

# Relationship of Restriction Fragment Length Polymorphisms (RFLP) at the Bovine Calpastatin Locus to Calpastatin Activity and Meat Tenderness<sup>1,2</sup>

S. M. Lonergan<sup>†</sup>, C. W. Ernst\*, M. D. Bishop\*<sup>3</sup>,  
C. R. Calkins<sup>†</sup>, and M. Koohmaraie\*<sup>4</sup>

\*Roman L. Hruska U.S. