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BEEF REPORT 2012-10

The influence of caspase-3 on the calpain enzyme system during meat aging[1]

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SUMMARY

Tenderness is a key component of palatability, which influences consumers' perception of meat quality. There are a variety of factors that contribute to variations in tenderness, 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. Additionally, 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 μ-calpain, μ-calpain + calpastatin, caspase-3 + calpastatin, or μ-calpain + caspase-3 + calpastatin for 0.25, 1, 3, 24, 48, or 72 hr at a pH of 6.8. Proteolysis of troponin T and calpastatin was evaluated using SDS-PAGE and western blotting techniques. Analysis of western blots confirmed significant degradation of calpastatin by caspase-3. Additionally, western blots revealed intact calpastatin disappeared rapidly due to digestion by μ-calpain. While caspase-3 did not significantly degrade troponin T, all μ-calpain digestion treatments resulted in substantial troponin T breakdown. Degradation of troponin T did not differ between the μ-calpain + calpastatin and μ-calpain + caspase-3 + calpastatin digestions. Results of this study indicate caspase-3 cleavage of calpastatin does not enhance *in vitro* degradation of myofibrillar proteins by μ-calpain.

INTRODUCTION

Tenderness is one of the key factors affecting a consumer's perception of beef palatability. However, due to the variety of factors that contribute to the development of this attribute, consistently tender beef has remained elusive (Koohmarie, 1995; Brooks et al., 1998). After rigor mortis is complete, specific muscle proteins are degraded by endogenous enzymes. This breakdown results in the tenderization of meat during storage (Wheeler and Koohmarie, 1994). Unfortunately, the enzyme systems responsible for this degradation and subsequent improvement in tenderness continue to be under investigation. Multiple 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). The caspase system has also been investigated due to its role in programmed cell death (Orlowski, 1999, Goll et al., 2008, Bernassola et al., 2010). However, research has provided little evidence of the involvement of the

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caspase enzymes in the direct breakdown of structural proteins of postmortem bovine skeletal muscle (Kemp et al., 2009; Mohrhauser et al., 2011). Still, caspase-3 may serve another role as it has been reported that caspase-3 can cleave calpastatin, the inhibitor of μ-calpain (Wang et al., 1998; Kemp et al., 2009). 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

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. A 10-g sample from each excised semitendinosus was vacuum packaged and frozen at −20°C to serve as a time 0 sample. 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 containing protease inhibitors to inhibit endogenous proteases. Samples were stored at 4°C overnight with constant stirring. Upon completion of rigor, myofibrils were isolated from semitendinosus muscle strips according to the procedure by Weaver et al. (2008) and sarcomere length determination was completed following the procedure of Mohrhauser et al. (2011).

Myofibrils from samples with similar sarcomere lengths were assigned to 1 of 4 digestion protocols: 1) digestion by 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). Following digestion samples were subjected to SDS-PAGE and western blotting to visualize the degradation of calpastatin and TnT.

RESULTS AND DISCUSSION

Calpastatin is a four-domain, specific inhibitor of the calpain enzymes that can bind calpain at each domain. Taylor et al. (1995) indicated μ-calpain and calpastatin are colocalized in postmortem muscle; thus, it could be conceived that any μ-calpain activity 24 hours postmortem 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. Previous research has shown the ability of caspase-3 to cleave calpastatin (Wang et al., 1998; Kemp et al., 2009). 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. Results from this study validated the breakdown of calpastatin by caspase-3 (Figure 1 and 2) as there was an obvious, significant difference between the relative abundance of intact calpastatin between 0 and 72 hr of enzymatic digestion ($p < 0.0001$). However, this rate of calpastatin degradation appears trivial when compared to μ-calpain's ability to degrade calpastatin (Figure 2). After only 0.25 hr, minimal amounts of intact calpastatin remained in the μ-calpain + calpastatin enzymatic digestion, while virtually all calpastatin had been degraded by 1 hr (Figure 2). These figures are negligibly different in the μ-calpain + caspase-3 + calpastatin digestion as calpastatin degradation rates were similar between the μ-calpain + calpastatin and μ-calpain + caspase-3 + calpastatin treatments ($P>0.05$).

