Bovine Skeletal Muscle Calpastatin: Cloning, Sequence Analysis, and Steady-State mRNA Expression[1]

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ABSTRACT: Calpastatin is a specific inhibitor of the calpains. Calpains play a key role in postmortem tenderization of meat and have been hypothesized to be involved in muscle protein degradation in living tissue. Isolation, cloning of complementary DNA, and nucleotide sequencing of bovine calpastatin from the longissimus muscle have been completed. Two clones were identified that encompass the entire coding sequence. Clone pCR41, derived by reverse transcription-PCR, covers domains L and 1; clone pBSA1, obtained from cDNA library screening, covers domains 2 through 4 in addition to the 3′-nontranslated region. Nucleotide sequence analysis of the cDNA for bovine calpastatin revealed an average nucleotide sequence identity of approximately 70 to 80% compared with published calpastatin nucleotide sequences of human, rabbit, and pig. Exon 3, corresponding to a highly conserved 22-amino acid region, was deleted from bovine calpastatin domain L. The calculated molecular weight of bovine skeletal muscle calpastatin of 706 amino acid residues (M_r 75,842) corresponds to the value of purified bovine skeletal muscle calpastatin as determined by SDS-PAGE (M_r 68,000). Northern blot analysis revealed the presence of multiple calpastatin mRNA transcripts having estimated sizes of 3.8, 3.0, and 1.5 kb in beef and 3.8, 3.0, 2.5, and 1.5 kb in sheep. Calpastatin mRNA expression was increased with β-adrenergic agonist-induced muscle hypertrophy.

Key Words: Calpastatin, Muscles, DNA, Sequences, Expressivity, Beta-Adrenergic Agonist

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Introduction

The calcium-dependent proteolytic system (calpains) is considered to be one of the major cytosolic proteolytic systems. Calpains have been hypothesized to be involved in a variety of cellular processes (for review see Croall and DeMartino, 1991). In living muscle, the calpains are considered to be the primary candidate for initiating the degradation of myofibrillar proteins (for review see Goll et al., 1992). Current evidence suggests that in postmortem tissue the calpains are responsible for proteolysis of those myofibrillar proteins whose degradation causes the weakening of the myofibrillar structure and, therefore, meat tenderization (for review see Koohmaraie, 1992a,b,c; Koohamaraie et al., 1993).

Calpastatin is an endogenous inhibitor of the calpains (EC 3.4.22.17, Ca^{2+}-dependent cysteine proteinase) inhibiting both forms of calpains (low-calcium-requiring form called μ-calpain and a high-calcium-requiring form called m-calpain). Calpastatin is, therefore, considered to be one of the major modulators of the calpains. Some studies have reported that in living muscle, when protein degradation is reduced, the activity of calpastatin is increased (Wheeler and Koohmaraie, 1992; Morgan et al., 1993). Additionally, there is ample evidence to suggest that calpastatin is a principal regulator of the calpains in postmortem proteolysis (for review see Koohmaraie et al., 1994). Because of these significant functions of calpastatin and because its sequence in skeletal muscle has not been determined, the objective of the present work was to clone and sequence the bovine skeletal muscle calpastatin and examine the steady-state expression of its messenger RNA in beef steers having differences in calpastatin activity.

[1]Names are 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 USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Materials and Methods

Tissue for cDNA Library Preparation. Muscle samples were collected from the longissimus muscle (**LM**) of a steer at the 12th-13th rib interface within 20 min of exsanguination. Muscle tissue was trimmed of fat, minced, rapidly frozen by immersion in liquid nitrogen, and stored at −70°C until it was processed.

RNA Preparation. Total muscle cellular RNA was isolated from 10 g of frozen sample according to the method of Chomczynski and Sacchi (1987). Total RNA was quantified by reading its absorbance at 260 nm and then stored at −80°C. Poly (A^+) RNA was isolated from muscle total RNA by oligo (dT)-cellulose chromatography as described by Aviv and Leder (1972) using two passages through separate 1-mL columns. The poly (A^+) RNA was quantified and stored as described above.

cDNA Synthesis and Library Construction. A cDNA library representing the bovine LM poly (A^+) RNA was constructed using 5 μg of poly (A^+) RNA in the Uni-ZAP™ XR cloning vector as described by the manufacturer (Stratagene, La Jolla, CA). Synthesized cDNA were size-fractionated on a Sepharose CL-4B column and larger cDNA populations (> 700 bp) were pooled. The cDNA was unidirectionally ligated into the Uni-ZAP™ XR vector arms, packaged in vitro, and amplified in the PLK-F′ (mcrA-, mcrB-) host strain.

Screening of cDNA Library. Approximately 5×10^6 recombinant phage were plated on 150-mm NZ-yeast broth (NZY) plates (5×10^3 plaque-forming units[pfu]/plate) with liquid broth (LB) top agar. Plates were incubated overnight at 37°C and then placed at 4°C for 4 h. Replicate plaque lifts were made, denatured, and UV-crosslinked (1,200 μJ/cm^2) to nylon membranes (MagnaGraph, Micron Separations, Westboro, MA). Membranes from each replicate lift were probed into ^{32}P-labeled random primed cDNA probes to rabbit calpastatin (λC1-21 and λC1-413; Emori et al., 1987; a generous gift to M. K. from Y. Emori) labeled to a specific activity of 1×10^9 cpm/μg of cDNA. Prehybridization and hybridization solutions consisted of 50% deionized formamide, 5× SSC (1× SSC is 150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0), 1× PE (50 m*M* Tris-HCl, pH 7.5; .1% wt/vol sodium pyrophosphate; 1% SDS; .2% polyvinylpyrolidone; .2% ficoll; 5 m*M* EDTA), and 150 μg/mL of denatured salmon sperm DNA. Membranes were prehybridized at 42°C for 3 h and then hybridized overnight at 42°C in fresh hybridization solution containing cDNA probes. Fifteen-minute membrane washes were performed at 65°C with two washes in 2× SSC, .1% SDS and two washes in .1× SSC, .1% SDS. Membranes were air-dried and positive signals visualized on Kodak X-OMAT™-AR film exposed overnight at −70°C. Clones giving positive signals were plaque-purified and rescreened two additional times to eliminate false positives.

