Ionic strength-induced inactivation of μ-calpain in postmortem muscle[1,2]

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ABSTRACT: The present study was conducted to study the stability of autolyzed μ-calpain activity and determine whether measurement of μ-calpain activity after anion exchange chromatography accurately reflects its activity in postmortem muscle. Ionic strength and pH affected the stability of partially autolyzed μ-calpain. Complete loss of activity was observed as a result of binding of autolyzed μ-calpain to DEAE-Sephacel when the large subunit of μ-calpain was autolyzed from 80 to 76 kDa. Therefore, determination of μ-calpain by standard anion exchange chromatography may underestimate μ-calpain activity in postmortem muscle. The activity of autolyzed μ-calpain was stabilized by inclusion of glycerol in the buffers, and this permitted us to investigate whether the apparent loss of μ-calpain activity in postmortem muscle is an artifact of the methodology. Despite the inclusion of glycerol in the buffers, a decrease in μ-calpain activity was observed during postmortem storage of muscle, even though the autolyzed enzyme was readily detectable by Western blotting in muscle extracts and column eluates. This result indicates that instability of autolyzed μ-calpain is a major cause for the decline in μ-calpain activity in postmortem muscle.

Key Words: Calpain, Inactivation, Ions, Postmortem Changes, Proteinases, Proteolysis

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Introduction

The calcium-dependent protease μ-calpain has been implicated as a major cause of postmortem tenderization (for review see Koohmaraie, 1996). Upon activation by calcium, μ-calpain not only degrades other proteins, but also autolyzes. Autolysis initially reduces the mass of the 80-kDa subunit to 76 kDa, through a 78-kDa intermediate, and the mass of the 28-kDa subunit to 18 kDa. This partial autolysis reduces the calcium requirement for proteolytic activity but does not affect the specific activity. Further autolysis leads to more extensive degradation of the large subunit and loss of activity (Inomata et al., 1985, 1986; Edmunds et al., 1991; Koohmaraie, 1992). To quantify μ-calpain activity in postmortem muscle, it has to be separated from calpastatin, its endogenous inhibitor, and m-calpain. A common separation technique is anion exchange chromatography (Koohmaraie, 1990). However, anion exchange chromatography of autolyzed calpain results in a great (Edmunds et al., 1991), or complete (Suzuki et al., 1981a,b; Koohmaraie et al., 1989), loss of activity. This loss of activity is likely due to instability of autolyzed calpain at an elevated ionic strength (Brown and Crawford, 1993; Elce et al., 1997). It was recently reported that a relatively large amount of μ-calpain can be immunologically detected in its autolyzed 78- and 76-kDa form in postmortem muscle extracts at a time postmortem when little μ-calpain activity can be detected after chromatography (Boehm et al., 1998; Geesink and Koohmaraie, 1999b). Furthermore, it has been suggested that instability of autolyzed μ-calpain may account for the loss of its activity in postmortem muscle (Geesink and Koohmaraie, 1999a). Therefore, our objective was to study the stability of autolyzed μ-calpain and determine whether measurement of μ-calpain activity after anion exchange chromatography accurately reflects its activity in postmortem muscle.

Material and Methods

Animals

The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in this study.

[1]Mention 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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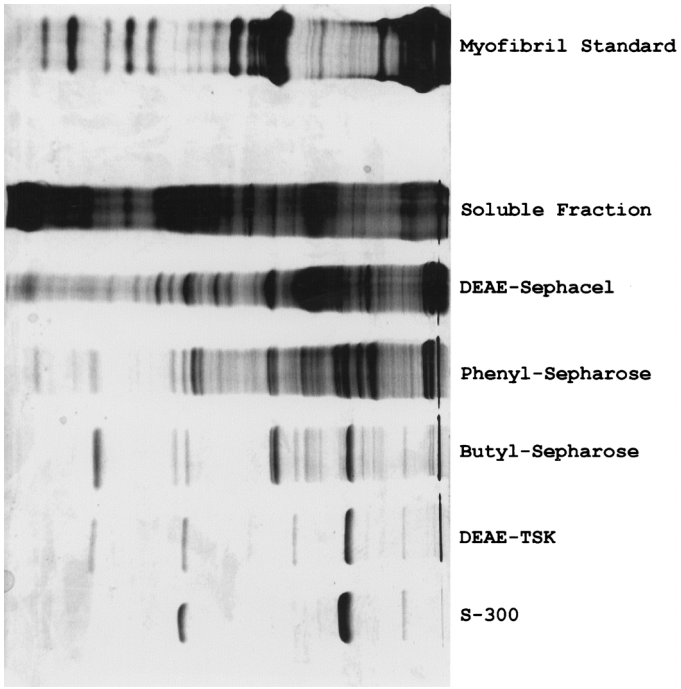

Figure 1. Purification of μ-calpain from porcine spleen. First to seventh lanes: Soluble extract, DEAE-Sephacel, phenyl-Sepharose, butyl-Sepharose, DEAE-TSK, S-300. Proteins were separated on a 12.5% gel and stained with Coomassie blue R-250. Amount of protein loaded was 30, 25, 15, 10, 3, and 3 μg for second to seventh lanes, respectfully.