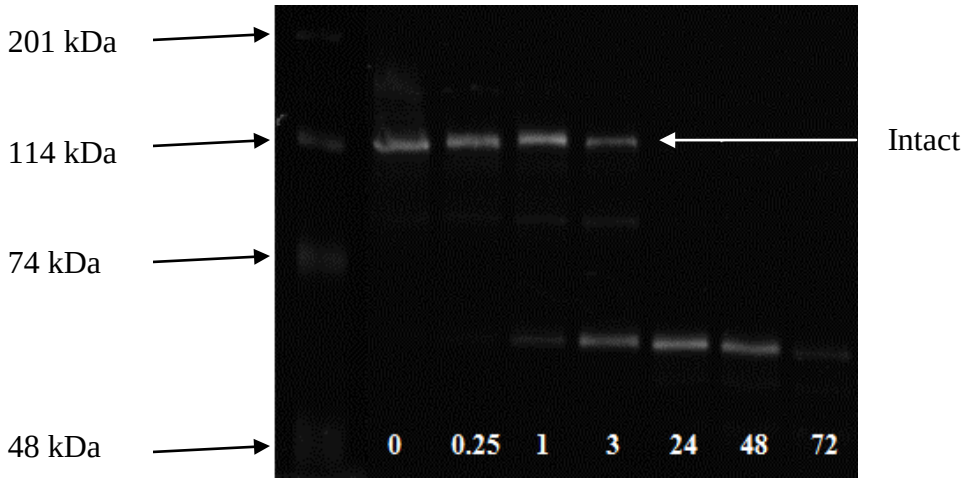


Figure 1. Western blots, prepared from 10% polyacrylamide resolving gels transferred to a polyvinylidene difluoride membrane of isolated myofibrils from bovine *semitendinosus* digested with caspase-3 + calpastatin for 0, 0.25, 1, 3, 24, 48, and 72. Blots were labeled with immunoreactive anti-calpastatin (Domain IV, 1F7E3D10, Calbiochem, Billerica, MA). Time is indicated at the bottom of the blot. Arrows indicate molecular weight of standards. Intact = location of intact calpastatin.

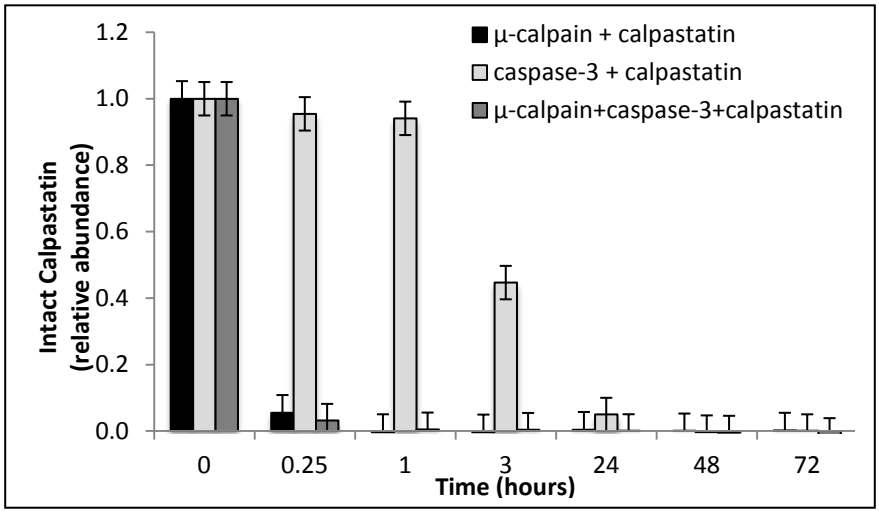
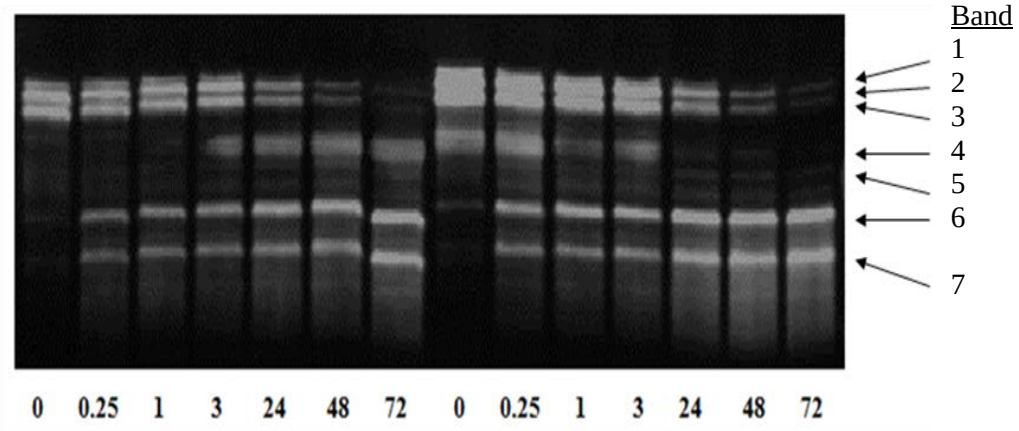