Conversion of ssDNA Phage into dsDNA Plasmid. The Uni-ZAP™ XR cDNA phage clones were converted into respective pBluescript plasmid counterparts by in vivo excision according to the manufacturer's protocols to produce double-stranded DNA pBluescript plasmids containing cDNA inserts. Double-stranded replicative DNA was isolated from host bacteria and the cDNA insert excised by double digestion of the plasmid with *Xho*I and *Eco*RI restriction enzymes. Complete digestion of pBluescript DNA was confirmed by agarose gel electrophoresis and positive calpastatin pBluescript clones were reconfirmed by screening dot blots (Sambrook et al., 1989) with ^{32}P-labeled random primed rabbit cDNA (lC1-21 and lC1-413) probes.

Nucleotide Sequencing of Bovine Calpastatin. Nucleotide sequencing was performed on double-stranded plasmid DNA according to the method of Sanger as described in the procedures provided with Sequenase Version 2.0 (United States Biochemicals, Cleveland, OH). Initial sequence data were generated using the forward and reverse M13 sequencing primers. Additional sequencing primers were designed from the derived nucleotide sequence until both strands were completely sequenced. Comparison and manipulation of bovine calpastatin sequence data were performed with the aid of the GCG program (Devereux et al., 1984).

Reverse Transcription-Polymerase Chain Reaction Cloning of the 5′-End of Bovine Calpastatin. A sense primer P1, CTCTGCTGCAGCIAGCAAGITCIII-CAGIATG (Lee et al., 1992b) as a universal calpastatin amino-terminal primer and an antisense primer P2, $(GA)_{10}$ ACTAGTCTCGAGAGTTCTGATTCACT-CAGGGG to derived sequence in bovine domain 2 were synthesized.

Reverse transcription (**RT**; adapted from ZAP-cDNA Synthesis Protocol, Stratagene, La Jolla, CA) was carried out in a final reaction volume of 50 μL of the following reaction mixture: 50 m*M* Tris-HCl (pH 8.3), 100 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol, .6 m*M* each dinucleotide triphosphate, 50 p*M* primer P2, 1 unit RNase Block II, 45 units of Moloney-Murine Leukemia Virus Reverse Transcriptase (**M-MuLVRT**), and 30 μg of bovine LM total RNA as template. Template and primer were annealled at room temperature for 15 min before addition of M-MuLVRT. The reaction was performed at 37°C for 1 h followed by heating to 95°C for 5 min and centrifugation at 14,000 × *g* for 5 min. The resultant supernatant was used as cDNA template for PCR amplification.

The PCR was carried out in a MJ Research Thermal Cycler (Watertown, MA) using the following conditions: P1- and P2-primed PCR was repeated for 30 cycles with a single initial denaturation at 94°C for 2 min followed by cycling conditions of 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min in a volume of 50 μL. Reaction mixture concentrations were 50 m*M* KCl, 10 m*M* Tris-HCl (pH 9.0), 1.5 m*M* $MgCl_2$, .01% gelatin (wt/vol), .1% Triton X-100, .2 m*M* each dNTP, 1.0 μ*M* each primer, and 2.5

μg of reverse transcription product. Ethidium bromide-stained DNA fragments were visualized on 1% agarose gels.

Cloning of 5′ End of Calpastatin Reverse Transcription-Polymerase Chain Reaction Products. For cloning, RT-PCR products were cloned into the pCRII cloning vector using the TA Cloning System Version 1.3 (Invitrogen, San Diego, CA). Briefly, 20 ng of PCR product was ligated to 50 ng of linearized vector at 4°C overnight. The ligation mixture was used to transform competent host cells. Transformants were selected on LB agar plates 525 containing 50 μg/mL of ampicillin.

Animals for Calpastatin mRNA Expression. Eight MARC III composite (1/4 each Hereford, Angus, Pinzgauer, and Red Poll) steers weighing approximately 350 kg were randomly assigned to control and β-adrenergic agonist-fed (**BAA**) treatment groups. Steers were allowed ad libitum access to a standard diet (17.3% CP) with or without 3 ppm of $L_{644,969}$, 6-amino-alpha-([(1-methyl-3-phenylpropyl)-amino]methyl)-3-pyridine methanol dicholoride (Merck, Sharp, and Dohme, Rahway, NJ) for 6 wk. For specifics of feed composition, feeding, animal handling, and carcass and muscle measurements, see Wheeler and Koohmaraie (1992). Sixteen crossbred wether lambs (1/2 Finnsheep, 1/4 Dorset, 1/4 Rambouillet), all within 2 wk of age, were randomly assigned to either control (18% CP) or BAA (4 ppm $L_{644,969}$) treatment groups for 6 wk. For results of growth, endogenous proteinase activities, and postmortem proteolysis, see Koohmaraie et al. (1991).

RNA Isolation and Northern and Slot Blot Analysis. Muscle samples were removed from LM and stored as described before. Isolation of total RNA and poly(A+) RNA were performed as described earlier. Northern blot analysis and slot blot analysis were done according to Sambrook et al. (1989) as described previously (Koohmaraie et al., 1991). ^{32}P random-primed cDNA probes used in calpastatin message analysis were derived from excised cDNA inserts encoding bovine calpastatin domains L-1 (clone pCR41) or domains 2 to 4 including the 3′ non-coding region (clone pBSA1).

Statistical Analysis. Data were analyzed by analysis of variance for a completely randomized design to test the effect of BAA treatment with the GLM procedures of SAS (1985). Data from beef and lamb studies were analyzed separately.

