

Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for Quantification of Skeletal Muscle Calpastatin^{1,2}

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ABSTRACT: An indirect antibody ELISA was developed for rapid and sensitive quantification of skeletal muscle calpastatin. Polyclonal antibodies were raised in rabbits against recombinant calpastatin, corresponding to domains 2, 3, and 4 of bovine skeletal muscle calpastatin. Western blot analysis revealed that these antibodies specifically recognize an immunoreactive calpastatin protein of approximately 130 kDa in prerigor skeletal muscle extracts. The intensity of the immunoreactive bands corresponds qualitatively with assayable calpastatin activity. For ELISA development, optimum dilutions of sample, primary anti-calpastatin antibody, and peroxidase-conjugated secondary antibody were determined by titration. A dilution optimum for coating of Immulon[®] 4 (Dynatech) plates was observed when

heated muscle extracts were diluted to 2 to 4 μg of protein/mL and incubated for 2 h at 37°C. Optimum primary (30 μg IgG/mL) and secondary (Sigma A-6154; 1:1000 dilution) antibody incubations were for 1 h at 37°C. Tetramethylbenzidine was used as substrate and A₄₅₀ of the stopped reaction product was recorded in an automated plate reader. Calpastatin ELISA results were linearly related to calpastatin activity (calpain inhibitory activity) of heated longissimus muscle homogenates from prerigor lamb ($r^2 = .89$; $n = 40$) and beef aged for 24 or 48 h ($r^2 = .90$; $n = 47$). Intra-assay CV was < 5% ($n = 8$) and inter-assay CV was < 6% ($n = 5$). This assay offers advantages of speed, simplicity, and sensitivity over conventional methodology for calpastatin quantification.

Key Words: Calpastatin, ELISA, Skeletal Muscle

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Introduction

¹Mention 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. The authors gratefully acknowledge partial support from the Cattleman's Beef Promotion and Research Board through the Beef Industry Council of the National Live Stock and Meat Board. We thank S. Hauver, B. Hasley, and T. Wycoff for technical assistance, P. Bures for photography, and M. Bierman for secretarial assistance.

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Calpastatin is an endogenous inhibitor of the calpain (EC 3.4.22.17, Ca²⁺-dependent cysteine protease) proteolytic system, which has been implicated in a multitude of cellular functions in a variety of tissues (reviewed by Croall and DeMartino, 1991). In skeletal muscle, the calpain system has the potential to regulate growth through involvement in myogenic cell differentiation (Schollmeyer, 1986; Kwak et al., 1993) and initiation of myofibrillar protein turnover (reviewed by Goll et al., 1992). It is also well established that calpains are primarily responsible for postmortem proteolysis, which results in meat tenderization (reviewed by Koohmaraie, 1992). Postrigor calpastatin activity, which is inversely proportional to postmortem tenderization, 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). However, current procedures for quantification of skeletal muscle calpastatin activity are laborious.

Bovine skeletal muscle calpastatin cDNA has been cloned and sequenced (Killefer and Koohmaraie, 1994). The availability of cDNA clones has made possible the production of recombinant skeletal muscle calpastatin. Production of recombinant calpastatin, which can be readily purified and used for antibody production, circumvents the need to purify significant quantities of calpastatin from skeletal muscle. The objectives of this research were 1) to express and purify recombinant bovine skeletal muscle calpastatin, 2) to raise polyclonal antibodies against recombinant calpastatin, and 3) to develop an enzyme-linked immunosorbent assay (**ELISA**) to quantify skeletal muscle calpastatin. These tools will improve our ability to study the involvement of calpastatin in muscle growth and meat tenderness.

Materials and Methods

Expression and Purification of Recombinant Calpastatin. *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) was the host strain for the recombinant plasmid. Procedures for purifying plasmid DNA, DNA manipulations, and bacterial transformations were as previously described (Sambrook et al., 1989). A 2.2-kb cDNA encoding domains 2, 3, 4, and the 3' untranslated region of bovine calpastatin (clone pBSA1; Killefer and Koohmaraie, 1994) was subcloned into the pGEX-5X-2 plasmid (Pharmacia, Piscataway, NJ). The plasmid pGEX-5X-2 contains the glutathione-S-transferase (**GST**) gene at the 5' end of the cloning site, which allows for expression of a GST-calpastatin fusion protein. Expression of the fusion protein offers ease of purification with glutathione sepharose affinity chromatography.

