**Immunoblot Analysis of Calpastatin Degradation: Evidence for Cleavage by Calpain in Postmortem Muscle**¹²

M. E. Doumit³ and M. Koohmaraie⁴

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

**ABSTRACT:** A negative correlation exists between calpastatin activity and meat tenderness. Therefore, it is important to determine the mechanism of calpastatin inactivation in postmortem skeletal muscle. Western immunoblot analysis was performed to determine the protease(s) responsible for degradation of muscle calpastatin during postmortem storage. To accomplish this, purified calpastatin was digested with different proteases in vitro, and their pattern of calpastatin degradation was compared with that of calpastatin degradation in postmortem muscle. Polyclonal antibodies raised in mice against recombinant bovine skeletal muscle calpastatin were used to monitor calpastatin degradation. Lamb longissimus was stored at 4°C and sampled at 0, 6, 12, 24, 72, 168, and 336 h postmortem. Postmortem storage produced a discrete pattern of calpastatin degradation products that included immunoreactive bands at approximately 100, 80, 65, 54, 32, and 29 kDa. Undegraded calpastatin (130 kDa) was barely detectable after 72 h of postmortem storage at 4°C, and no immunoreactive calpastatin was observed by 336 h postmortem. For in vitro proteolysis, lamb longissimus calpastatin (0 h postmortem) was purified using Affi-Gel Blue chromatography. Calpastatin was digested with m-calpain, μ-calpain, cathepsin B, proteasome, trypsin, or chymotrypsin. Each of these enzymes degraded calpastatin. Immunoreactive fragments resulting from digestion of calpastatin with m- and μ-calpain were similar to each other and closely resembled those observed during postmortem aging of lamb longissimus at 4°C. Digestion of calpastatin with μ-calpain reduced calpastatin activity. Degradation of calpastatin by other proteases resulted in unique patterns of immunoreactive fragments, distinct from that observed in longissimus. Thus, m- and(or) μ-calpain seem to be responsible for calpastatin degradation during postmortem storage of meat.

Key Words: Calpains, Calpastatin, Degradation, Tenderness

©1999 American Society of Animal Science. All rights reserved. J. Anim. Sci. 1999. 77:1467-1473

**Introduction**

Calpastatin is an endogenous inhibitor of the ubiquitous calpain (EC 3.4.22.17; Ca²⁺-dependent cysteine proteinase) proteolytic system, which has been implicated in a multitude of cellular functions (reviewed by Croall and DeMartino, 1991; Goll et al., 1992). Current evidence indicates that postmortem tenderization is primarily a result of μ-calpain-mediated degradation of key myofibrillar and cytoskeletal proteins (for review see Goll et al., 1983, 1992; Koohmaraie, 1992a, 1996; Ouali 1990, 1992; Koohmaraie et al., 1995a). Calpastatin activity at 24 h postmortem is inversely proportional to postmortem tenderization (reviewed by Koohmaraie, 1992a) and accounts for a greater proportion of the variation in beef tenderness (~40%) than any other single measure (Whipple et al., 1990; Shackelford et al., 1994). Koohmaraie et al. (1995a) hypothesized that μ-calpain is responsible for postmortem degradation of skeletal muscle calpastatin. The basis for this hypothesis was that 1) infusion of carcasses or injection of cuts of meat with a solution of calcium chloride, which activates calpains, inactivates calpastatin (Koohmaraie et al., 1988, 1989, 1990) and 2) infusion of carcasses with zinc chloride, which inhibits calpains, also prevents calpastatin inactivation in postmortem muscle (Koohmaraie, 1990a).
Because calpastatin accounts for such a large proportion of the variation in meat tenderness, it is important to determine the mechanism of calpastatin inactivation in postmortem muscle. Understanding calpastatin degradation may provide insight for development of improved methods for meat tenderization. The objective of the experiments described here was to determine the protease(s) responsible for degradation of muscle calpastatin during postmortem storage. This was accomplished using Western immunoblot analysis as a qualitative means to compare muscle calpastatin degradation products resulting from postmortem storage and in vitro digestion with purified proteases.