Purification of μ-Calpain

μ-Calpain was purified from porcine spleen according to the procedure described by Edmunds et al. (1991) with minor modifications. Spleen is an abundant and inexpensive source of μ-calpain (Koohmaraie, unpublished data). Briefly, spleen was trimmed of visible fat within 30 min postmortem and homogenized in 3 volumes of extraction buffer (100 m*M* Tris/HCl, pH 7.5, 10 m*M* EDTA, 0.05% 2-mercaptoethanol [**MCE**], 100 mg/L ovomucoid, 2 m*M* phenylmethylsulfonyl fluoride [**PMSF**], and 6 mg/L leupeptin). After centrifugation, μ-calpain was purified from the supernate (Figure 1) using successive chromatography over DEAE-Sephacel (Amersham Pharmacia Biotech, Piscataway, NJ), phenyl-Sepharose (Amersham Pharmacia Biotech), butyl-Sepharose (Amersham Pharmacia Biotech), DEAE-TSK (Supelco, Bellefonte, PA), and Sephacryl S-300 (Amersham Pharmacia Biotech). The purified calpain (28 U/mL, 0.56 mg/mL) was stored in 40 m*M* Tris/HCl, pH 7.35, 0.5 m*M* EDTA, and 0.5% MCE (**TEM**). Calpain activity was determined according to Koohmaraie (1990).

Preparation of Autolyzed μ-Calpain and Activity Assays

Purified μ-calpain (28 U/mL, 0.56 mg/mL) was autolyzed for 2 min at 25°C by addition of 5 m*M* $CaCl_2$ (from a 100 m*M* stock solution). Autolysis was stopped by addition of 20 m*M* EDTA (from a 200-m*M* stock solution). This treatment resulted in autolyzed μ-calpain with its large subunit predominantly in the 76-kDa form. Autolysis was performed immediately before the start of each experiment, and SDS-PAGE was used to verify that autolysis was effectively stopped during the respective incubations. Remaining activity of autolyzed μ-calpain was assayed in duplicate according to Koohmaraie (1990) by adding 35 to 200 μL of autolyzed μ-calpain to 2 mL of assay media (50 m*M* Tris/HCl, pH 7.5, 3.5 mg/mL casein, 5 m*M* $CaCl_2$, 0.4% MCE, and 0.5 m*M* NaN_3).

Effect of Salt Concentration on the Stability of Autolyzed μ-Calpain

Autolyzed calpain was mixed with an equal volume of TEM with 0, 100, 200, 300, 400, or 600 m*M* NaCl and incubated for 5, 15, 30, 60, or 120 min at 25°C. Remaining activity was determined after the respective incubation periods.

Effect of pH on the Stability of Autolyzed μ-Calpain

Experiment 1. Autolyzed μ-calpain was mixed with 10 volumes of buffer (100 m*M* Mes/NaOH and 333 m*M* NaCl, pH 5.7, 6.2, or 7.0), and incubated for 5, 15, 30, 45, or 60 min at 25°C. Remaining activity was determined after the respective incubation periods.

Experiment 2. Autolyzed μ-calpain was mixed with 10 volumes of buffer (100 m*M* Mes/NaOH and 333 m*M* NaCl, pH 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, or 7.6) and incubated for 30 min at 25°C. Remaining activity was determined after the incubation period. The pH

of the assay media was only slightly affected by the pH of the added incubation buffer and ranged from 7.3 to 7.5.

Effect of Glycerol on μ-Calpain Activity and the Stability of Autolyzed μ-Calpain

Experiment 1. μ-Calpain activity was determined according to Koohmaraie (1990) in the presence of up to 40% glycerol in the assay mixture.

Experiment 2. Autolyzed μ-calpain was mixed with an equal volume of TEM containing 300 m*M* NaCl and 0, 10, 20, 30, 40, 50, 60, 70, or 80% glycerol and incubated for up to 42 h at 25°C. Remaining activity was assayed after the incubation periods.

Experiment 3. Autolyzed μ-calpain was mixed with an equal volume of TEM containing 300 m*M* NaCl and 50% glycerol and incubated for up to 62 h at 4°C. Remaining activity was assayed after the incubation periods.

