Caspase-3 does not enhance in vitro bovine myofibril degradation by μ-calpain

D. A. Mohrhauser,1 S. A. Kern, K. R. Underwood, and A. D. Weaver
Department of Animal Science, South Dakota State University, Brookings 57007

ABSTRACT: Tenderness is a key component of palatability, which influences consumers’ perception of meat quality. There are a variety of factors that contribute to the tenderness of beef carcasses, including postmortem proteolysis. A more complete understanding of this biological mechanism regulating tenderness is needed to ensure consistently tender beef. Numerous reports indicate μ-calpain is primarily responsible for the degradation of proteins postmortem. Meanwhile, it has been shown that caspase-3 can cleave calpastatin, the inhibitor of μ-calpain. Therefore, the objective of this study was to determine if in vitro degradation of calpastatin by caspase-3 can enhance the postmortem breakdown of myofibrillar proteins by μ-calpain. Bovine semitendinosus muscles were excised from two carcasses 20 min postmortem. Muscle strips were dissected from the semitendinosus, restrained to maintain length, and placed in a neutral buffer containing protease inhibitors. Upon rigor completion, myofibrils were isolated from each strip, and sarcomere length was determined. Samples with similar sarcomere lengths were selected to minimize the effect of sarcomere length on proteolysis. Myofibrils were then incubated at 22°C with either μ-calpain, μ-calpain + calpastatin, μ-calpain + caspase-3 + calpastatin, or caspase-3 + calpastatin for 0.25, 1, 3, 24, 48, or 72 h at a pH of 6.8. Proteolysis of troponin T (TnT) and calpastatin was evaluated using SDS-PAGE and Western blotting techniques. Analysis of Western blots confirmed significant degradation of calpastatin by caspase-3 (P < 0.05). Additionally, Western blots revealed intact calpastatin disappeared rapidly as a result of digestion by μ-calpain. Although caspase-3 did not significantly degrade TnT (P > 0.05), all μ-calpain digestion treatments resulted in substantial TnT breakdown (P < 0.05). Degradation of TnT did not differ between the μ-calpain + calpastatin and μ-calpain + caspase-3 + calpastatin digestions (P > 0.05). Results of this study indicate caspase-3 cleavage of calpastatin does not enhance in vitro degradation of TnT by μ-calpain.

Key words: caspase, μ-calpain, calpastatin, proteolysis, tenderness

INTRODUCTION

Tenderness remains one of the most important factors influencing consumers’ perception of beef quality. However, although some influences on tenderness are well understood, the mechanisms involved in postmortem proteolysis remain unclear. Following rigor mortis, muscle proteins are degraded by enzymes, resulting in the tenderization of meat during storage (Wheeler and Koohmaraie, 1994). However, the enzyme systems responsible for improvements in tenderness during aging remain under debate. Numerous reports indicate μ-calpain is primarily responsible for the degradation of proteins postmortem (Taylor et al., 1995; Geesink et al., 2006; Mohrhauser et al., 2011). Meanwhile, the caspase system has been investigated because of its role in programmed cell death (Orlowski, 1999; Goll et al., 2008; Bernassola et al., 2010). Still, research has not provided direct evidence of the involvement of the caspase enzymes in the breakdown of postmortem skeletal muscle (Kemp et al., 2009; Mohrhauser et al., 2011). It has been suggested that there may be an interaction between the calpain and caspase systems (Wang et al., 1998; Kemp and Parr, 2012). Of note, activity at 1 d postmortem of the calpain-specific inhibitor, calpastatin, is highly correlated with meat tenderness (Whipple et al., 1990). Meanwhile, it has

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2Corresponding author: dustin.mohrhauser@sdstate.edu
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been demonstrated that caspase-3 can cleave calpastatin (Porn-Ares et al., 1998; Wang et al., 1998), yet it is unclear if calpastatin cleavage results in reduced inhibition of µ-calpain. Further investigation is required to determine if degradation of calpastatin by caspase-3 can enhance the postmortem breakdown of muscle proteins by µ-calpain. Thus, preceded by the hypothesis that caspase-3 breakdown of calpastatin can enhance µ-calpain activity and indirectly improve tenderness, the objective of this study was to determine if in vitro degradation of calpastatin by caspase-3 can enhance the postmortem breakdown of myofibrillar proteins by µ-calpain.

MATERIALS AND METHODS

Animal care and experimental protocols were not submitted for approval by the Animal Care and Use Committee because samples were collected from the state-inspected South Dakota State University Meat Laboratory.