**Materials and Methods**

**Antibodies.** Recombinant bovine calpastatin was expressed and purified as previously described (Doumit et al., 1996). Polyclonal antirecombinant calpastatin antibodies were raised in mice at the Monoclonal/Polyclonal Antibody Core Facility, Center for Biotechnology (University of Nebraska, Lincoln). Ascites fluid containing monoclonal antibodies was used in immunoblotting experiments.

**Calpastatin Degradation in Postmortem Muscle.** Lamb longissimus was stored at 4°C and sampled at 0, 6, 12, 24, 72, 168, and 336 h postmortem. Longissimus samples (from six lambs) were homogenized in 1 volume of 100 mM Tris, 10 mM EDTA (pH 8.3) and heated at 95°C for 15 min. Supernatant containing heat-stable proteins, including calpastatin, was mixed with 2× treatment buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% mercaptoethanol, pH 6.8), and proteins were separated by SDS-PAGE as described below.

**Calpastatin Purification.** Prerigor lamb longissimus samples were homogenized in 3 volumes of 50 mM Tris, 10 mM EDTA, pH 8.3, containing 100 mg/L of ovomucoid, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 6 mg/L of leupeptin. Heated supernatant containing calpastatin was obtained as described by Shackelford et al. (1994), with the modifications of Koohmaraie et al. (1995b). Calpastatin was further purified by Affi-gel blue affinity gel chromatography (Bio-Rad Laboratories, Hercules, CA), using a method adapted from Melgren et al. (1988). Briefly, supernatant was loaded onto a 1.5-× 20-cm Affi-gel blue column. The column was washed with 20 volumes of 40 mM Tris, .5 mM EDTA, 120 mM NaCl, pH 7.35, and eluted with wash buffer containing 500 mM NaCl, pH 7.35. Fractions (5 mL) were collected and assayed for calpastatin activity as described by Koohmaraie (1990b). Active fractions were subjected to Western immunoblot analysis to verify that a single, undegraded calpastatin band was present, and fractions were pooled.

**Calpastatin Degradation.** In vitro calpastatin degradation was performed by incubation of purified calpastatin with m-calpain, μ-calpain, cathepsin B (28 U/mg protein; Sigma Chemical Co., St. Louis, MO), proteasome, trypsin (1:250 Gibco BRL, Gaithersburg, MD), or chymotrypsin (40 to 60 U/mg protein; Sigma). Although it is preferable to conduct these experiments under postmortem conditions, we chose the optimum assay conditions for each of the proteases used. The reason for doing so was that it is impossible to reproduce postmortem conditions with respect to temperature, pH, ionic strength, regulators, activators, inhibitors, and other biomolecules. Therefore, the most efficient way to study the effect of proteases on a given substrate is to use the optimal conditions for expression of proteolytic activity. Calpains were purified as described by Koohmaraie (1992b). Calpastatin was incubated with m- and μ-calpain at an inhibitor: protease activity ratio of 1:1 and 4:1, respectively. Calpain digestion of calpastatin was performed at pH 7.5 and 4°C. Each reaction mixture consisted of approximately .8 U calpastatin, 7 mM CaCl2, 40 mM Tris, and .5 mM EDTA. Control calpain incubations contained an additional 1 mM EDTA and no CaCl2. Calpastatin was incubated with all other proteases on a protein basis (micrograms of calpastatin:micromagrams of protease) as follows: cathepsin B (2:1), protease (1:1), trypsin (50:1), and chymotrypsin (30:1). Prior to digestion of calpastatin with cathepsin B, the buffer containing calpastatin was adjusted to pH 6.0 by adding .2 volumes of 100 mM sodium acetate, 200 mM NaCl (pH 5.0). Cathepsin B digests were at 37°C. Proteasome was purified according to Koohmaraie (1992b). Calpastatin degradation by proteasome was performed at pH 7.5 and 45°C in buffer containing .25 mM SDS. Trypsin and chymotrypsin digestions were performed at pH 7.5 and 4°C. At indicated times, 40 or 50 μL of digestion reaction was removed, immediately mixed with hot 2× protein denaturing buffer (20 or 30 μL, respectively), and boiled for 5 min. All calpastatin incubation experiments were repeated five times.