For expression of fusion protein, *E. coli* XL1-Blue cells containing the calpastatin expression vector were grown to $A_{600} = .6$ in LB medium (Sambrook et al., 1989) containing 50 $\mu\text{g}/\text{mL}$ ampicillin. Fusion protein expression was induced by addition of isopropyl- β -D-thio-galactopyranoside (**IPTG**) to 5 mM. Additionally, phenylmethylsulfonyl fluoride (**PMSF**) was added to a final concentration of 2 mM at the time of induction. After 24 h, cells were harvested by centrifugation and suspended in 40 mM Tris, .5 mM EDTA, 2 mM PMSF, pH 7.35 (1/30 culture volume). Cells were lysed by sonication (three repetitions of 4 s at 100% output) with a sonic disruptor (Tekmar Co., Cincinnati, OH). Triton X-100 was added to a final concentration of 1.0%, and the lysate was incubated on ice for 20 min. The slurry was clarified by centrifugation ($39,800 \times g$) for 30 min at 4°C. The supernatant was adjusted to 500 mM NaCl and applied to glutathione-Sepharose 4B. The resin was extensively washed first with 40 mM Tris, .5 mM EDTA, 500 mM NaCl, pH 7.35, followed by three washes with the same buffer containing 1 M urea. The resin was equilibrated with 40 mM Tris, .5 mM EDTA, pH 7.35, prior to elution

with 40 mM Tris, .5 mM EDTA, 10 mM glutathione, pH 7.35.

Glutathione-S-transferase-calpastatin was further purified using preparative SDS-PAGE. Briefly, 1 mg of affinity-purified GST-calpastatin was fractionated by SDS-PAGE (Laemmli, 1970) in a Mini-Protean II (Bio-Rad Laboratories, Hercules, CA) using 1.5-mm spacers. Ten percent separating gels (37.5:1 ratio of acrylamide to N,N'-methylenebis [bis-acrylamide]) and 4% (37.5:1) stacking gels were used. A vertical strip containing molecular weight standards and a portion of the sample lane was cut and stained to visualize the protein. The portion of the gel that contained the fusion protein was then removed, crushed in 40 mM Tris, .5 mM EDTA, 150 mM NaCl, pH 7.35, and agitated for 12 h at 4°C to passively elute the fusion protein from the acrylamide gel. Removal of SDS was as described by Huang et al. (1993).

Calpastatin Assay. Longissimus samples (10 g) were homogenized in three volumes of 50 mM Tris (prerigor) or 100 mM Tris (postrigor), 10 mM EDTA, pH 8.3, containing 100 mg/L of ovomucoid, 2 mM PMSF, and 6 mg/L of leupeptin. The homogenate was centrifuged at $37,500 \times g$ for 90 min, then dialyzed overnight against 40 mM Tris, 5 mM EDTA, pH 7.4. After dialysis, the extract was heated at 95°C for 15 min, cooled on ice, and centrifuged at $37,500 \times g$ for 60 min. Calpastatin activity of heated extracts and GST-calpastatin was determined as described by Koohmaraie (1990).

Production of Antibodies. Polyclonal rabbit anti-recombinant calpastatin antibodies were raised at the Monoclonal/Polyclonal Antibody Core Facility, Center for Biotechnology (University of Nebraska, Lincoln). The IgG fraction of rabbit serum was purified using a protein-G affinity column (MAbTrap G, Pharmacia).

Immunoblotting. For immunoblotting, soluble proteins from heated muscle homogenates were resolved by SDS-PAGE on .75-mm-thick 10% (37.5:1) separating gels, with 4% (37.5:1) stacking gels. Proteins were electrophoretically transferred to nitrocellulose for 2.5 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 90 min. Antibodies were diluted in blocking buffer and incubations were for 1 h at room temperature. Membranes were incubated with primary rabbit anti-calpastatin antibody (2 μg IgG/mL), followed by an alkaline phosphatase conjugated anti-rabbit IgG diluted 1:1,000 (Sigma Chemical Company, St. Louis, MO). Membranes were washed three times with blocking buffer after each incubation. Antibody binding was visualized by exposure to BCIP/NBT (Bio-Rad).

Table 1. Purification of GST-calpastatin

Stage of purification	Calpastatin activity, units/mg protein	Total protein, mg	Fold purification
Cell lysate	15.0	3,230.0	—
Glutathione-sepharose 4B	200.0	56.0	13.3
10% Preparative gel	432.0	9.3	28.8

ELISA Procedure. The method developed is an indirect antibody procedure. Longissimus muscle extracts were prepared as described for the calpastatin activity assay. Protein concentrations of longissimus muscle extracts were determined by the method of Bradford (1976) using premixed reagents (Bio-Rad). Unless otherwise stated, dialyzed and heated skeletal muscle homogenates were serially diluted to 4, 2, and 1 $\mu\text{g}/\text{mL}$ in TBS. Initial sample dilution (4 $\mu\text{g}/\text{mL}$) was done directly in low-protein-binding microtiter wells (Immulon[®] 1 strips, Dynatech Laboratories, Chantilly, VA). Serial dilution was performed by transferring 100 μL of initially diluted sample directly into Immulon 4 wells (Dynatech) containing 100 μL TBS, mixing, and transferring 100 μL /well of this dilution to the next well. Samples (100 μL /well) were incubated in Immulon 4 microtiter wells for 2 h at 37°C. Wells were emptied and washed five times with TBS containing .01% Tween-20 (TTBS), then incubated for 1 h at 37°C with 100 μL /well rabbit anti-calpastatin antibody diluted to 30 $\mu\text{g}/\text{mL}$ IgG in 1%

BSA-TTBS. Wells were then washed five times with TTBS and goat anti-rabbit IgG-peroxidase conjugate (1:1,000 dilution; Sigma A-6154) in 1% BSA-TTBS was applied for 1 h at 37°C. Following five washes with TTBS, 100 μL /well tetramethylbenzidine (TMB; Kirkegaard and Perry Laboratories, Gaithersburg, MD; 50-76-00) substrate was applied, and this resulted in formation of a soluble blue product. After approximately 10 min at room temperature, the reaction was stopped by adding 100 μL /well of 1 M phosphoric acid. The A_{450} of each stopped yellow reaction product was determined using an automated plate reader (Bio-Tek Instruments, Winooski, VT).