Sample Collection

Two A maturity steers were slaughtered at the South Dakota State University Meat Laboratory using standard procedures. The semitendinosus from the left side of each carcass was removed approximately 20 min postmortem. Muscle strips (1 cm wide × 25 cm long) were then excised from the superficial portion of the muscle parallel to muscle fiber orientation and attached to wooden applicator sticks to maintain sarcomere length. The muscle samples were then placed in a neutral rigor buffer [RB: 75 mM KCl, 10 mM imidazole, 2 mM MgCl₂, 2 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 mM NaN₃] containing protease inhibitors [0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM Ac-DEVD-CHO (caspase-3 inhibitor), 16 mg/L of aprotinin, and 16 mg/L of leupeptin] to inhibit endogenous protease activity. Samples were stored at 4°C overnight with constant stirring.

Myofibril Preparation

Twenty-four hours postmortem, myofibrils were isolated from semitendinous muscle strips according to the procedure by Weaver et al. (2008). Approximately 5 g of muscle were minced with a knife and homogenized in 35 mL of RB for two 15-s bursts using an Ultra-Turrax T25 homogenizer (Janke and Kunkel GmbH and Co., KG, Staufen, Germany) at medium speed. After the sample was centrifuged at 1,000 × g for 10 min at 4°C, the supernatant was decanted, and the remaining pellet was homogenized in 35 mL of RB for two 15-s bursts at medium speed. Again, the suspension was centrifuged at 1,000 × g for 10 min at 4°C, the supernatant was decanted, and the remaining pellet was resuspended by shaking in 35 mL of RB and centrifuged at 1,000 × g for 10 min at 4°C. Final myofibril pellets were resuspended in 20 mL of RB plus 0.1 mM phenylmethylsulfonyl fluoride and prepared for sarcomere length determination.

Enzymatic Digestion of Myofibrils

Myofibrils from samples with similar sarcomere lengths were assigned to 1 of 4 digestion protocols: digestion by 1) µ-calpain, 2) µ-calpain + calpastatin, 3) caspase-3 + calpastatin, or 4) µ-calpain + caspase-3 + calpastatin. All treatments were subjected to enzymatic digestion at room temperature (~22°C) using a modified procedure from Weaver et al. (2009). Eight milliliters of glycerinated myofibrils were centrifuged at 3,100 × g for 6 min at 4°C and washed with 4 mL of 5 mM Tris-HCl (pH 8.0). The samples were then centrifuged at 3,100 × g for 6 min at 4°C and washed with 4 mL of 50 mM MES-Tris (MES = 2-[N-Morpholino]ethanesulfonic acid; pH 6.8). Samples were centrifuged twice more at 1,100 × g for 6 min at 4°C and washed and resuspended with 4 mL of 50 mM MES-Tris (pH 6.8). Protein concentration was determined using the biuret procedure. 3.5 mL of myofibrils were placed in 15-mL glass test tubes, and protein concentration was adjusted to 4 mg/mL with 50 mM MES-Tris (pH 6.8). Calcium chloride (100 μM) and 15 mM 2-mercaptoethanol were added to each reaction tube. An aliquot was removed from each tube at time 0 and mixed with 0.5 vol of pyronin Y sample buffer [3 mM EDTA, 3% (wt/vol) SDS, 30% (vol/vol) glycerol, 0.003% pyronin Y, 30 mM Tris-HCl, pH 8.0] and 0.1 vol