Stability of Autolyzed μ-Calpain on DEAE-Sephacel in the Presence of Glycerol

Autolyzed μ-calpain (2.3 U) was mixed with an equal volume of TEM with or without 50% glycerol and loaded on a 1-mL DEAE-Sephacel column at 4°C. Calpain was left on the column for up to 3 d and eluted with 8 column volumes (2-mL fraction volume) TEM with 200 m*M* NaCl in the presence or absence of 25% glycerol.

Determination of Calpain and Calpastatin Activities in Postmortem Muscle Using Anion Exchange Chromatography in the Presence or Absence of Glycerol

Experiment 1. After slaughter and dressing (within 30 min postmortem), porcine longissimus muscle was removed from the left side of a carcass for determination of calpain and calpastatin activities (d 0). At 24 h postmortem, the longissimus muscle was removed from the right side of the carcass, and calpain and calpastatin activities were determined immediately (d 1) and after 3 d of postmortem storage at 4°C (d 3).

Extracts were prepared from 100 g of muscle. At-death muscles, trimmed of visible fat and connective tissue, were homogenized in 3 volumes of prerigor extraction buffer (50 m*M* Tris/HCl, pH 8.3, 10 m*M* EDTA, 0.05% [vol/vol] MCE, 100 mg/L ovomucoid, 2 m*M* PMSF, and 6 mg/L leupeptin, 4°C) with or without 25% (vol/vol) glycerol using a Waring Blender (two times at high speed for 20 s, with a 30-s cooling period between bursts). The homogenate was centrifuged at 30,000 × *g* for 1 h at 4°C. The supernate was filtered over glass wool and dialyzed overnight against dialysis buffer (40 m*M* Tris/HCl, pH 7.35, 5 m*M* EDTA, and 0.05% [vol/vol] MCE) with or without 25% (vol/vol) glycerol. Extracts were prepared similarly from postrigor samples, with the difference that postrigor extraction buffer (100 m*M* Tris/HCl, pH 8.3, 10 m*M* EDTA, 0.05% [vol/vol] MCE, 100 mg/L ovomucoid, 2 m*M* PMSF, and 6 mg/L leupeptin, 4°C) with or without 25% glycerol was used. After dialysis, the extracts were clarified by centrifugation at 30,000 × *g* for 1 h at 4°C and loaded on a 2.5- × 40-cm DEAE Sephacel column (Amersham Pharmacia Biotech). Calpains and calpastatin were eluted and their activities were determined according to Koohmaraie (1990). Briefly, the columns were washed with 5 volumes of elution buffer (40 m*M* Tris/HCl, pH 7.35, 0.5 m*M* EDTA, and 0.05% [vol/vol] MCE) with or without 25% (vol/vol) glycerol. The bound proteins were then eluted with a continuous gradient from 0 to 400 m*M* NaCl in 800 mL of elution buffer with or without 25% (vol/vol) glycerol at 60 mL/h, and 140 fractions (5.7 mL each) were collected.

Experiment 2. Triceps brachii muscle from four pork carcasses was excised at 1 d postmortem and stored for 6 d at 4°C. Preparation of the muscle extracts and chromatography with or without glycerol was performed as described for Exp. 1 with the following differences: 25 g of muscle and 1.5- × 30-cm columns were used, the total elution volume was 500 mL, and 3-mL fractions were collected.

SDS-PAGE and Immunoblotting

Electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) on .75-m*M*-thick 12.5% or 8% acrylamide (37.5:1) separating gels with 4% acrylamide (37.5:1) stacking gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked with 5% nonfat, dry milk in Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 (**TTBS**) for 1 h. The primary anti-μ-calpain monoclonal antibody was produced according to standard protocols (Harlow and Lane, 1988) against the gel-purified 80-kDa subunit of bovine skeletal muscle μ-calpain and was characterized by Geesink and Koohmaraie (1999b). The secondary antibody was alkaline phosphatase conjugated anti-mouse IgG (Sigma, St. Louis, MO). Antibodies were diluted in blocking buffer and incubated with the blots 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 Laboratories, Hercules, CA).