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

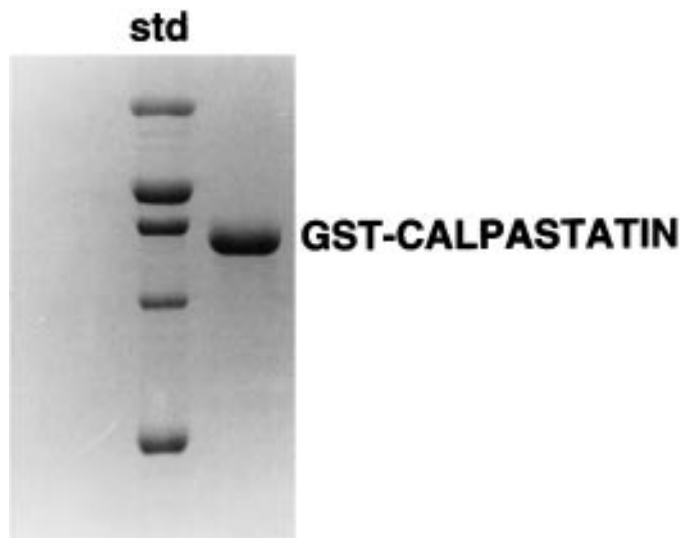


Figure 1. SDS-PAGE analysis of recombinant bovine skeletal muscle calpastatin. The first lane corresponds to molecular weight standards consisting of myosin (200 kDa), b-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). Lane 2 contains a representative 10- μg sample of purified recombinant calpastatin.

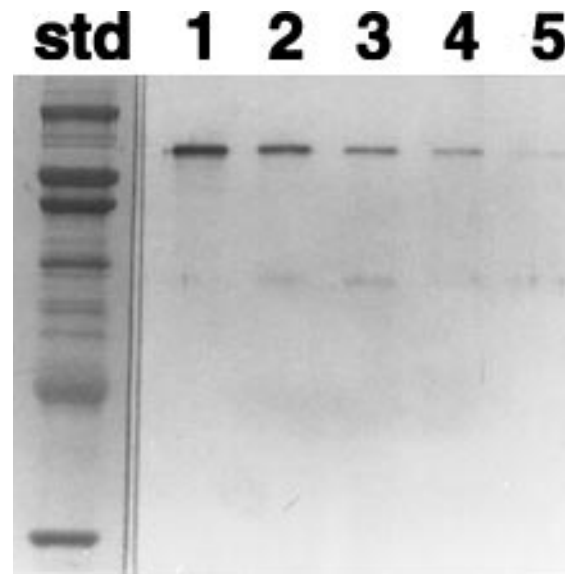


Figure 2. Western immunoblot analysis of lamb longissimus muscle calpastatin. Proteins were resolved on a 10% polyacrylamide gel, then transferred onto Nitrocellulose. The first lane (std) corresponds to molecular weight standards as described in Figure 1 (with the inclusion of carbonic anhydrase, 31 kDa) stained with amido black. Lanes 1-5 represent heated prerigor skeletal muscle extract diluted to contain 40, 20, 10, 5, and 2.5 mU of calpastatin activity per lane, respectively. Membrane was probed with rabbit anti-calpastatin as described in Materials and Methods.