Figure 2. *In vitro* degradation of calpastatin by μ-calpain+calpastatin, caspase-3+calpastatin, and μ-calpain+caspase-3+calpastatin. Abundance of intact calpastatin is expressed relative to the abundance of intact calpastatin at 0 hr.

Troponin T (TnT) has 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 (Koohmaraie, 1994). Although it is questionable whether TnT degradation has a direct effect on meat tenderness due to its regulatory function, the disappearance of intact TnT and appearance of 28-32 kDa degradation products are the most noticeable changes that take place during postmortem aging as measured by Western blots (Koohmaraie, 1994). Figure 3 shows representative images of Western blots labeled with a monoclonal antibody against TnT. Visual assessment of the blots reveal a qualitative decrease of intact TnT (bands 1-3) over time in myofibrils subjected to incubations with μ-calpain (Figure 3a). Meanwhile, visual evaluation of Western blots for the caspase-3 + calpastatin digestion indicate the slight appearance of TnT degradation product at 48 and 72 hr (Figure 3b, bands 4-5). However, no significant differences in intact TnT were indicated in this treatment (P>0.05; Figure 4). This coincides

with previous research showing little involvement by caspase-3 in the direct postmortem degradation of structural proteins (Mohrhauser et al., 2011; Kemp et al., 2009).

a) μ-Calpain + Calpastatin μ-Calpain+Caspase-3+Calpastatin



b) Caspase-3 + Calpastatin

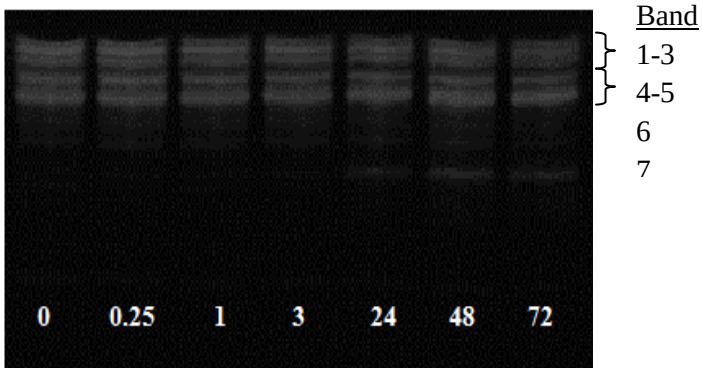


Figure 3. Western blots, prepared from 15% polyacrylamide resolving gels transferred to a polyvinylidene difluoride membrane of isolated myofibrils from bovine *semitendinosus* digested for 0, 0.25, 1, 3, 24, 48, and 72 hr with a) μ-calpain + calpastatin and μ-calpain + caspase-3 + calpastatin, and b) caspase-3 + calpastatin. Blots were labeled with immunoreactive anti-troponin T (JLT-12, Sigma-Aldrich, St. Louis, MO). Time is indicated at the bottom of the blots. Arrows indicate molecular weight of immunoreactive bands. Bands 1, 2, and 3 indicate the location of intact troponin T.

These results were verified through quantification as can be seen in Figure 4. As anticipated, μ-calpain rapidly degraded TnT over time (P<0.05; Figure 4). This degradation was slowed considerably when myofibrils were digested with μ-calpain + calpastatin (P<0.05; Figure 4). Thus, 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, 1990; Koohmaraie et al., 1991), this study found that utilizing a lower amount of calpastatin *in vitro* produces desired inhibiting effects on μ-calpain. Unfortunately, the relative amount of intact TnT remaining for the μ-calpain + calpastatin digestion compared to the μ-calpain + caspase-3 + calpastatin digestion were not different throughout the course of the enzymatic incubation (P>0.05), although the addition of caspase-3 resulted in a lower numerical amount of intact TnT at several time points (Figure 3 and 4).

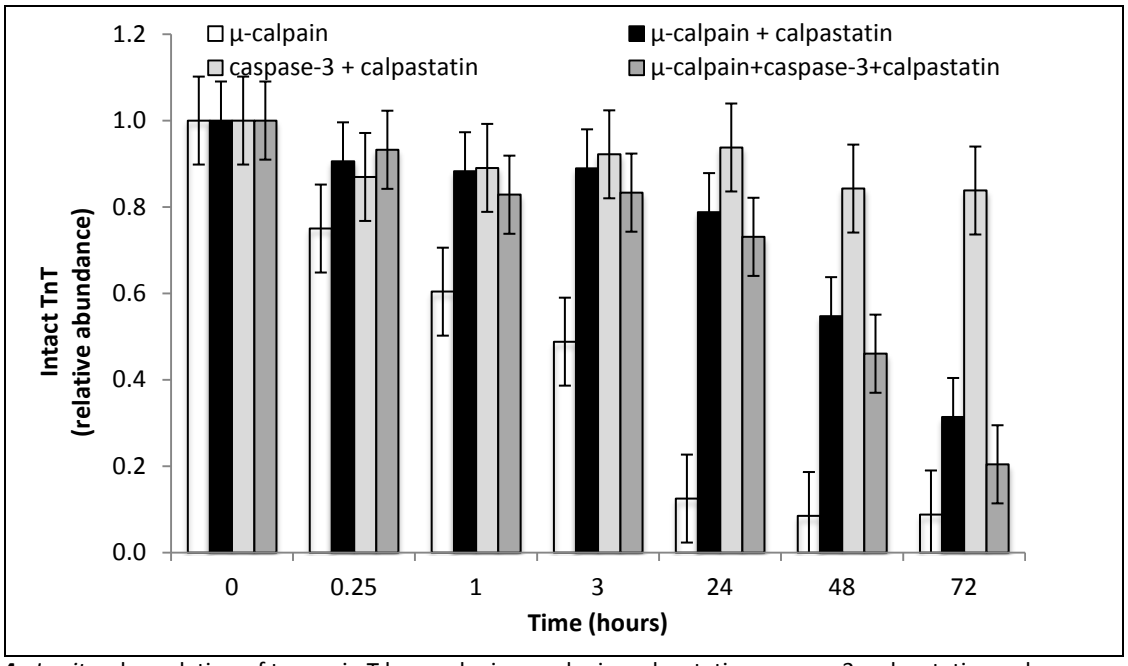


Figure 4. *In vitro* degradation of troponin T by μ-calpain, μ-calpain+calpastatin, caspase-3+calpastatin, and μ-calpain+caspase-3+calpastatin. Abundance of intact troponin T is expressed relative to the abundance of intact troponin T at 0 hr.

IMPLICATIONS

In conclusion, this study confirms that although both caspase-3 and μ-calpain cleave the inhibitor of μ-calpain, calpastatin, it provides support to previous research that caspase-3 is not significant in the direct breakdown of myofibrillar proteins, and that μ-calpain should be considered the primary protease responsible for the degradation of these key proteins during beef aging. Finally, this study provides evidence that caspase-3 does not enhance *in vitro* myofibril degradation by μ-calpain by significantly degrading calpastatin.

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