Results and Discussion

In agreement with the results of Elce et al. (1997) for m-calpain, the stability of autolyzed μ-calpain in the absence of Ca^{2+} was dependent on ionic strength, and not, as suggested by Dransfield (1998), an effect of EDTA on autolyzed μ-calpain. In accordance with our earlier results (Geesink and Koohmaraie, 1999a), the activity of autolyzed μ-calpain decreased (Figure 2) as the ionic strength was increased, despite its apparent stability as determined by SDS-PAGE (data not shown). Sodium acetate (0 to 1 *M*) and ammonium sulfate (0 to

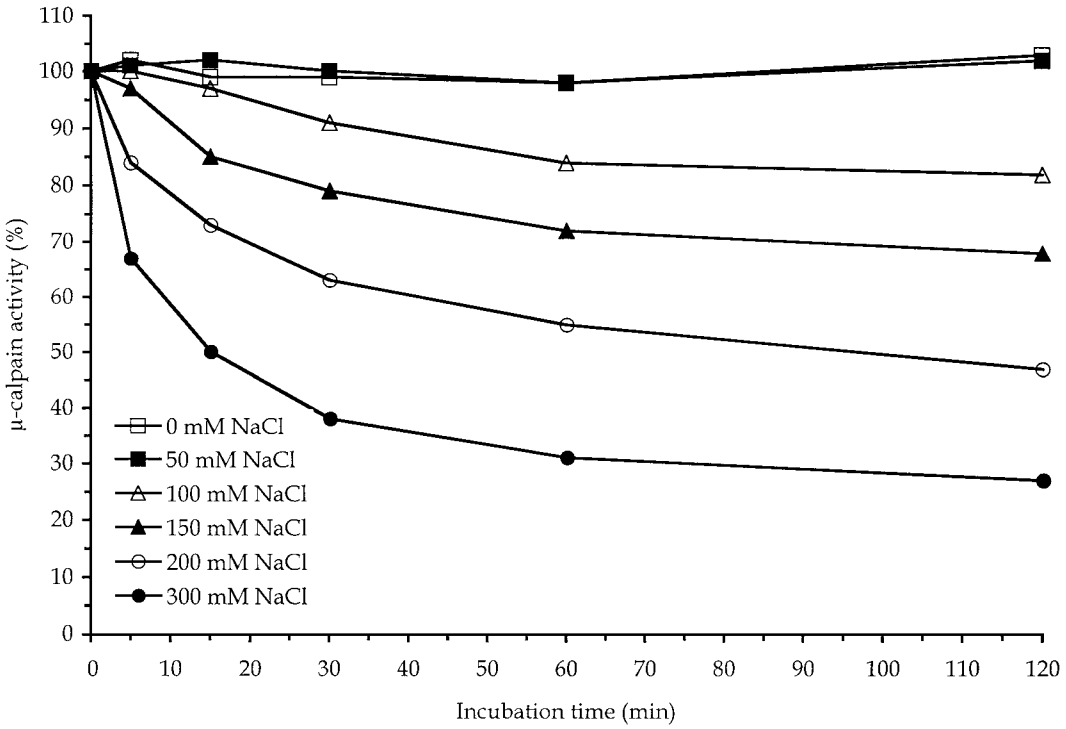

Figure 2. Effect of salt concentration (m*M* NaCl) on the stability of autolyzed μ-calpain at 25°C (SEM = 1.74).

300 m*M*) had a similar effect, indicating that the loss of activity was solely dependent on ionic strength, and not on a specific effect of sodium chloride (data not shown). Because the activity assays were performed at low ionic strength, it is evident that the loss of activity was not readily reversible by reducing the ionic strength. Removal of salt by dialysis also did not result in recovery of activity of inactivated autolyzed μ-calpain (data not shown).

The pH of postmortem muscle ranges between 5.5 and 7, and the ionic strength in postmortem muscle is equivalent to 0.2 to 0.3 *M* NaCl (Winger and Pope, 1980-81). We were, therefore, interested to know whether pH values in this range would affect the stability of autolyzed μ-calpain. Surprisingly, the activity of autolyzed μ-calpain was more stable at pH 6.2 than at pH 7.0 (Figure 3). This result prompted us to determine the optimum pH for stability of autolyzed μ-calpain. The stability was strongly pH-dependent, with an optimum pH between 6.2 and 6.4 (Figure 4). However, even in this pH range, autolyzed μ-calpain lost 30% of its activity within 30 min under the incubation conditions.