CALPASTATIN ELISA

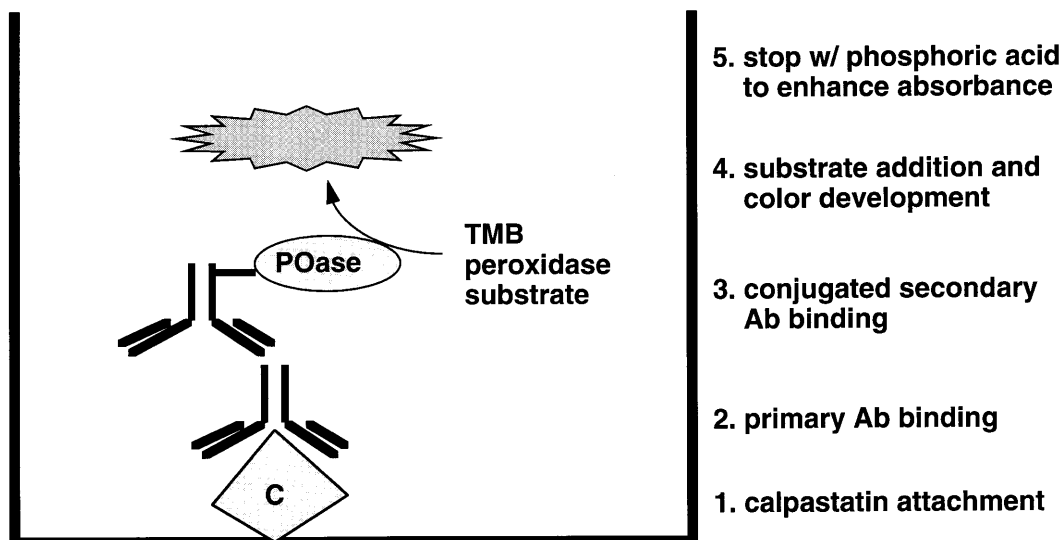


Figure 3. Illustration of steps used to perform the indirect antibody ELISA procedure.

mus muscle samples were taken from carcasses of crossbred lambs (Dorset \times Romanov) and crossbred steers and heifers (25 to 50% *Bos indicus*).

Results

Purification of Bovine Skeletal Muscle Calpastatin. Recombinant calpastatin corresponding to domains 2, 3, and 4 of bovine skeletal muscle calpastatin was expressed in *E. coli* as a 90-kDa fusion protein with glutathione-S-transferase (GST-calpastatin). This fusion protein retained the calpain inhibitory activity characteristic of calpastatin (Table 1). Purification of the fusion protein using glutathione-sepharose 4B chromatography followed by preparative polyacrylamide gel electrophoresis and subsequent elution increased calpastatin specific activity by 28.8-fold (Table 1). Figure 1 illustrates the purity of recombinant calpastatin fractions used for polyclonal antibody production.

Polyclonal Anti-Calpastatin Antibody Characterization. Monospecific polyclonal antibodies were raised in rabbits using recombinant GST-calpastatin as antigen. Western blot analysis revealed that these antibodies recognize a predominant immunoreactive calpastatin band of approximately 130 kDa in prerigor skeletal muscle and, in some samples, interact slightly with a 60-kDa band of unknown origin (Figure 2). The intensity of the calpastatin bands corresponds qualitatively with assayable calpastatin activity in prerigor skeletal muscle (Figure 2) and in beef and lamb meat aged at -1.1°C for 24 h (unpublished observations).

ELISA Development. The ELISA described is an indirect antibody procedure (Figure 3). Optimal concentrations of sample protein, primary antibody, and secondary antibody were determined by titration. Dilution of heated lamb longissimus muscle extracts to 2 to 4 mg of protein/mL of TBS was optimal for coating of Immulon 4 microtiter wells with immunoreactive calpastatin (Figure 4). A set of standards was developed by pooling skeletal muscle extracts with high calpastatin specific activity (mean = 12 U/mg) and extracts with low calpastatin specific activity (mean = 5.2 U/mg), then combining these pooled samples to yield standards with similar protein concentrations, but variable calpastatin specific activity. These standards were used to determine the kinetics of calpastatin binding to Immulon 4 microtiter wells (Figure 5). We selected a sample incubation of 2 h at 37°C , although a 1-h incubation may be used with only a slight ($\approx 8\%$) decrease in total binding. Immulon 2 and 4 plates were found to produce similar results, but Immulon 3 plates were not suitable for detection of calpastatin using this method (data not shown). Blocking the plates for up to 100 min with 1% BSA in TTBS before antibody incubation did not reduce the background signal, presumably caused by nonspecific antibody binding (data not shown). Therefore, a blocking step was not included in the final protocol. Antibody dilutions and incubation times chosen were those resulting in the maximum slope of the calpastatin standard curve (Figure 6a). Conditions chosen for primary anti-calpastatin antibody incubation were $32\ \mu\text{g}$ of IgG/mL (Figure 6b) for 1 h at 37°C (Figure 6c). For optimum binding of secondary antibody, we selected a dilution