Sarcomere Length Determination

Sarcomere length determination was completed following the procedure of Mohrhauser et al. (2011). Isolated myofibril samples were incubated with monoclonal anti-α-actinin antibody (A7811, Sigma, St. Louis, MO). Samples were then rinsed and incubated with a donkey anti-mouse fluorescein isothiocyanate–conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) to allow visualization of Z lines. The distance between labeled Z lines was measured using a microscope (Olympus AX70, Leeds Precision Instruments Inc., Minneapolis, MN) equipped with a fluorescence filter at 40× magnification, and images were captured using an Olympus DP71 camera (Leeds Precision Instruments Inc.). Myofibril images were analyzed, and samples with similar sarcomere lengths between the two carcasses were selected for use in the study to minimize the effect of sarcomere length on proteolysis. Myofibrils were then stored in an equal volume of glycerol at -20°C for later use.
The µ-calpain + calpastatin digestion reaction was then initiated by the addition of active µ-calpain to myofibrils (0.45 units µ-calpain/mL of myofibrils). The µ-calpain + calpastatin digestion reaction was then initiated by the addition of active µ-calpain to myofibrils (0.45 units µ-calpain/mL of myofibrils) along with active calpastatin [0.45 units calpastatin/mL of myofibrils; µ-calpain (48.2 units/mg protein) and calpastatin (262.8 units/mg protein) were kindly provided by S. M. Lonergan and E. Huff-Lonergan according to the procedure of Thompson and Goll (2000) with minor modifications as described by Maddock et al. (2005)]. The caspase-3 + calpastatin digestion reaction was then initiated by the addition of active caspase-3 [caspase-3, human recombinant (Invitrogen, Carlsbad, CA), activity of 100 units (1 unit caspase-3/µg calpastatin), where 1 unit is defined as the amount of enzyme that cleaves 1 nM DEVD-pNA (Asp-Glu-Val-Asp-p-nitroaniline) in 1 h at 37°C] along with active calpastatin (0.45 units calpastatin/mL of myofibrils). The µ-calpain + caspase-3 + calpastatin digestion reaction was then initiated by the addition of active µ-calpain (0.45 units µ-calpain/mL of myofibrils), active caspase-3 (1 unit caspase-3/µg calpastatin), and active calpastatin (0.45 units calpastatin/mL of myofibrils) to myofibrils. Aliquots were removed from each reaction tube at desired time points (0.25, 1, 3, 24, 48, and 72 h) and placed in 0.5 vol pyronin Y sample buffer and 0.1 vol 2-mercaptoethanol. Samples were denatured at 100°C for 5 min, cooled, and stored at -20°C.

Gel Electrophoresis and Western Blotting of Calpastatin and Troponin T

Following digestion, 20 µL of each sample were loaded on precast 10% polyacrylamide resolving gels (Ready Gel, Tris-HCl gels; Bio-Rad Laboratories, Hercules, CA) and placed in a running buffer consisting of 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 0.1% (wt/vol) SDS to evaluate the degradation of calpastatin. Gels were electrophoresed at a constant 200 V for 90 min at 4°C. After electrophoresis, gels were equilibrated for 30 min at 4°C in transfer buffer [25 mM Tris, 192 mM glycine, and 15% (vol/vol) methanol] and transferred to polyvinylidene difluoride membranes at 4°C at a constant 90 V for 45 min.

Upon the completion of transfer, membranes were equilibrated for 60 min at 4°C in PBS. After equilibration, membranes were blocked in Odyssey blocking buffer (OBB; Li-Cor, Lincoln, NE) and PBS (1:1 OBB:PBS) for 60 min at room temperature. Blots were coincubated for 90 min at room temperature with rabbit anti-actin (1:8,000; anti-actin 20-33 antibody, Sigma-Aldrich, St. Louis, MO) and monoclonal anti-calpastatin (Domain IV, 1F7E3D10, Calbiochem) diluted 1:5,000 in OBB and PBS with Tween-20 (PBST; 1:1 OBB:PBST). Actin was labeled to correct for any variation due to loading differences. Blots were washed 4 times with PBST (5 min per wash) and then labeled with secondary antibodies, goat anti-mouse and goat anti-rabbit (Li-Cor), diluted 1:8,000 and 1:20,000, respectively, in OBB and PBST for 45 min at room temperature. Blots were again washed 4 times in PBST (5 min per wash), rinsed in PBS, and placed in PBS. Immunoreactive calpastatin and actin were visualized, and calpastatin was quantified by measuring the disappearance of intact calpastatin using a Li-Cor Odyssey scanner. Intensities of protein bands were expressed relative to the total intensity of actin to minimize variation due to loading. The relative intensity of bands was then normalized to the relative intensity of two reference samples found on each gel (loaded across each gel to help correct for differences in transfer) and then compared to intact calpastatin at 0 h to determine the relative abundance of calpastatin at all time points.

This procedure was modified to evaluate the degradation of troponin T (TnT). Following digestion, 5 µL of each sample were loaded on precast 15% polyacrylamide resolving gels (Ready Gel, Tris-HCl gels; Bio-Rad Laboratories). Blots were incubated with monoclonal TnT (JLT-12, Sigma-Aldrich) and rabbit anti-actin at a dilution of 1:5,000 and 1:8,000, respectively. Blots were then labeled with secondary antibodies, goat anti-mouse and goat anti-rabbit (Li-Cor), diluted 1:20,000 in OBB and PBST. Troponin T degradation was identified and quantified using the same procedure as calpastatin.

Statistical Analysis

Data were analyzed using repeated measures (PROC MIXED DATA) procedures (SAS Inst. Inc., Cary, NC). Means for each treatment were tested to a predetermined significance level of 0.05, evaluating the difference of relative intact protein over time.

