

Because the pH optimum of m-calpain activity is approximately 7.5 (Dayton et al., 1976), most reports use this pH in determining calpain activity. However, under typical postmortem storage, a gradual decline in the pH of the tissue from 7.0 to approximately 5.6 occurs during a 24-h period. At the same time, free calcium concentration in postmortem muscle could reach 100 μM due to its release from sarcoplasmic reticulum (Nakamura, 1973; Koohmaraie, 1992). These conditions may change the capacity of the calpains to act in normal postmortem muscle. Our results show that at pH 5.7, m-calpain caseinolytic activity decreased to 10 to 17% of the activity at pH 7.0, which agrees with the findings of Reddy et al. (1975), who demonstrated that m-calpain activity between pH 5.5 and 5.9 was 15 to 25% of maximum, and with the results of Koohmaraie et al., (1986), who showed that μ-calpain at pH 5.5 was 21% of maximum.

As for the effect of ionic strength on m-calpain activity, Tan et al. (1988) using bovine cardiac muscle found that the ability of m-calpain to hydrolyze casein declined with increasing ionic strength. Also, Wang and Jiang (1991) using tilapia muscle showed that m-calpain proteolysis of casein was hindered by increased ionic strength, which was explained as possibly being due to enzyme aggregation or decreased stability of the Ca^{2+}-calpain complex. Our results indicate that m-calpain activity was essentially stable from 50 to 200 mM and then declined somewhat out to 400 mM. Additionally, the degree to which activity diminished with increases in ionic strength was dependent on pH. Activities declined at a much faster rate as pH increased. This may be a result of a pH × ionic strength interaction on the enzyme itself or a loss of casein solubility as pH decreased (pI = 4.6). Increasing the ionic strength of the casein buffers would aid in the solubilization of the casein, especially at the lower pH values. Thus, the decreased activity could be affected by casein availability at the low pH values.

A pH × ionic strength interaction was observed for calpastatin activity determined by caseinolytic assays ($P < .01$). Inhibition of m-calpain by calpastatin was not affected by pH or by ionic strength alone; it was only when the pH was lowered to 5.7 and ionic strength increased over the equivalent of 200 mM KCl that a decrease in calpastatin activity was observed. These results indicate that calpastatin has a broad pH optimum and agree with the results of Otsuka and Goll (1987), who found that the ability of calpastatin to inhibit calpains does not change in the pH range of 6.2 to 8.6 at low ionic strength (100 mM KCl). A decrease in m-calpain activity observed with decreased pH and increased ionic strength was also observed in the presence of calpastatin. It seems that the optimum pH and ionic strength for m-calpain activity were unaffected by the presence of calpastatin.

In the presence of isolated myofibrils, rather than casein, the effect of decreased pH on m-calpain activity was less evident as revealed by SDS-PAGE. Koohmaraie et al. (1986) demonstrated that μ-calpain retained a higher percentage of activity at lower pH values with myofibrils as the substrate rather than casein media, suggesting that the calpains are more active in the presence of myofibrils than in the presence of casein. General protein solubilization and m-calpain-induced proteolysis increased as pH and ionic strength increased. Despite the decreased m-calpain activity in the casein media due to treatment, there seemed to be a synergistic relationship between ionic strength effects on the myofibrils and the action of m-calpain, which resulted in a significant solubilization or degradation of myofibrillar proteins. Based on SDS-PAGE of incubated myofibrils it seems that m-calpain is not significantly affected by a change in ionic strength over the range normally observed in postmortem muscle (100 to 300 mM KCl). Therefore, postmortem ionic strength does not seem to be a significant factor affecting m-calpain activity. Myofibrillar proteolysis was observed in the presence of calpastatin. In an attempt to detect a possible increase in calpastatin activity due to increased ionic strength with myofibrils as substrate, the calpastatin:m-calpain ratio was selected to yield a 50% reduction in m-

calpain activity. Because m-calpain inhibition was only partial, sufficient enzyme remained active to bring about all the changes observed when myofibrils were incubated with m-calpain in the absence of calpastatin. In particular, desmin was absent in all pellet preparations. Any increase in calpastatin activity due to increased ionic strength was not of sufficient magnitude to decrease m-calpain activity significantly, nor was a decrease in calpastatin activity detectable under these conditions.

During postmortem storage of carcasses, numerous changes occur in skeletal muscle that result in loss of structural integrity of this tissue (for review see Koohmaraie, 1992). Clearly, m-calpain can reproduce most of the changes observed during postmortem storage (Figures 6d-f): 1) disappearance of troponin-T and simultaneous appearance of polypeptides with molecular weights of 28 to 32 kDa; 2) degradation of desmin; 3) degradation and(or) release of titin; 4) removal of α-actinin; and 5) no detectable effect on myosin and actin. However, the effects of pH and ionic strength on the substrates alone did not reproduce all these changes. Desmin degradation is considered the best indicator of the degree of structural integrity of myofibrils at the Z-disk level and the subsequent increase in meat tenderness. Desmin was absent in pellet preparations from m-calpain-treated samples, except for pH 5.7 at 50 m*M* KCl. However, desmin is visible in all pellet preparations from controls, even at the lower pH and higher ionic strength. Therefore, although general solubilization of myofibrillar proteins occurred in the presence of high ionic strength, this alone could not be responsible for the myofibrillar changes observed during normal postmortem storage. Conversely, m-calpain has been demonstrated to hydrolyze the same proteins that μ-calpain hydrolyzed (Koohmaraie et al., 1986; Whipple and Koohmaraie, 1991), and postmortem free calcium concentrations are sufficient to activate μ-calpain. Therefore, comparison of these results with the SDS-PAGE of myofibrils extracted from postmortem muscle (Koohmaraie et al., 1984a,b,c) and myofibrils treated with μ-calpain (Koohmaraie et al., 1986) strongly suggests that calpains could be responsible for the changes observed during postmortem storage. Also, the postmortem tenderization effect observed with infusion of carcasses with $CaCl_2$ (Koohmaraie et al., 1989, 1990) is probably due to the activation of the calpain system and not due to an elevation of ionic strength.

Implications

The results of these in vitro studies with m-calpain and calpastatin indicated that both the protease and inhibitor retained at least partial activity within a range of pH and ionic strengths, including conditions similar to those present in muscle cells postmortem. Thus, both m-calpain and calpastatin may be active in influencing the loss of structural integrity of the myofibrils in postmortem muscle even at elevated ionic strengths and decreased pH.

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