Glycerol is commonly used to stabilize proteins, and the presence of glycerol in buffers does not interfere with anion exchange chromatography (Scopes, 1988). We therefore decided to test whether glycerol can stabilize autolyzed μ-calpain. Preliminary experiments indicated that glycerol did stabilize autolyzed μ-calpain for several hours. Our standard calpain activity determination procedure alone takes 2 to 3 d (extraction, dialysis, chromatography, and assays), and the maximum salt concentration to which μ-calpain is exposed is about 150 m*M*. This means that, for our purposes, glycerol must stabilize autolyzed μ-calpain for several days at an ionic strength equivalent to 150 m*M* NaCl. The minimal concentration of glycerol to achieve maximum stabilization of autolyzed μ-calpain was 20% (Figure 5). Addition of 25% glycerol to the buffer stabilized autolyzed μ-calpain to the extent that over 90% of its activity remained after 40 h of incubation at 4°C (Figure 6). A drawback of the inclusion of glycerol in the buffers is that the viscosity increased and, therefore, the pressure over the chromatography columns increased, and that

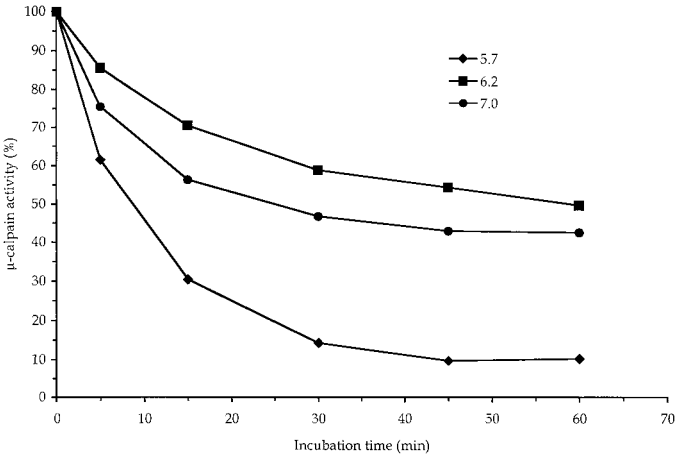

Figure 3. Stability of autolyzed μ-calpain at pH 5.7, 6.2, and 7.0 in the presence of 300 m*M* NaCl at 25°C (SEM = 1.45).

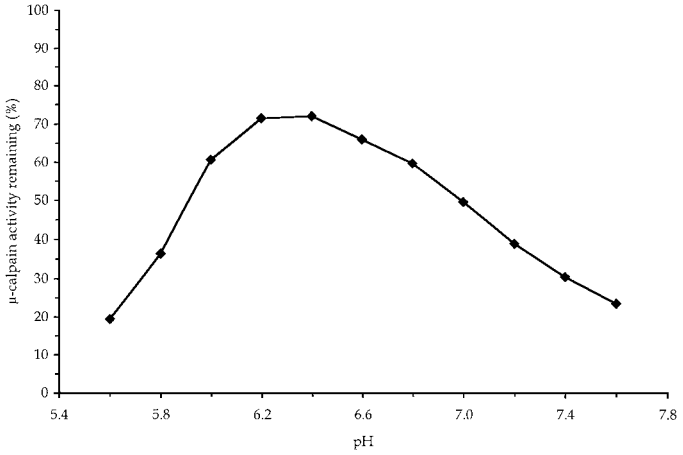

Figure 4. pH effect on the stability of autolyzed calpain in the presence of 300 m*M* NaCl during 30-min incubation at 25°C (SEM = 1.42).

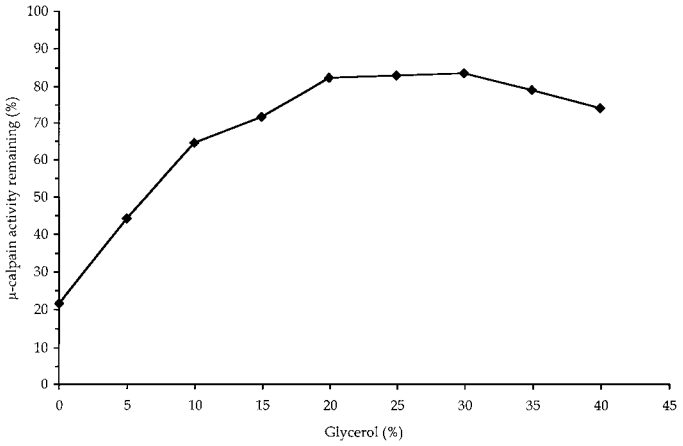

Figure 5. Effect of glycerol on the stability of autolyzed μ-calpain during 42 h of incubation at 25°C in the presence of 150 m*M* NaCl (SEM = 1.29).