**SDS-PAGE and Immunoblotting.** Samples (25 μL) containing calpastatin and/or calpastatin degradation products were resolved by SDS-PAGE (Laemmli, 1970) on 7.5-mm-thick 12.5% (37:5:1) separating gels, with 4% (37:5:1) stacking gels. Proteins were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 2 h at 4°C and 200 mA in buffer containing 25 mM Tris, 193 mM glycine, and 15% methanol (Towbin et al., 1979). Lanes containing molecular weight markers were stained with amido black. To prevent nonspecific antibody binding, membranes were incubated with blocking buffer (3% BSA in Tris buffered saline [TBS] containing .05% Tween-20, pH 7.4) for ≥ 1 h. Membranes were incubated with primary mouse anti-calpastatin antibodies (1:1,000), followed by an alkaline phosphatase conjugated anti-mouse IgG diluted 1:1,000 (Sigma). Antibodies were diluted in blocking buffer, and incubations were for 1 h at room temperature with
gentle rocking. Membranes were washed three times with blocking buffer after each incubation. Antibody binding was visualized by exposure to BCIP/NBT (Bio-Rad).

**Assay of Calpastatin Activity After Incubation with μ-Calpain.** To determine the activity of calpastatin after incubation with μ-calpain, purified skeletal muscle calpastatin was incubated with purified μ-calpain as described. After 0, 5, 15, 30, 60, and 120 min, the reaction was stopped by increasing the EDTA concentration to 25 mM. The samples were then heated at 95°C for 3 min (to inactivate μ-calpain), cooled on ice, and centrifuged at 14,000 × g. The calpastatin activity in the supernatant was measured as described by Koohmaraie (1990b). Prior to stopping the reaction, 500 μL (of 2 mL total reaction volume) was removed and brought up to 10 mM EDTA, and its absorbance (turbidity) was measured at 490 nm. After turbidity measurement, protein denaturing buffer was added to an aliquot. This sample was then processed for electrophoresis on 12.5% separating SDS-PAGE. To the remainder of the sample used for the turbidity assay (1.5 mL), 1.5 mL of 5% TCA was added. This sample was then centrifuged, and the absorbance of the TCA-soluble fraction was measured at 278 nm.

**Animals.** The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in this study. Longissimus muscle samples were taken from carcasses of crossbred lambs (Dorset × Romanov).

**Statistical Analysis.** The calpastatin activity data were analyzed by analysis of variance using GLM procedures of SAS (1988) for a completely randomized design. The model included the main effects of incubation time (0, 5, 15, 30, 60, and 120 min) and assay method (absorbance of TCA-soluble materials at 278 nm and turbidity) and their interaction. Least squares means were separated using the PDIF procedure (pair-wise t-test).

**Results and Discussion**

**Postmortem Proteolysis of Skeletal Muscle Calpastatin.** The monoclonal antibody raised in mice against recombinant bovine skeletal muscle calpastatin recognized an undegraded, ∼130-kDa protein in lamb longissimus taken immediately after slaughter (Figure 1, d 0). This is consistent with reported values for the molecular mass of muscle calpastatin (Croall and DeMartino, 1991). Storage of lamb longissimus at 4°C resulted in proteolytic breakdown of calpastatin within 6 h. Little undegraded calpastatin was detectable at 3 and 7 d of postmortem storage (Figure 1). Koohmaraie et al. (1995b) demonstrated that lamb longissimus calpastatin activity decreases by ∼62% during the first 7 d of postmortem storage (3.2 to 1.2 U/g muscle). Postmortem storage produced a discrete pattern of calpastatin degradation products that included immunoreactive bands at approximately 100, 80, 65, 54, 32, and 29 kDa (Figure 1). Likewise, Mellgren et al. (1986) reported distinct calpastatin degradation products of 100, 90, and 60 kDa in bovine heart extracts. It is possible that these products correspond to the larger proteolytic fragments observed in the current study.

**Degradation of Calpastatin by Selected Proteases.** Undegraded calpastatin was digested with m-calpain, μ-calpain, cathepsin B, proteasome, trypsin, or chymotrypsin (Figure 2). Each of these enzymes degraded calpastatin. Immunoreactive fragments (apparent molecular masses between 100 and 29 kDa) resulting from digestion of calpastatin with m- and μ-calpain were similar to each other (Figure 2, top), and they closely resembled those observed during postmortem storage of lamb longissimus at 4°C (Figure 1). Calpastatin was more readily degraded by μ-calpain than by m-calpain; therefore, a higher ratio (4:1 vs 1:1) of calpastatin to μ-calpain was used. Initial experiments revealed that no undegraded calpastatin remained after a 15-min incubation with μ-calpain at
an activity ratio of 2:1 (unpublished observations). Di Lisa et al. (1995) demonstrated that \( \mu \)-calpain was at least 10 times more active than m-calpain in degrading troponin-I and troponin-T, both in vitro and in situ. However, Koohmaraie (1992b) indicated that m-calpain was twice as active as \( \mu \)-calpain in degrading casein. Thus, \( \mu \)-calpain may be more active against native substrates than m-calpain.