Results and Discussion

Identification and Isolation of Bovine Clones for Calpastatin. A cDNA library was produced representing the mRNA from bovine LM. Two cDNA clones developed to rabbit heart calpastatin (Emori et al., 1987) were used as probes for screening the bovine cDNA library. Screening at a high stringency using rabbit calpastatin cDNA as a probe failed to detect any calpastatin-containing clones in the bovine skeletal muscle cDNA library, suggesting that there is some divergence in DNA sequence among calpastatins from different species (Emori et al., 1987; Tankano et al., 1988; Asada et al., 1989; Maki et al., 1991). Variation in calpastatin primary sequence among species is further supported by the low level of hybridization of rabbit cDNA probes to bovine LM RNA upon Northern blot analysis (data not shown). Approximately 5×10^6 plaques of the bovine cDNA library were screened, resulting in numerous false positives as determined by dot blotting and sequencing. Careful attention to hybridization conditions and washing stringencies resulted in three clones that could be identified as containing the bovine calpastatin sequence. From restriction enzyme digestion determination of insert cDNA size, a single clone containing a cDNA insert of approximately 2.2 kb was isolated (clone pBSA1, accession no. L14450) and used in subsequent studies. Nucleotide sequencing strategy is shown in Figure 1. Nucleotide sequencing and database comparisons of pBSA1 verified that a bovine calpastatin cDNA clone had been isolated. Sequence analysis also indicated that pBSA1 contained only domains 2 through 4 and the 3′-nontranslated region, and that the 5′-end of bovine calpastatin was not contained in pBSA1. The lack of domains L and 1 suggested that either bovine LM messenger RNA calpastatin is truncated as in anucleated erythrocytes (Maki et al., 1991) or that pBSA1 contained only a portion of the full length message. A RT-PCR strategy was devised and implemented that used a degenerate universal calpastatin primer, P1, located upstream of the expected translational start site and a second primer, P2, designed to elucidate the bovine sequence contained at the border of domains 1 and 2 (Figure 1). Analysis of RT-PCR products on 1% agarose gels

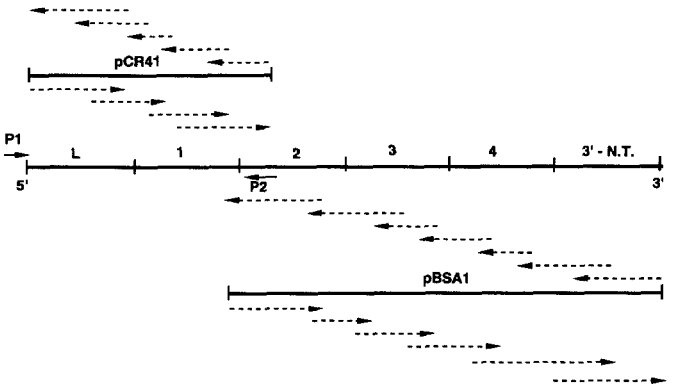

Figure 1. Schematic illustrating the sequencing strategy, position of reverse transcription-Polymerase Chain Reaction (RT-PCR) primers, and domain structure of the cDNA clones to bovine skeletal muscle calpastatin. Clone pBSA1 was obtained through screening of bovine LM cDNA libraries. Clone pCR41 was obtained through RT-PCR amplification of bovine LM RNA and subsequent cloning into the pCRII vector (Invitrogen). Arrows indicate length and direction of sequencing.

indicated the presence of two PCR products, one a major product of approximately 1.2 kbp and the other a minor product of approximately 650 bp. Cloning of the larger RT-PCR product (1.2 kbp), subsequent restriction enzyme analysis, and sequencing indicated a clone containing the appropriate 1.2-kb insert cDNA (pCR41, accession no. L14450). Sequencing of RT-PCR products was performed on two separate cloning reactions to confirm sequence data.

Amino Acid Sequence of Calpastatin. The longest open reading frame deduced from the nucleotide sequence codes for 706 amino acid residues (Figure 2). Comparisons with other reported calpastatin cDNA sequences indicated that domain L of bovine calpastatin contains a 22 amino acid deletion (Figure 2) corresponding to exon 3 of human calpastatin (Lee et al., 1992b). The significance of this deletion has not been ascertained, but it is speculated that deletions

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-27  TCT GCT GCA GCG AGC AAG TCG GGC AGG ATG AAT CCC ACA GAA GCC AAG GCT GTA AAA ACA GAA CCT GAG AAG AAG CCA CAA TCA TCT AAG CCA TCT GTG GTT CAT    77
 -9                              Met Asn Pro Thr Glu Ala Lys Ala Val Lys Thr Glu Pro Glu Lys Lys Pro Gln Ser Ser Lys Pro Ser Val Val His    25

 78  GAG AAA AAA ACC CAA GAA GTA AAG CCA AAG GAA CAC ACA GAG CCA AAA AGC CTA CCC AAG CAC TCA TCA GAT ACA GGA GTC AAG CAT GCT CCT AAG GAA AAA GCC   182
 26  Glu Lys Lys Thr Gln Glu Val Lys Pro Lys Glu His Thr Glu Pro Lys Ser Leu Pro Lys His Ser Ser Asp Thr Gly Val Lys His Ala Pro Lys Glu Lys Ala    60

183  GTT TCC AAA TCA AGT GAG CAG CCA CCA TCA GAG AAA TCA ACA AAA CCA AAG ACC AAG TCA CAG GAC AAG ATC TCC GGT GGT GGA AAG AGC ACT GTT CCT GCT GCT   287
 61  Val Ser Lys Ser Ser Glu Gln Pro Pro Ser Glu Lys Ser Thr Lys Pro Lys Thr Lys Ser Gln Asp Lys Ile Ser Gly Gly Gly Lys Ser Thr Val Pro Ala Ala    95

288  GCT GCT GCA GCA TCT GCC GAA CCA GCT GAC AAG AAT AAA GAA AAT AAA TTG TTA ACA TCG GCC GTA CCA GCT GAA TCT AAA CCA AGT AAA CCA TCT GGA AAG TCA   392
 96  Ala Ala Ala Ala Ser Ala Glu Pro Ala Asp Lys Asn Lys Glu Asn Lys Leu Leu Thr Ser Ala Val Pro Ala Glu Ser Lys Pro Ser Lys Pro Ser Gly Lys Ser   130

393  GAC ATG GAC ACT GCT CTG GAT GAC TTA ATA GAC ACT TTA GGA GAA CCT GAA GAG ATG AAA GAA GAT AAC ACA ACA TAT ACC GGA CCG GAA GTG TCG GAT CCG ATG   497
131  Asp Met Asp Thr Ala Leu Asp Asp Leu Ile Asp Thr Leu Gly Glu Pro Glu Glu Met Lys Glu Asp Asn Thr Thr Tyr Thr Gly Pro Glu Val Ser Asp Pro Met   165

498  AGT TCT ACC TAC ATA GAG GAA CTG GGT AAA AGA GAA TCC ACA CCT CCT CCA AAA TAT AAG GAA CTT CTG AAT AAA GAA GAA GGG ATC GCA GGG CCT CCT CCA GAC   602
166  Ser Ser Thr Tyr Ile Glu Glu Leu Gly Lys Arg Glu Ser Thr Pro Pro Pro Lys Tyr Lys Glu Leu Leu Asn Lys Glu Glu Gly Ile Ala Gly Pro Pro Pro Asp   200

603  TCC TTG AAA CCC CTG GGG CCC AAT GAT GCC ATC GAT GCC TTG TCA TCC GAC TTC ACC TGC AGT TCC CTA CAG CTG ACT ACC TGC AGT CCT ACA GCT GAT GGC AAG   707
201  Ser Leu Lys Pro Leu Gly Pro Asn Asp Ala Ile Asp Ala Leu Ser Ser Asp Phe Thr Cys Ser Ser Leu Gln Leu Thr Thr Cys Ser Pro Thr Ala Asp Gly Lys   235

708  GAA ACT GAG AAA GAG AAA TCT ACA GAA GAG GCT TTA AAA GCT CAG TCA GCT GGG GTG ATC AGA AGT GCT GCT CCA CCC AAA GAG AAA AGA AGA AAA GTG GAA GAG   812
236  Glu Thr Glu Lys Glu Lys Ser Thr Glu Glu Ala Leu Lys Ala Gln Ser Ala Gly Val Ile Arg Ser Ala Ala Pro Pro Lys Glu Lys Arg Arg Lys Val Glu Glu   270

813  GAT ACC ATG ACT GAG CAA GCC CTC CAG GCC CTG TCT GCC TCC CTT GGC ACC CGG AAG CCA GAG CCG GAG CTC GAC CCC AGC TCC ATT AGG GAG GTC GAT GAG GCA   917
271  Asp Thr Met Thr Glu Gln Ala Leu Gln Ala Leu Ser Ala Ser Leu Gly Thr Arg Lys Pro Glu Pro Glu Leu Asp Pro Ser Ser Ile Arg Glu Val Asp Glu Ala   305

918  AAA GCC AAA GAA GAG AAA GTA AAG AAA TGT GGT GAA GAT GAG GAA ACA GTC CCA TCG GAG TAC AGA TTA AAA CCG GCC ACA GAT AAA GAT GGA AAA CCA CTC TTG  1022
306  Lys Ala Lys Glu Glu Lys Val Lys Lys Cys Gly Glu Asp Glu Glu Thr Val Pro Ser Glu Tyr Arg Leu Lys Pro Ala Thr Asp Lys Asp Gly Lys Pro Leu Leu   340

1023  CCA GAG GCT GAA GAA AAA CCC AAG CCC CTG AGT GAA TCA GAA CTC ATT GAT GAA CTC TCA GAA GAT TTT GAC CAG TCT AAG CCA ACT GAA AAA CAA TCT AAG CCA  1127
341  Pro Glu Ala Glu Glu Lys Pro Lys Pro Leu Ser Glu Ser Glu Leu Ile Asp Glu Leu Ser Glu Asp Phe Asp Gln Ser Lys Pro Thr Glu Lys Gln Ser Lys Pro   375

1128  ACA GAA AAA ACA GAG GCA TCC CCT GCC GCT GCC CCG TAC CCC GTG GCA GAG GAC GTG CCT CGG ACC TCT ATG TGT TCC CTG CAG TCG GCT CCG CCC ACA GCA GCT  1232
376  Thr Glu Lys Thr Glu Ala Ser Pro Ala Ala Ala Pro Tyr Pro Val Ala Glu Asp Val Pro Arg Thr Ser Met Cys Ser Leu Gln Ser Ala Pro Pro Thr Ala Ala   410

1233  CCA GCG AAG GGC ATG GTG CCA GAC GAT GCT GTC GAA GCG CTT GCT GGA AGC CTC CCG AAA AAG GAA GCA GAT CCA GAA GAC GGA AAG CCT GTG GAG GAT AAA GTC  1337
411  Pro Ala Lys Gly Met Val Pro Asp Asp Ala Val Glu Ala Leu Ala Gly Ser Leu Pro Lys Lys Glu Ala Asp Pro Glu Asp Gly Lys Pro Val Glu Asp Lys Val   445

1338  AAG GAG AAA GCC AAA GAA GAG GAT CGT GAG AAC TTC GGT GAA AAG GAA GAA ACG ATT CCT CCT GAT TAC AGA TTA GAA GAA GCC AAG GAT AAA GAC GGA AAA CCA  1442
446  Lys Glu Lys Ala Lys Glu Glu Asp Arg Glu Asn Phe Gly Glu Lys Glu Glu Thr Ile Pro Pro Asp Tyr Arg Leu Glu Glu Ala Lys Asp Lys Asp Gly Lys Pro   480

1443  CTG CTG CCA AAA GAG GTC AAG GAA CCG CTC CCA CCC TTG AGT GAA GAC GTC CTC CTC GAT GCT CTG TCC AAG GAC TTC ACT GTC CCC TCA GAC ACA TCA TCG CCT  1547
481  Leu Leu Pro Lys Glu Val Lys Glu Pro Leu Pro Pro Leu Ser Glu Asp Val Leu Leu Asp Ala Leu Ser Lys Asp Phe Thr Val Pro Ser Asp Thr Ser Ser Pro   515

1548  CAA TTT GAA GAT GCT AAA CTT TCA GCT GTC GTC TCT GAA GTG GTT TCC CAA ACC CCA GCT CCA ACC ACC CAG GCA GCC GGT CCA CCC CCA GAC TGC GCA CGT GAC  1652
516  Gln Phe Glu Asp Ala Lys Leu Ser Ala Val Val Ser Glu Val Val Ser Gln Thr Pro Ala Pro Thr Thr Gln Ala Ala Gly Pro Pro Pro Asp Cys Ala Arg Asp   550

1653  AAC AAA GAA CTT GAC GAT GCC CTG GAT CAA CTT TCT GAC ACT CTC GGG CAA AGA CAG CCT GAT CCA GAT GAG AAT AAA CCC GTA GAG GAT AAA GTC AAG GAA AAA  1757
551  Asn Lys Glu Leu Asp Asp Ala Leu Asp Gln Leu Ser Asp Thr Leu Gly Gln Arg Gln Pro Asp Pro Asp Glu Asn Lys Pro Val Glu Asp Lys Val Lys Glu Lys   585

1758  GCC AAA GCT GAA CAC AGA GAC AAG CTG GGA GAA AGA GAT GAC ACC ATC CCA CCT AAA TAC CAA CAT CTT TTG GAT GAC AAC AAG GAG GGC ACA CCC GGG AAG CCA  1862
586  Ala Lys Ala Glu His Arg Asp Lys Leu Gly Glu Arg Asp Asp Thr Ile Pro Pro Lys Tyr Gln His Leu Leu Asp Asp Asn Lys Glu Gly Thr Pro Gly Lys Pro   620

1863  AAG GAT CAG AGA GCC CAA GGC ATC AGG AAC TGC GGA GAG AAA CCT GCA GGT GCC CAG GAC CCC ATT GAT GCC CTC TCA GGG GAC TTT GAC AGC TGT CCC TCG ACT  1967
621  Lys Asp Gln Arg Ala Gln Gly Ile Arg Asn Cys Gly Glu Lys Pro Ala Gly Ala Gln Asp Pro Ile Asp Ala Leu Ser Gly Asp Phe Asp Ser Cys Pro Ser Thr   655

1968  ACA GAA ACC TCG ACA GAC ACA CCA AAG GAC AAA GAC AAG AAG CCT GCT TCA GTG CCG AAG CAC CTA GGG AAT GGC GGG AAA GCA AAG GAT TCC ACA AAG GCA AAG  2072
656  Thr Glu Thr Ser Thr Asp Thr Pro Lys Asp Lys Asp Lys Lys Pro Ala Ser Val Pro Lys His Leu Gly Asn Gly Gly Lys Ala Lys Asp Ser Thr Lys Ala Lys   690

2073  GAG GAA ACT TCC AAG CCA AAA GCT GAT GGA AAA AGT ACA AGT TAA AAG TTC ACA CTA TTT GGT ATC TGC ATA TAA AAT CTT CAG CGG GTA GAT GGT GAC TTT CTG  2177
691  Glu Glu Thr Ser Lys Pro Lys Ala Asp Gly Lys Ser Thr Ser End                                                                              725

2178  AAG AAG AAA AGG CTT TGA TAA CAG AAA CAA TTT CTG GGT GGC TTG GAG ACA GTG GTA TTT GCT GAG TCT TTT GAC CTC CTA AAC ATT GTC TGT TAT TCT TTT CCT  2282

2283  GAA AAG AAA CTG AAT TTG TCT GGT TCA CCT GTG TTA TTC TAC TGA GTA TTG ATA AAC TTT AAA TTT TCG AAG AAA CGA TTG TCT GGT CAC TGT GTA TCT ACT GAG  2387

2388  TAT GAT AAC TTA ATT TAA ATG CCT CGT TGG AGA GAA GGA ACT TAT ATT CTA GAG ATA CAT TGA TAG TTC TAA AGC AGC ACA CAA AAA GAA AAC CTT GCA AAC TTT  2492

2493  TGC ACA TTC TCC CCA CAG TGC CTG TAA ATC TCA TTA GTA TTT TCA ATT TGC ACT TAA TTT TGT TGT TAG CAT TTG GAA AAC GAT GCC TCA CGT GTT CTT CAG TGT  2597

2598  TCT GAT TTC TCA TGA CCC CTT TCC TCT TGG ACT TGT GGG ACT GTG TTT GAT GTT TCT CTG GGT TTG TGT TAT AAG ACA TAA ATA CTG TGC ATT GGG CAC ATG TCT  2702

2703  CCT CTT GAG CTG CTA ATC GTA GAT GAC CCT GAC AGA CAG GAA GGG GGC ACC CCC CCT TTC AGG TTG AAC TTC TAT TTG CCA GAG GTT AGA CTT CCA CAC CTT GTC  2807

2808  TGC AGC GAC CCA CCG GGG ACT TGA CTC AAG TCA GAA GAG CAA AAC GCA GAC TCC TGT CAC TGC ATA GCA AGT TTC AAG AAT AAC AAA ACC TAA TGC TTA AGA TAC  2912

2913  TCA AAT GGC TTG ATC TCT GCC GCA CTG CCT TAA GCT GGG CTT GCC ATC CTC TCT CTG TGA ACC CGA GCA GCC GCT GTT AGC GCA GAG CCA TTG CCA CTT CAG TCT  3017

3018  TGC GCT GTA CAG CAT GTG GCT TAA TGG AAG TTG GGT TTA TTG CCA AAA ACA AAA ACA AAC TTC TGC TTA AAA ATA AAG AAA AAA GAT CTT TCT AAC ATT  3116
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Figure 2. Nucleotide sequences of cDNA clones pBSA1 and pCR41 and the predicted amino acid sequence of bovine skeletal muscle calpastatin. The deduced amino acid sequence is shown below the derived nucleotide sequence. Boundaries between the five domains of calpastatin are marked by inverted arrows. The central consensus sequence of each domain is marked by double underlines. The sequence marked by a single underline indicates a polyadenylation signal sequence (AATAAA). The diamond indicates the position of the 22 amino acid deletion (corresponding to exon #3 of human calpastatin) in domain L.

and modifications to domain L may affect the calpain inhibitory activity of calpastatin. Modifications to calpastatin domain L may impart tissue specificity, cellular localization, and responsiveness to specific physiological conditions, as suggested by the truncated forms found in erythrocytes (Maki et al., 1991) and the multiple forms found in the rat (Lee et al., 1992a). The recent reporting of the binding of calpastatin to membranes (Mellgren et al., 1989; Adachi et al., 1991) also suggested the involvement of domain L in this process. Taken together, the presence of multiple calpastatin transcript forms may suggest variations in the selection of inhibitory sites for calpain, which has also been shown to bind to membranes (Lee et al. 1990; Suzuki and Ohno, 1990). Deletions contained in domain L evidently are not responsible for all the observed differences in calpastatin mRNA transcript length, but they may have an effect on post-transcriptional and(or) translational modifications of the message or product. Evidence of such processes was previously demonstrated by Emori et al. (1987), who showed that the actual amino-terminal amino acids of purified rabbit calpastatin were coded for in the mRNA beginning at residue 240, suggesting that an N-terminal 80 amino acid sequence from domain L is cleaved from calpastatin either before or after the translational process.

Analysis of Amino Acid Composition. Comparison of the amino acid compositions of bovine skeletal muscle calpastatin with that of bovine cardiac calpastatin (Otsuka and Goll, 1987) and that derived from other species (Ishiura et al., 1982; Takano and Murachi, 1982; Emori et al., 1987; Takano et al., 1988) revealed that substantial differences can be present in the relative levels of many amino acids, whereas the levels for tryptophan, methionine, cysteine, and phenylalanine were consistently low (Table 1). The calculated isoelectric point of 4.75 for bovine calpastatin agrees well with the estimated isoelectric point value of 4.85 to 4.95 for bovine cardiac calpastatin (Otsuka and Goll, 1987).

Northern Blot Analysis. When probed with cDNA from the pBSA1 clone, skeletal muscle calpastatin mRNA is present as three isoforms (3.8, 3.0, and 1.5 kb) in beef and four isoforms (3.8, 3.0, 2.5, and 1.5 kb) in sheep (Figure 3). The predominance of each isoform varies. In beef the 3.0, and in sheep the 3.8, isoform is the most predominant message, as verified

Table 1. Comparison of the amino acid composition of bovine skeletal muscle calpastatin with that of bovine cardiac calpastatin and calpastatins of other species

Amio acid	Bovine skeletal muscle calpastatin[a]	Bovine skeletal muscle calpastatin[b]	Bovine cardiac calpastatin[c]	Chicken skeletal muscle calpastatin[d]	Human erythrocyte calpastatin[e]	Porcine cardiac calpastatin[f]	Rabbit calpastatin[g]
Gly	34	48.17	48.70	114.40	52.90	47.62	51.47
Glu	83	117.56	152.10[h]	156.90[h]	149.30[h]	114.85	119.61
Asp	63	89.23	113.30[i]	86.60[i]	126.00[i]	93.83	82.06
Val	30	42.49	47.80	71.90	32.70	44.82	34.76
Ala	64	90.65	99.40	76.80	94.90	79.84	112.65
Arg	16	22.66	20.70	35.90	31.10	25.21	33.37
Ser	66	93.48	84.60	85.00	96.40	102.24	95.97
Lys	95	134.56	134.10	58.80	121.30	147.06	132.12
Asn	12	17.00	—	—	—	9.80	9.73
Met	7	9.92	10.30	11.40	10.90	8.40	13.91
Ile	12	17.00	19.50	31.00	21.80	18.20	20.85
Thr	49	69.41	57.30	62.10	49.80	70.03	58.42
Trp	0	0	0	ND[j]	0	0	0
Cys	7	9.92	9.96	ND	1.24	7.00	5.56
Tyr	7	9.92	8.05	29.40	9.33	8.40	8.34
Leu	46	65.16	72.40	76.00	79.30	68.63	62.59
Phe	6	8.50	7.36	13.10	12.40	7.00	5.56
Gln	21	29.74	—	—	—	26.61	27.81
His	7	9.92	5.77	29.40	9.33	15.40	15.30
Pro	80	113.32	108.40	70.30	101.10	103.64	108.49

[a]Values expressed are the actual number of each amino acid for bovine skeletal muscle calpastatin as predicted by the coding region cDNA.
[b]Expressed as residues/1,000.
[c]From Otsuka and Goll (1987) expressed as residues/1,000.
[d]From Ishiura et al. (1982) expressed as residues/1,000.
[e]From Takano and Murachi (1982) expressed as residues/1,000.
[f]From Takano et al. (1988) expressed as residues/1,000.
[g]From Emori et al. (1987) expressed as residues/1,000.
[h]Indicates the sum of Glu + Gln.
[i]Indicates the sum of Asp + Asn.
[j]Not determined.

by densitometric analysis of Northern blots (data not shown). Probing with a cDNA insert derived from domains L and 1 (clone pCR41) confirmed the observed banding patterns. The beef pattern and isoform sizes are in agreement with the values reported for rabbit calpastatin isoforms (Emori et al., 1987). Variation in calpastatin transcript length may be the result of alternate polyadenylation sites (Emori et al., 1987) and(or) differences in exon splicing (Lee et al., 1992b) in the protein coding region.

Comparisons with Other Calpastatins. A broad range of molecular weights were previously reported for purified calpastatin (from M_r of 68,000 to 170,000) as determined by SDS-PAGE mobility (Otsuka and Goll, 1987; Takano et al., 1988; Kendall et al., 1993). The anomalies observed in determination of calpastatin molecular weight have been attributed to post-translational modifications and(or) proteolytic cleavage encountered during purification (Goll et al., 1989). The nucleic acid sequence of bovine calpastatin cDNA encodes a 706-amino acid protein with a predicted M_r of 75,842. Although difficulty has been encountered in the determination of the amino terminal residues of calpastatin, calpastatin has been shown to be truncated at the amino terminal end when isolated from its native state (Maki et al., 1991). Determined positions of truncation range from amino acid residue 2 for bovine heart (Mellgren et al., 1989), position 80 for rabbit liver (Emori et al., 1987), and position 290 for anucleated erythrocytes (Imajoh et al., 1987). The amino terminal residues of bovine skeletal muscle calpastatin have yet to be determined. It is interesting to speculate that the occurrence and(or) position of truncation may have an effect on the calpain inhibitory activity of calpastatin.

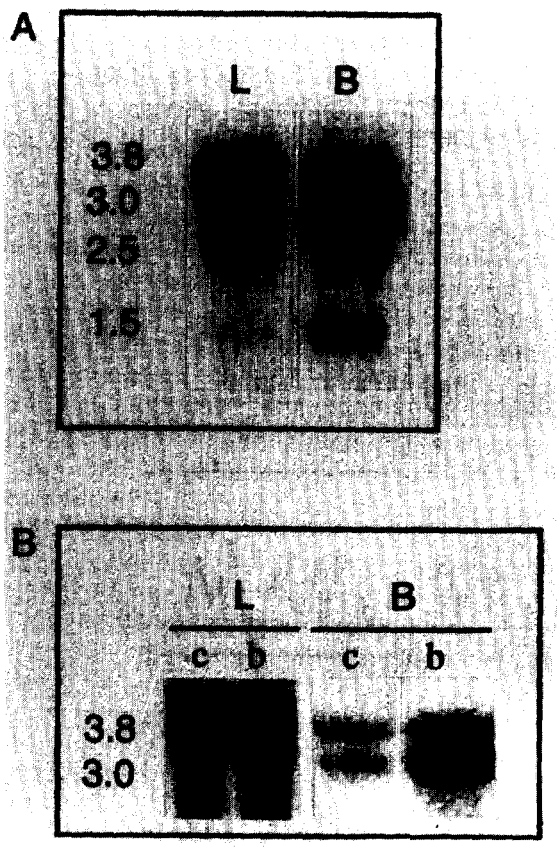

Figure 3. Autoradiographs of Northern blots containing either 3 μg of poly(A+) RNA/lane (panel A) or 25 μg of total RNA/lane (panel B) from either lamb (L) or beef (B) longissimus muscle. RNA was separated on a 1% agarose gel and transferred to nylon membrane. Panel A represents control animals. Panel B represents control (c) or β-adrenergic agonist (b) treatment groups. The blot was hybridized to a radiolabeled bovine calpastatin cDNA probe encoding domains 2 through 4 plus the 3′-nontranslated region. To compensate for the greater bovine calpastatin signal strengths on the autoradiographs, exposure times were 18 h at −70°C and 6 h at −70°C for lamb and beef samples, respectively.

Lengths of encoded amino acid chains for muscle calpastatins as derived from cloned cDNAs vary slightly, ranging from 718 in rabbit (Asada et al., 1989) to 713 for porcine cardiac (Takano et al., 1988) to 706 in bovine skeletal muscle. Comparison of the amino acid composition deduced from the cDNA for bovine skeletal muscle calpastatin with that obtained from the amino acid analysis of purified bovine cardiac calpastatin (Otsuka and Goll, 1987) indicates a very high degree of agreement (Table 1). It was observed that none of the reported cDNAs nor the amino acid analyses of the purified calpastatin indicated the presence of tryptophan. Furthermore, calpastatins all possessed consistently low levels of phenylalanine, methionine, and cysteine amino acid residues. All other amino acids seem to have greater degrees of variability associated with their relative levels. As reported by Otsuka and Goll (1987), the low levels of tryptophan and phenylalanine support the finding of very low A_{280} values for purified calpastatin. The relatively conserved low levels of methionine and cysteine (the sulphur-containing amino acids) in calpastatin may suggest the importance of these residues in calpastatin activity, possibly through involvement in secondary structure and disulfide bridge formation. The hydrophilic nature of the amino acids that compose calpastatin is in agreement with the proposed and demonstrated predominance of calpastatin in the cytosolic fraction of the cell (Lee et al., 1990; Mellgren and Richard, 1990; Suzuki and Ohno, 1990). The hydrophilic nature of calpastatin seems to be a highly conserved characteristic (Figure 4), particularly in the domains that possess inhibitory activity (Maki et al., 1987; Emori et al., 1988; Maki et al., 1988; Uemori et al., 1990). Domain L, which lacks inhibitory activity but is thought to contain regulatory properties, shows much greater variability in hydropathic structure. It is possible that variability in domain L may alter selection of splice site, message,

and product stability, resulting in regulation of calpastatin inhibitory activity. These variations may be characteristic of species, tissue, and physiological state.

Expression of Calpastatin mRNA. Numerous studies have reported effects of BAA on the components of the skeletal muscle calpain system. One of the most consistent effects of BAA feeding is the significant increase in calpastatin activity (Higgins et al., 1988; Wang and Beermann, 1988; Forsberg et al., 1989; Kretchmar et al., 1990; Koohmaraie et al., 1991; Wheeler and Koohmaraie, 1992; Pringle et al., 1993). Two general levels of gene expression regulation that could possibly account for the reported increase in calpastatin activity are pre- and post-translational control. To determine whether pretranslational levels of calpastatin mRNA are increased with BAA treatment, the level of calpastatin mRNA was determined using cDNA probes homologous to bovine calpastatin. The samples were obtained from two previous studies (Koohmaraie et al., 1991; Wheeler and Koohmaraie, 1992). Those studies reported increases in LM area and extractable LM calpastatin activity (Table 2). The BAA treatment was found to increase the amount of total calpastatin mRNA in both beef and lamb to levels more than double those of their respective controls (Table 2). Discrepancies in the percentage increase of calpastatin mRNA levels (> 100%) and the activity levels (60%) may be attributable to BAA-induced isoform selection and its possible effects on translational efficiency and(or) product activity.

To determine whether the observed increase in total calpastatin mRNA was due to a general increase in all calpastatin mRNA isoforms or whether the different isoforms show varying responsiveness to BAA, Northern blots were run on the control and BAA samples and subjected to scanning densitometry. Results indicated that the calpastatin isoforms are independently regulated with respect to BAA ($L_{644,969}$) stimulation (Figure 3). The BAA treatment was shown to increase the level of expression of the 3.0 and the 3.8 kb isoforms in beef, but of particular interest is the effect of BAA treatment on the ratio of the 3.0:3.8 kb isoforms. The ratio (3.0:3.8) is different between control and BAA treatment groups in both beef (control = .96 ± .12, BAA = 1.48 ± .14, $P < .05$) and

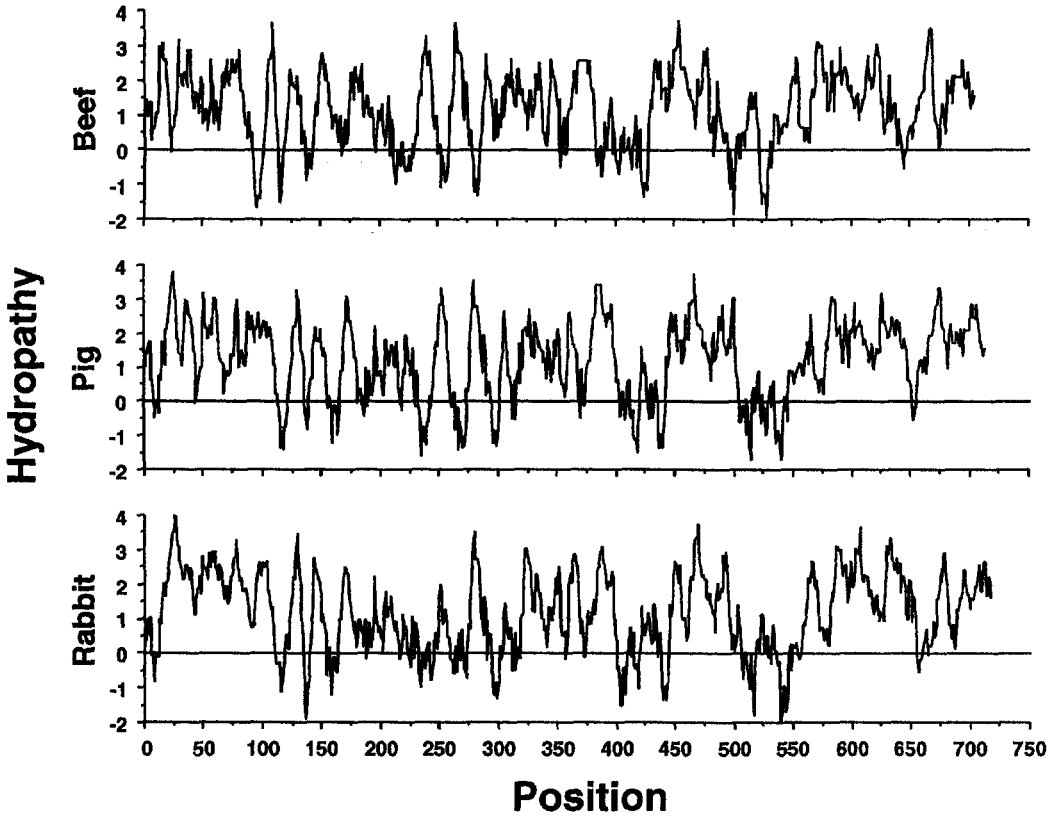

Figure 4. Diagram showing the hydropathy plots of the amino acid sequences deduced from cloned cDNAs for bovine skeletal muscle, porcine cardiac, and rabbit calpastatins. Position 0 indicates the start methionine and the plots end with the last amino acid prior to the termination codon. Hydrophilic and hydrophobic amino acid values are indicated by positive and negative hydropathic values, respectively. Domain borders (L, 1–4) are indicated by verticle arrows and the central consensus sequence of each of the active domains is indicated by the horizontal line segment.

Table 2. Effect of β-adrenergic agonist (BAA) on muscle area, calpastatin activity, and total calpastatin mRNA/milligram of total RNA in beef and lamb

Item	Longissimus muscle area, cm^2[a]		Calpastatin activity[a]		Total calpastatin mRNA, mRNA/mg of total RNA[b]	
	Beef	Lamb	Beef	Lamb	Beef	Lamb
Control	60.0[c]	12.0	3.72	2.30	9167	4454
BAA	74.6	14.6	5.95	3.74	19868	9361
SEM	2.5	.7	.48	.25	3317	604
P	.01	.05	.05	.05	.06	.0001
% Change	+24.3	+21.7	+60.0	+62.6	+116.7	+112.2

[a]The longissimus muscle area and calpastatin activity data were compiled from data previously reported (Koohmaraie et al., 1991; Wheeler and Koohmaraie, 1992). One unit of calpastatin is defined as the amount that inhibits one unit of m-calpain activity.
[b]Total calpastatin mRNA expression levels normalized to absorbance units/microgram of total RNA. Values were determined from the linear portion of individual slot blot standard curves. ^{32}P-labeled probe was bovine calpastatin cDNA encoding domains 2 through 4 and the 3′-nontranslated region (p^{BSA1}).
[c]Number of observations per treatment were 4 for beef and 8 for lamb.

lamb (control = .41 ± .03, BAA = .50 ± .03, $P < .05$). These results are in general agreement with those reported for Friesian cattle (Parr et al., 1992). The size discrepancies between our study and that of Parr et al. (1992) cannot be explained without further experimentation.

To our knowledge, this is the first report of the complete sequence of bovine skeletal muscle calpastatin cDNA and selective isoform responsiveness to BAA-induced muscle hypertrophy.

Implications

Bovine skeletal muscle calpastatin cDNA probes have been cloned, sequenced, and analyzed, allowing for the development of cDNA probes that are compatible for use in both bovine and ovine studies. Calpastatin messenger RNA was demonstrated to exist in multiple isoforms that were differentially effected by β-adrenergic agonist feeding. Complementary DNA probes to calpastatin will allow for examination of this gene's involvement in regulation of muscle protein degradation during growth and postmortem storage and its potential in marker-assisted selection.

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