RESULTS AND DISCUSSION

According to Koohmaraie (1988), a protease must meet certain criteria to be considered effective at degrading postmortem muscle and eliciting the aging response. It must be located within skeletal muscle cells, must have the capability to reproduce postmortem changes in myofibrils in vitro, and, finally, must have access to substrates (Koohmaraie, 1988). There have been many enzyme systems identified within skeletal muscle (for a review of enzyme systems in muscle, see Goll et al., 2008), but one system that appears to fit these requirements is the calpain system. Much work has pointed to the calpain sys-
Caspase-3 does not enhance µ-calpain activity

tem as the primary system responsible for the postmortem tenderization process due to its access to myofibrils, its lack of requirement for ATP, and its ability to cause postmortem changes in vitro (Koohmaraie, 1988; Huff-Lonergan et al., 1996; Geesink et al., 2006). In skeletal muscle, the calpain system consists of two ubiquitous calpains, µ-calpain and m-calpain, along with their inhibitor, calpastatin (Goll et al., 2003). The Ca^{2+} requirement for half-maximal activity of these two enzymes is drastically different, with µ-calpain requiring 3–50 µM Ca^{2+}, far less than the 400–800 µM Ca^{2+} needed for m-calpain activity (Goll et al., 2003). Of these proteases, µ-calpain is believed to be responsible for postmortem tenderization and not m-calpain because of the amount of Ca^{2+} required vs. actual concentrations present in muscle (Geesink et al., 2006). However, it has been argued that postmortem conditions do not fully favor µ-calpain activity. It has been discovered that only about 20% to 60% of µ-calpain and 20% to 70% of calpastatin remain active 24 h postmortem, with calpastatin activity decreasing less rapidly than µ-calpain during postmortem storage (Boehm et al., 1998). Additionally, calpastatin is a 4-domain, specific inhibitor of the calpain enzymes that can bind calpain at each domain, and Taylor et al. (1995) indicated µ-calpain and calpastatin are colocalized in postmortem muscle. Thus, it could be conceived that any µ-calpain activity after 24 h may be completely inhibited by calpastatin (Boehm et al., 1998). Therefore, it has been suggested that proteases other than µ-calpain could play a role in meat tenderization.

Of these, the caspase system has been investigated most recently because of its involvement in programmed cell death (Kemp et al., 2006; Goll et al., 2008; Mohrhauser et al., 2011). Previous research has indicated the capability of caspase-3 to cleave specific myofibrillar proteins (Communal et al., 2002; Kemp and Parr, 2008). Additionally, it has been shown that caspase-3 can cleave calpastatin (Porn-Ares et al., 1998; Wang et al., 1998). With this premise, it is postulated that caspase-3 could play an indirect role in the improvement of meat aging by enhancing the activity of µ-calpain through the cleavage of the calpain inhibitor, calpastatin.

Results from the present study validated the degradation of calpastatin by caspase-3 (Figs. 1a and 2) as there was an obvious, significant difference between the relative abundance of intact calpastatin between 0 and 72 h of enzymatic digestion (P < 0.0001). However, this rate of calpastatin degradation appears inconsequential when compared to µ-calpain’s ability to degrade calpastatin (Figs. 1b and 2). After only 0.25 h, minimal amounts of intact calpastatin remained in the µ-calpain + calpastatin

![Figure 1](image-url). Western blots, prepared from 10% polyacrylamide resolving gels transferred to a polyvinylidene difluoride membrane of isolated myofibrils from bovine semitendinosus digested with a) caspase-3 + calpastatin, b) µ-calpain + calpastatin, c) µ-calpain + caspase-3 + calpastatin. Blots were labeled with immunoreactive anti-calpastatin (Domain IV, 1F7E3D10, Calbiochem, Billerica, MA). Intact = location of intact calpastatin.
enzymatic digestion, whereas virtually all calpastatin had been degraded by 1 h (Fig. 2). These figures are negligibly different in the µ-calpain + caspase-3 + calpastatin digestion (Fig. 1c) as calpastatin degradation rates were similar between the µ-calpain + calpastatin and µ-calpain + caspase-3 + calpastatin treatments (Fig. 2; \( P > 0.05 \)).

Doumit and Koohmaraie (1999) reported similar results in that µ-calpain and m-calpain significantly degraded calpastatin in vitro. Although all proteases they tested caused calpastatin degradation, it was concluded that µ-calpain and m-calpain caused calpastatin breakdown that most closely resembled the calpastatin degradation patterns normally found in aging lamb muscle; however, that study was conducted before the investigation of the caspase enzyme system’s involvement in meat aging. The present study validated that caspase-3 can cleave calpastatin, but at levels that may not be relevant since µ-calpain caused a much greater degradation of the inhibitor.

Despite the fact that the contribution of caspase-3 in calpastatin degradation might be minimal, we evaluated the importance of the enzyme in the breakdown of myofibrillar proteins to determine if µ-calpain activity had been altered as a result of caspase-3 influencing the ability of calpastatin to inhibit µ-calpain. To do so, we evaluated the degradation of TnT. Troponin T has long been the hallmark protein evaluated in postmortem proteolysis as its degradation appears to be an excellent indicator of the extent of protein degradation in myofibrils during aging (Wheeler and Koohmaraie, 1994; Huff-Lonergan et al., 1996). Although it is questionable whether TnT degradation has a direct effect on meat tenderness because of its regulatory function, the disappearance of intact TnT and appearance of 28 to 32 kDa degradation products are notable changes that take place during postmortem aging as measured by Western blots (Wheeler and Koohmaraie, 1994; Huff-Lonergan et al., 1996). Figure 3 shows representative images of Western blots labeled with a monoclonal antibody against TnT. Visual assessment of the Western blots reveals a qualitative decrease of intact TnT over time in myofibrils subjected to incubations with µ-calpain (Fig. 3a). However, visual evaluation of Western blots for the caspase-3 + calpastatin digestion indicates the slight appearance of TnT degradation product at 48 and 72 h (Fig. 3b); how-

Figure 2. In vitro degradation of calpastatin by µ-calpain + calpastatin, caspase-3 + calpastatin, and µ-calpain + caspase-3 + calpastatin. Abundance of intact calpastatin is expressed relative to the abundance of intact calpastatin at 0 h. Bars signify mean ± SE. Means without a common letter across all time points and treatments differ \( (P < 0.05) \).

Figure 3. Western blots, prepared from 15% polyacrylamide resolving gels transferred to a polyvinylidene difluoride membrane of isolated myofibrils from bovine semitendinosus digested with a) µ-calpain, b) caspase-3 + calpastatin, c) µ-calpain + calpastatin, and d) µ-calpain + caspase-3 + calpastatin. Blots were labeled with immunoreactive anti-troponin T (JLT-12, Sigma-Aldrich, St. Louis, MO). Arrows indicate molecular weight of immunoreactive bands.
Caspase-3 does not enhance µ-calpain activity

However, no significant differences in intact TnT were indicated in this treatment ($P > 0.05$; Fig. 4).

Limited research has been conducted evaluating the degradation of myofibrillar proteins by caspase-3 within bovine skeletal muscle. Most notably, Huang et al. (2011) reported that caspase-3 could degrade titin and nebulin, as well as TnT and desmin, and thus, caspase-3 may be involved in postmortem proteolysis. However, although there appears to be formation of degradation product due to caspase-3 incubation, quantified intact TnT and desmin do not change over time in that study. Those results would agree with the lack of significant changes in intact TnT due to caspase-3 incubation in the present study and previous findings from our lab (Mohrhauser et al., 2011).

The results for TnT degradation were verified through quantification, as shown in Fig. 4. As anticipated, µ-calpain rapidly degraded TnT over time ($P < 0.05$; Fig. 4), coinciding with the results of numerous researchers (Huff-Lonergan et al., 1996; Geesink et al., 2006; Mohrhauser et al., 2011). In agreement with many reports (Geesink and Kooohmaraie, 1999; Maddock Carlin et al., 2006), this degradation was slowed considerably when myofibrils were digested with µ-calpain + calpastatin ($P < 0.05$; Fig. 4). Although physiological activity ratios of µ-calpain:calpastatin are typically 1:4 in beef, 1:2.5 in lamb, and 1:1.5 in pork (Ouali and Talmant, 2011), these observations, the caspase system appears to have little to no effect on postmortem proteolysis and tenderization of meat.

Implications

In conclusion, this study confirms that both caspase-3 and µ-calpain cleave the inhibitor of µ-calpain, calpastatin. Additionally, it provides support that µ-calpain should be considered the primary protease responsible for the direct breakdown of the myofibrillar protein TnT while agreeing with previous research indicating that caspase-3 does not appear to be significant in the direct breakdown of TnT. Finally, this study provides evidence that caspase-3 degradation of calpastatin likely does not enhance in vitro myofibril degradation by µ-calpain.

LITERATURE CITED