Figure 2. Degradation of skeletal muscle calpastatin by m-calpain (top, left), \( \mu \)-calpain (top, right), cathepsin B (middle, left), proteasome (middle, right), trypsin (bottom, left), or chymotrypsin (bottom, right). Calpastatin was digested with each protease as described in the Materials and Methods section. Samples (25 \( \mu \)L) containing calpastatin were resolved on 12.5% polyacrylamide gels then transferred onto Immobilon-P membrane and subjected to immunoblot analysis. On each blot, the first lane (std) corresponds to molecular weight standards as described in Figure 1.
As indicated earlier, it is well documented that calpastatin loses its activity during postmortem storage and that the rate of calpastatin inactivation is correlated with meat tenderization that occurs during postmortem storage of meat (for review see Koohmaraie et al., 1995a). The results presented here suggest that calpains are the best candidate for postmortem degradation of calpastatin. These results are in agreement with several other studies with regard to degradation of calpastatin by calpains. However, most of these previous studies have indicated that calpain-induced degradation of calpastatin does not result in the loss of calpastatin’s ability to inhibit calpain activity (Goll et al., 1983; Mellgren et al., 1986; Nakamura et al., 1989; Mellgren and Lane, 1990).

With the exception of Mellgren and Lane (1990), none of the previous reports quantified the loss in calpastatin activity. The conclusion that calpain degradation of calpastatin does not affect its inhibitory activity seems to have been made based on qualitative assays. However, Mellgren and Lane (1990) reported that incubation of calpastatin (measured by inhibition of caseinolytic activity of m-calpain) with 10-fold molar excess of m-calpain reduced calpastatin activity by 50%. To determine the activity of calpastatin after its degradation by m-calpain, purified calpastatin was incubated with purified m-calpain. At various times of incubation, an aliquot was removed, heated to inactivate calpain, and reassayed to measure its m-calpain inhibitory activity (Table 1). Results indicate that even after 120 min of incubation, calpastatin retained 57.2% of its initial activity when m-calpain activity was measured as the A_{280} of the TCA-soluble fraction.

When calpain is incubated with casein (Hammarsten, United States Biochemical Corp., Cleveland, OH), the reaction mixture is a clear solution at the beginning of the reaction and gradually becomes turbid as a result of casein hydrolysis by calpain. The relationship between the extent of turbidity (absorbance at 490 nm) and calpain activity is linear (Jiang et al., 1996). However, with increasing enzyme concentration or incubation time due to extensive degradation of casein, the reaction mixture becomes clear again, thus, limiting utility of turbidity as a viable method to assay calpains (personal observation).

While conducting the assays to measure activity of m-calpain-degraded calpastatin, we noticed that although the reaction mixtures representing a time of 5, 15, 30, 60, and 120 min of degradation of calpastatin by m-calpain became turbid (i.e., reduced

### Table 1. Inhibition of m-calpain by calpastatin after various incubation time with μ-calpain

<table>
<thead>
<tr>
<th>Incubation time, min</th>
<th>% Inhibition (TCA-soluble fraction method)</th>
<th>% Inhibition (turbidity method)</th>
<th>Difference, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–16.3</td>
</tr>
<tr>
<td>5</td>
<td>67.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–65.3</td>
</tr>
<tr>
<td>15</td>
<td>66.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–65.0</td>
</tr>
<tr>
<td>30</td>
<td>65.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–67.1</td>
</tr>
<tr>
<td>60</td>
<td>61.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–87.4</td>
</tr>
<tr>
<td>120</td>
<td>57.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–82.0</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Means in a column and row without a common superscript differ (P < .05).
Figure 3. Effect of μ-calpain-degraded calpastatin on hydrolysis of casein by m-calpain. Calpastatin was incubated with μ-calpain as described in the Materials and Methods section. At indicated times, an aliquot was removed, heated to inactivate μ-calpain, centrifuged, and then assayed for inhibition of caseinolytic activity of m-calpain. After 60 min of incubation, an aliquot was removed and electrophoresed on 12.5% (37.5:1) polyacrylamide gels as described in the Materials and Methods section. The first lane (std) corresponds to molecular weight standards as described in Figure 1. The casein assay blank is designated as blank, and it represents the assay mixture that contains casein alone in the assay buffer (i.e., no m-calpain). Calpain-Ca and calpain-EDTA represents the assay mixture that included m-calpain with casein and 5 mM CaCl₂ (Calpain-Ca) or 10 mM EDTA (Calpain-EDTA).