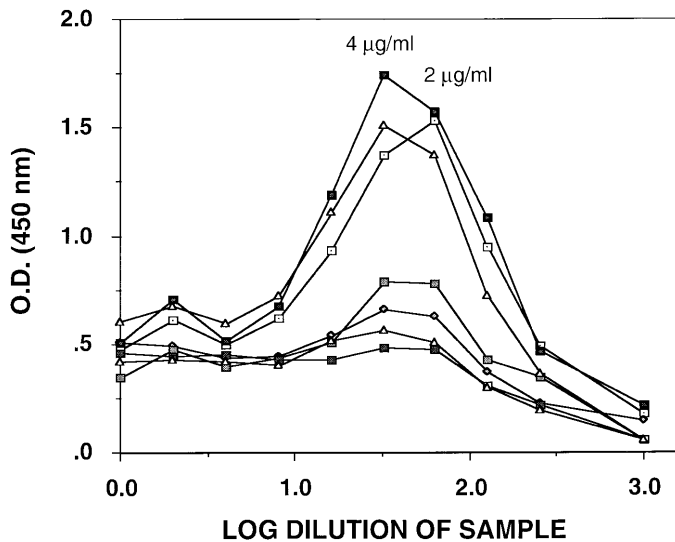


Figure 4. Titration of heated and dialyzed longissimus muscle homogenates results in a dilution optimum for detection of calpastatin by indirect antibody ELISA. Heat-stable proteins from eight randomly chosen lamb longissimus homogenates (mean = $128.8 \pm 21.1 \mu\text{g}$ protein/mL) were serially diluted. Samples ($100 \mu\text{L}$) were incubated in Immulon[®] 4 microtiter wells for 2 h at 37°C , and ELISA was performed as described in Materials and Methods. Each line represents dilution of an individual homogenate.

of 1:1,000 (Figure 7a) and a 1-h incubation at 37°C (Figure 7b). The peroxidase substrate, TMB, was chosen for sensitivity. Following TMB incubation at room temperature for 10 min, the reaction was stopped by addition of 1 M phosphoric acid. Acidification amplified the absorbance of the reaction product approximately threefold (data not shown).

Calpastatin ELISA results were linearly related to calpastatin activity (calpain inhibitory activity) of heated longissimus muscle homogenates from prerigor lamb skeletal muscle ($r^2 = .89$; Figure 8) and postrigor beef samples aged for 24 or 48 h ($r^2 = .90$; Figure 9a). These results were highly repeatable ($r^2 = .95$; Figure 9b). Intraassay CV was $< 5\%$ ($n = 8$) and interassay CV was $< 6\%$ ($n = 5$) for samples with high (12 U/mg) or low (5.2 U/mg) calpastatin specific activity.

Discussion

The calpain proteolytic system plays an important role in postmortem proteolysis and subsequent tenderness of meat. Due to the inverse relationship between postrigor calpastatin activity and meat tenderness, quantification of calpastatin activity is commonly performed by researchers interested in meat tenderness. Calpastatin quantification in skeletal muscle, measured as m-calpain inhibitory activity, was tradi-

Calpastatin activity

(U/mg)

- 12.0
- ◇ 10.6
- 9.3
- ◆ 7.9
- ▣ 6.6
- 5.2
- △ 0.0

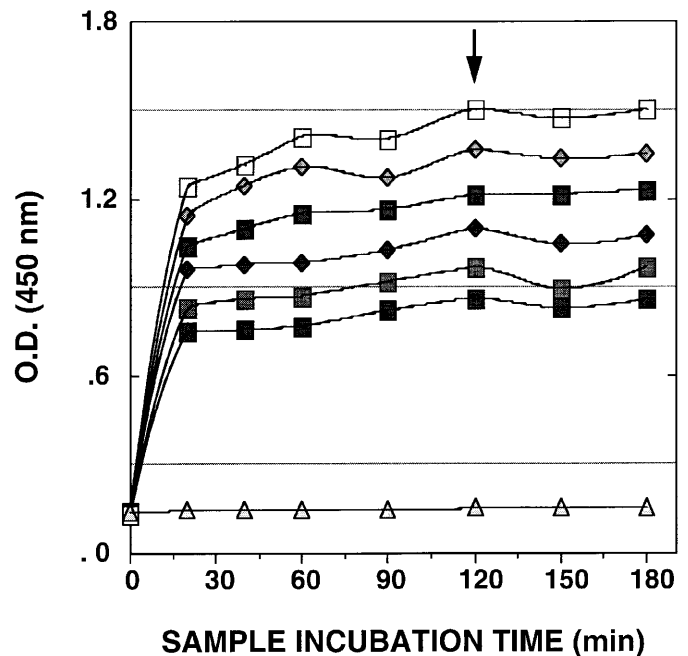


Figure 5. Time course for calpastatin binding to Immulon[®] 4 microtiter wells. Samples containing from 5.2 to 12 U/mg calpastatin specific activity were placed in microtiter well strips at a concentration of $4 \mu\text{g}$ protein/mL. Samples were incubated at 37°C for the indicated times, then wells were washed and ELISA was performed as described in Materials and Methods. Arrow indicates the incubation time chosen for use in the final assay.

tionally performed on samples subjected to ion-exchange chromatography (Koochmariae, 1990). Modification of the method for calpastatin quantification has included heating muscle homogenates to partially purify calpastatin, followed by enzymatic analysis (Shackelford et al., 1994). The current study describes the expression and purification of recombinant bovine skeletal muscle calpastatin suitable for the production of specific polyclonal antibodies. Additionally, we describe the development of a calpastatin immunoassay that accurately predicts calpain inhibitory activity in prerigor and postrigor skeletal muscle. This ELISA requires 10- to 100-fold less sample volume than the conventional and tedious enzymatic