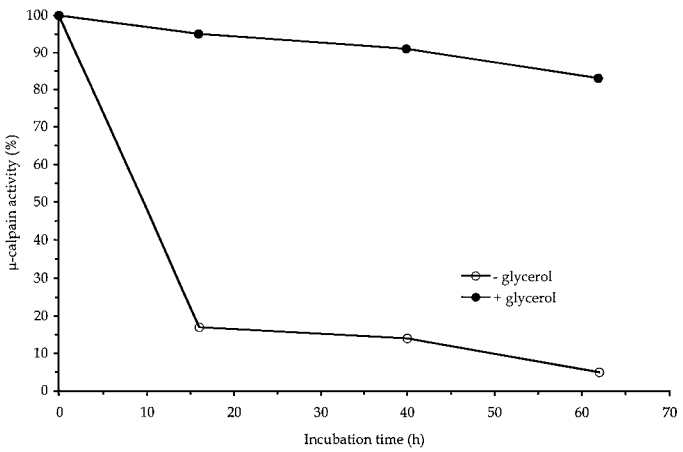

Figure 6. Stability of autolyzed μ-calpain in the presence of 150 m*M* NaCl with or without 25% glycerol at 4°C (SEM = 2.28).

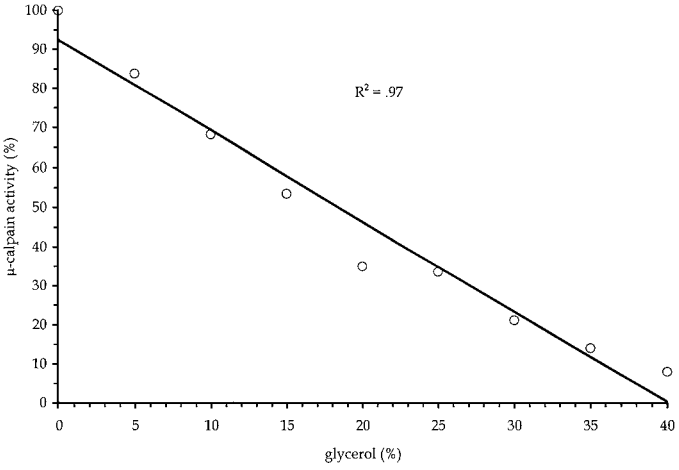

Figure 7. Effect of glycerol concentration on μ-calpain activity (SEM = .84).

it depressed calpain activity in the assays (Figure 7). On the basis of these results, we included 25% glycerol in the buffers used to test the stability of autolyzed μ-calpain on DEAE-Sephacel columns.

Binding of autolyzed μ-calpain to DEAE-Sephacel apparently also had a destabilizing effect. Although the autolyzed calpain was exposed to 200 m*M* NaCl for only approximately 30 min, chromatography of autolyzed calpain in the absence of glycerol resulted in complete loss of activity. In the presence of 25% glycerol, a 40% loss of activity was observed when the columns were eluted directly. The activity loss increased to approximately 60% when the columns were eluted 3 d later (Figure 8).

The stabilizing effect of glycerol on autolyzed μ-calpain permitted us to investigate whether the apparent loss of μ-calpain activity in postmortem muscle occurs mainly in the tissue or is an artifact of extraction and

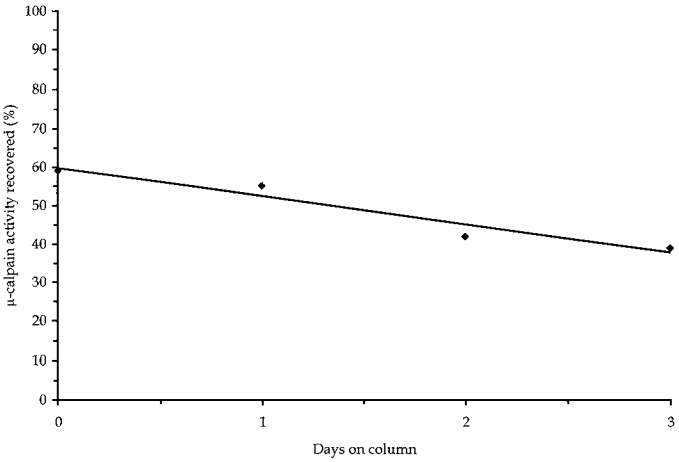

Figure 8. Stability of autolyzed μ-calpain bound to DEAE-Sephacel in the presence of 25% glycerol at 4°C (SEM = .84).