calpastatin inhibition of m-calpain), the A278 of the TCA-soluble fraction showed that calpastatin was very active (Table 1). We hypothesized that in the presence of degraded calpastatin the hydrolysis of casein, although it occurs, is not as complete as casein degradation in the absence of calpastatin, resulting in precipitation of large casein fragments at 2.5% TCA. Greenberg and Shipe (1979) demonstrated that precipitation of hydrolysates and protein with TCA was a function of the size of the precipitant and concentration of TCA. For example, even though 86% of the β-lactoglobulin was soluble at 1% TCA, only 2% was soluble at 10% TCA (Greenberg and Shipe, 1979).

Therefore, these experiments were repeated and the results were evaluated by different methods. Again, calpastatin was incubated with μ-calpain and after 0, 5, 15, 30, 60, and 120 min an aliquot was removed, heated to inactivate μ-calpain, centrifuged, and assayed for inhibition of m-calpain. After 60 min of incubation of degraded calpastatin with m-calpain, the turbidity (A₄₉₀ using a microtitier plate reader) of a 150-μL aliquot was determined. This aliquot was, thereafter, used for SDS-PAGE analysis of the extent of casein degradation (Figure 3). To the remaining 1.85 mL an equal volume of 5% TCA was added, the mixture was centrifuged, and the A₂₇₈ of the TCA-soluble fraction was measured (Table 1). Results indicated that there is a significant difference between the two assays (Table 1).

After 120 min of incubation with μ-calpain, calpastatin retained 57.2% of its activity with the A₂₇₈ assay and only 10.3% of its activity with the A₄₉₀ assay. These results are supported by SDS-PAGE analysis, which indicated that even though degradation of casein by m-calpain in the presence of degraded calpastatin was extensive, it was not as extensive as degradation of casein in the reaction that did not include calpastatin (Figure 3). Regardless of the method used to measure the activity of degraded calpastatin, there was a significant decrease in calpastatin activity after 5 min of incubation with μ-calpain, and there was little or no additional loss of activity with further incubation (Figure 3 and Table 1).

Considerable evidence indicates that the calpains are responsible for postmortem proteolysis in skeletal muscle that results in meat tenderization (for review see Goll et al., 1983, 1992; Koohmaraie, 1992a, 1996; Ouali, 1990, 1992; Koohmaraie et al., 1995a). Furthermore, the current study indicates that m- and(or) μ-calpain are responsible for calpastatin degradation during postmortem storage of meat. These conclusions are supported by Sorimachi et al. (1997), who reported that calpastatin degradation during reperfusion of rat heart after ischemia could be reproduced in vitro by m-calpain. In addition, reperfusion in the presence of a calpain inhibitor inhibited calpastatin degradation. However, because the calcium concentration in postmortem muscle is high enough to activate m-calpain, but not m-calpain, it seems reasonable to conclude that μ-calpain is responsible for postmortem degradation of calpastatin. Degradation of calpastatin by μ-calpain reduces calpain-inhibitory activity and is probably an important event in regulation of postmortem proteolysis, and, thus, meat tenderness.

Implications

Immunoblot analysis was used to compare longissimus muscle calpastatin degradation products resulting from postmortem storage and in vitro digestion.
with purified proteases. This study indicates that skeletal muscle calpastatin is degraded by calpains during postmortem storage of meat. Calpastatin degradation by calpains potentially plays an important role in regulating postmortem proteolysis, which influences the degree of meat tenderization.

**Literature Cited**


Utrecht, The Netherlands.