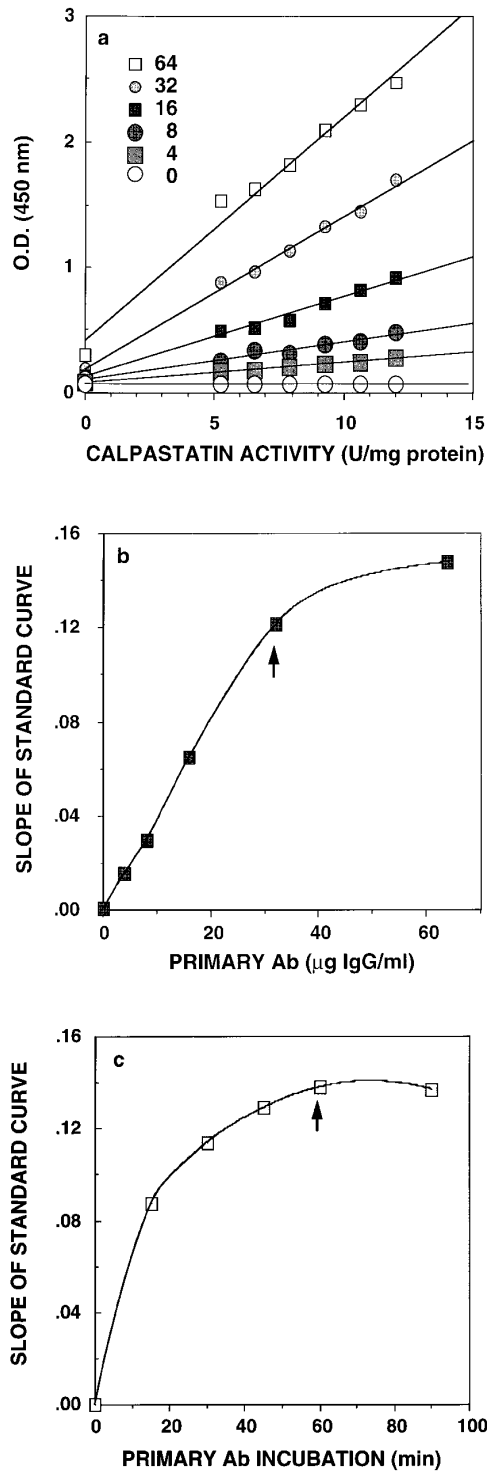


Figure 6. Optimization of primary antibody binding conditions. Samples ranging from 5.2 to 12 U/mg calpastatin served as standards, and standard curves (a) were assayed using primary antibody concentrations ranging from 0 to 64 $\mu\text{g IgG/mL}$ TTBS containing 1% BSA. The slope of each standard curve was plotted against primary antibody concentration (b). Primary antibody incubation time (c) was optimized by quantification of standards using 32 $\mu\text{g IgG/mL}$ incubated for up to 90 min at 37°C. Arrows indicate conditions chosen for use in the final assay. Assay conditions not being tested were as described in Materials and Methods.