chromatography. Extraction of the muscles with or without glycerol yielded similar quantities of intact and autolyzed μ-calpain, as judged by Western blotting (Figure 9). At 1 d postmortem, μ-calpain activity in the samples extracted with and without glycerol had declined to approximately 75% of the at-death activity (Figure 10), whereas clearly more than 25% of the μ-calpain was autolyzed at this time postmortem (Figure 9). This result seems to contradict the results discussed previously. However, the results of the in vitro experiments discussed previously pertain to μ-calpain with its large subunit autolyzed to 76 kDa. At 1 d postmortem, the autolyzed μ-calpain was mostly in its 78-kDa form (Figure 9). Comparison of the Western blots and the activity data suggests that autolyzed calpain with its large subunit in the 78-kDa form represents an active protease and loses little or none of its activity as a result of chromatography. At 3 d postmortem, most of the μ-calpain was autolyzed to its 76-kDa form (Figure 9). The μ-calpain activity in the sample extracted without glycerol had declined to 6% of its at-death activity, whereas 28% of its activity remained when the samples were processed in the presence of glycerol (Figure 10). Taking into account that even in the presence of glycerol autolyzed μ-calpain loses some of its activity during chromatography (Figure 8), this result could indicate that a relatively large amount of autolyzed μ-calpain is still active in situ at 3 d postmortem. To determine whether the decline in μ-calpain activity in postmortem muscle is indeed significantly slower than observed using standard methodology (Koohmaraie, 1990), porcine triceps brachii muscle was processed at 7 d postmortem with or without glycerol. At this time postmortem, little or no activity was expected using standard procedures. Detecting a significant amount of μ-calpain activity in the presence of glycerol would indicate that autolyzed μ-calpain retains part of its activity after 7 d in situ and that the observed loss of activity in postmortem muscle is partially an artifact of extraction and chromatography. The means (standard deviations) for m-calpain activities were 1.1 (0.1) without glycerol and 0.8 (0.1) with glycerol (12.5% in the assay). The difference between the two methods can be explained by the effect of glycerol on calpain activity (Figure 7). Although the autolyzed μ-calpain eluted at its expected location in the gradient as verified by Western blotting (data not shown), no μ-calpain activity was detected regardless of the addition of glycerol. This result indicates that the loss of μ-calpain activity largely occurred in situ, and was only to a limited extent an artifact of the methodology.

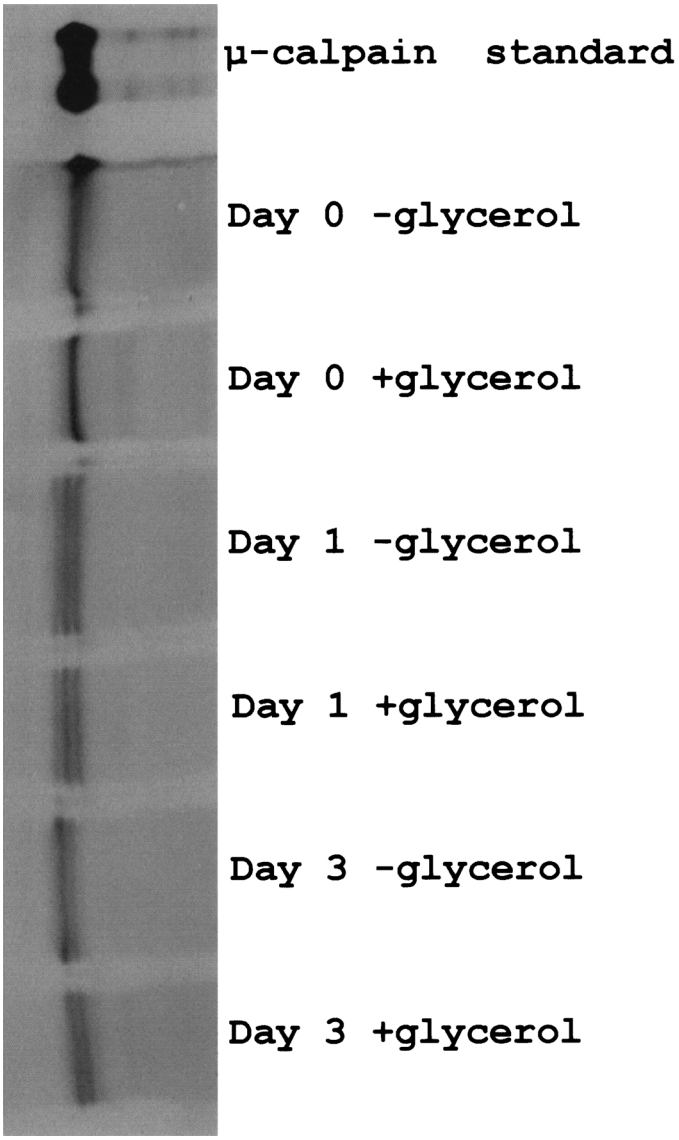

Figure 9. Western blot analysis of the soluble fraction (after dialysis and before chromatography) with and without glycerol at 0, 1, and 3 d postmortem. Proteins (30 μg) were separated on a 7.5% gel.