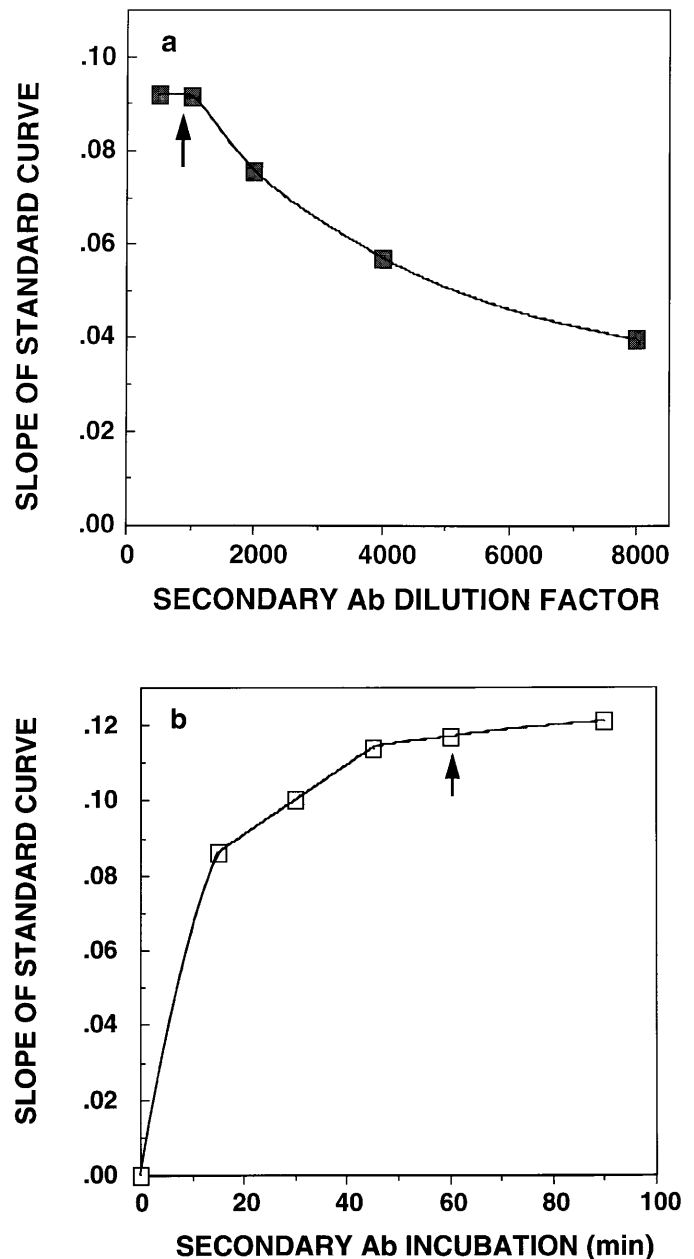


Figure 7. Optimization of secondary antibody binding conditions. Standards used were as described in the legend of Figure 6. Secondary antibody dilutions were from 1:500 to 1:8,000, and the slope of the standard curve was plotted against each secondary antibody concentration tested (a). Secondary antibody (diluted 1:1,000) was incubated at 37°C for the times indicated (b). Arrows indicate conditions chosen for use in the final assay. Assay conditions not being tested were as described in Materials and Methods.

methodology for calpastatin quantification. Furthermore, it is possible to rapidly and simultaneously quantify calpastatin in several hundred samples using the ELISA method with relatively little automation.

In the current study, calpastatin antibodies were raised against a fusion protein containing domains

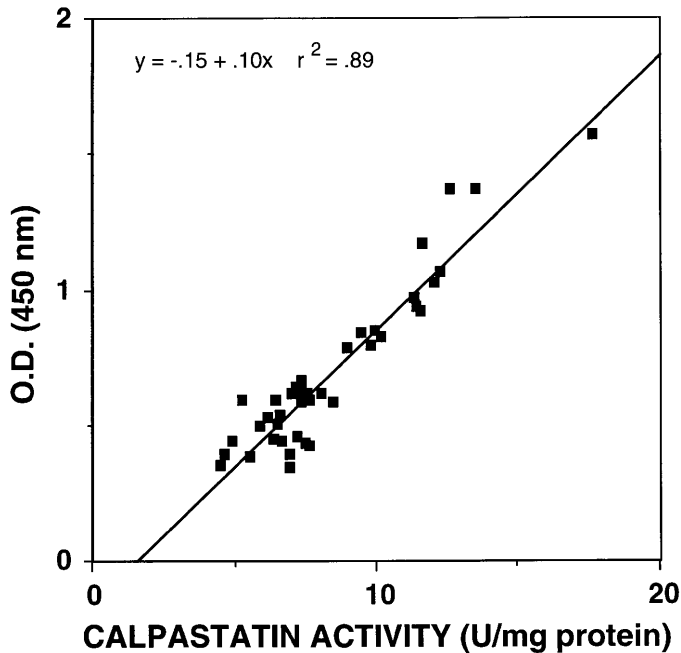


Figure 8. Relationship between calpastatin ELISA and conventional enzymatic assay of dialyzed and heated homogenates ($n = 40$) from prerigor lamb longissimus. Assays were performed as described in Materials and Methods. A strong, positive relationship ($r^2 = .89$) exists between ELISA results and enzymatic assay of calpastatin activity.

2–4 of recombinant bovine calpastatin. Production and purification of recombinant skeletal muscle calpastatin alleviated the more difficult task of purifying this protein from skeletal muscle. Polyclonal antibodies raised against the recombinant calpastatin fusion protein specifically recognize a 130-kDa protein in prerigor skeletal muscle homogenates. This is consistent with reported values for the molecular mass of muscle calpastatin (Croall and DeMartino, 1991). Additionally, these antibodies recognize calpastatin derived from bovine, ovine, and porcine skeletal muscle (our unpublished observations).

Immunoassays for quantification of calpastatin have been described previously. Takano et al. (1984) described a sandwich enzyme immunoassay for measuring erythrocyte calpastatin using polyclonal antibodies. We initially attempted to develop a sandwich immunoassay using the rabbit polyclonal anti-calpastatin antibodies described in the current study as capture antibodies bound to Immulon 3 microtiter wells. Biotinylated rabbit anti-calpastatin antibodies, subsequently detected with avidin-peroxidase, or polyclonal antibodies raised in mice against the same recombinant calpastatin antigen served as the second antibody. Unfortunately, these sandwich immunoassays did not afford sufficient sensitivity to consistently detect differences in skeletal muscle calpastatin. We attribute the lack of sensitivity of these assays to

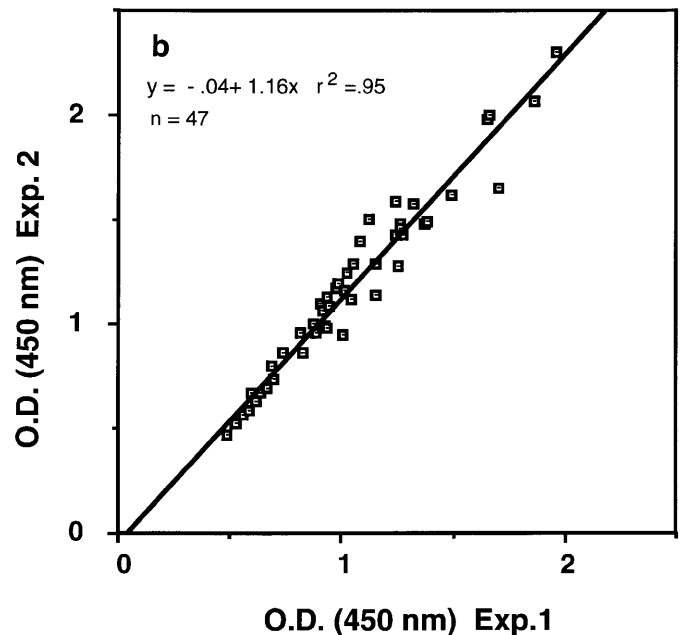
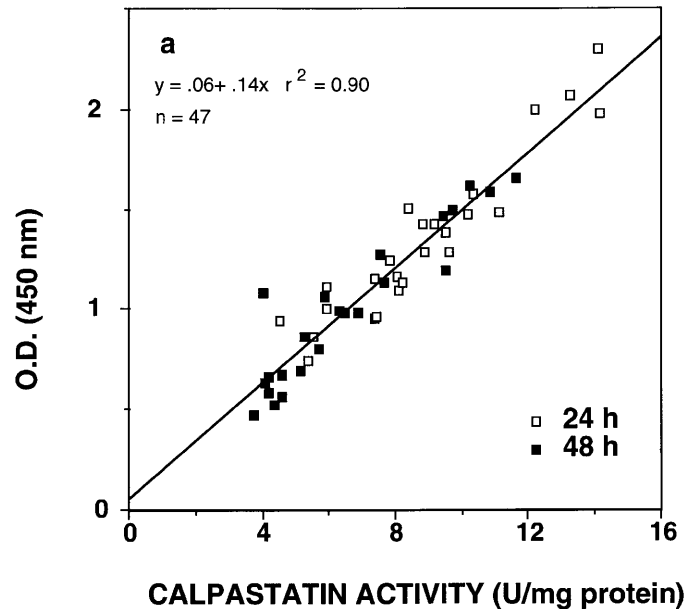


Figure 9. Relationship between calpastatin ELISA and conventional enzymatic assay of heated and dialyzed homogenates from postrigor beef longissimus. Beef longissimus samples were taken at 24 h (open squares, $n = 24$) or 48 h (filled squares, $n = 23$) postmortem. Assays were performed as described in Materials and Methods. A strong positive relationship exists between ELISA results and enzymatic assay of calpastatin activity (a), and this relationship was similar for both sampling times. Results of ELISA performed on these samples on separate days indicate that the assay is highly repeatable (b).

competition for common epitopes, since the capture and secondary antibodies were raised against the same antigen.

Two-antibody sandwich ELISA that measure human calpastatin subtypes have been reported (Yokota et al., 1991). However, it is unclear whether these assays are suitable for quantifying calpastatin in postmortem skeletal muscle of meat animal species. Additionally, the cost of antibodies required for these assays may be prohibitive, when calpastatin data on numerous samples are desired.

Two-antibody sandwich ELISA are the most commonly used immunoassays due to their speed, accuracy, and ability to quantify specific proteins present in complex mixtures of proteins. In contrast, the antibody capture ELISA described herein is contingent on the binding of calpastatin to microtiter wells having high protein binding capacity. Because the binding of proteins to microtiter wells is assumed to be nonspecific (e.g., minimal preference for binding of one protein species vs another), it is essential that samples of interest be uniformly extracted to yield similar protein concentrations, and that equal quantities of protein be loaded into microtiter wells. The necessity for uniform sample preparation is the only apparent drawback of the indirect antibody method described, when compared to the more common two-antibody sandwich ELISA. Despite this limitation, Doumit and Koohmaraie (1996) found that sample preparation suitable for calpastatin quantification and comparison among samples can be readily accomplished, using only a 250-mg muscle sample, and no sample dialysis. Furthermore, the method described effectively predicts calpastatin activity at 24 to 48 h postmortem, at which time degraded, but active, forms of calpastatin are present. It is unclear whether this could be accomplished with a two-antibody sandwich ELISA, which requires the presence of two specific epitopes for detection of the protein. We have continued to develop the indirect antibody approach because to date we have been unable to identify other polyclonal or monoclonal antibodies that recognize the degraded forms of calpastatin as effectively as the antibodies used in this study.

Implications

Polyclonal antibodies raised against recombinant bovine skeletal muscle calpastatin are suitable for detection of calpastatin by Western blot analysis and accurate quantification of calpastatin by enzyme immunoassay. These tools will complement studies designed to assess the antagonistic relationship be-

tween skeletal muscle calpastatin activity and calpain-induced proteolysis during growth and postmortem meat tenderization.

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