The present results indicate that determination of μ-calpain activity in postmortem muscle using anion-exchange chromatography does not reflect the activity of μ-calpain with its large subunit autolyzed to 76 kDa. At present, it is unclear how long autolyzed μ-calpain remains active in postmortem muscles, but the results presented herein indicate that the half-life for proteolytically active μ-calpain autolyzed to its 76-kDa form is limited to hours rather than days.

Koohmaraie et al. (1987) examined the effect of postmortem storage on the activity of the components of the calpain proteolytic system in bovine skeletal muscle. Their results indicated that μ-calpain and calpastatin gradually lost their activities, whereas m-calpain activity was very stable and did not decline during postmortem storage. Based on these observations and others (Koohmaraie, 1992, 1996), we have suggested that μ-calpain rather than m-calpain is primarily responsible for postmortem proteolysis that results in meat tenderization. Koohmaraie et al. (1987) suggested that autolysis is the mechanism of inactivation of μ-calpain in postmortem muscle. The results presented herein do not change our earlier conclusions but expand upon them. Based on these and previous experiments, we

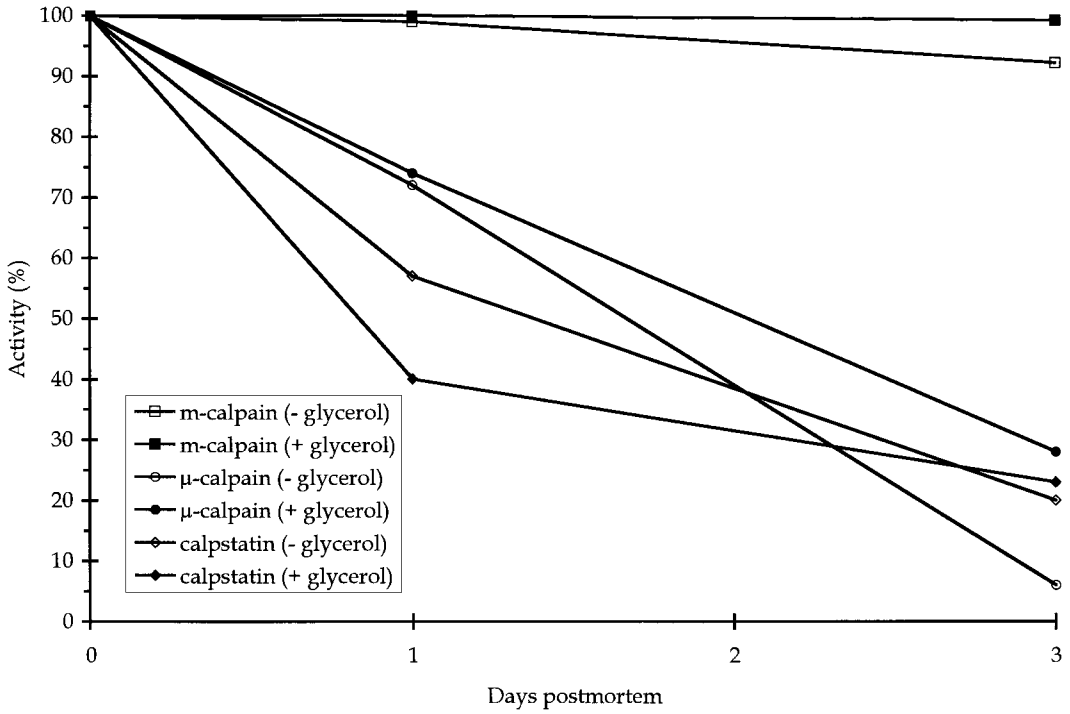

Figure 10. Calpain and calpastatin activities in porcine longissimus extracts processed with and without 25% glycerol at 0, 1, and 3 d postmortem. The 100% for calpastatin, μ-calpain, and m-calpain is 1.02, 0.96, and 1.73 with glycerol and 0.56, 0.57, and 0.97 U/g without glycerol, respectively.

believe that μ-calpain-mediated degradation of key myofibrillar and cytoskeletal proteins is primarily responsible for meat tenderization. Furthermore, autolysis and instability of the autolyzed enzyme lead to inactivation of μ-calpain in postmortem muscle.

Implications

The results of the present study indicate that determination of μ-calpain activity using previously described standard chromatography procedures does not reflect the activity of μ-calpain after it is autolyzed to its 76-kDa form. Furthermore, they, coupled with our previous studies, indicate that instability of partially autolyzed μ-calpain, and not extensive autolysis, is a major cause of the loss of μ-calpain activity in postmortem muscle. Thus, the rate and extent of postmortem proteolysis, and the resulting tenderization, seem to be limited by instability of autolyzed μ-calpain and inhibition of μ-calpain activity by calpastatin.

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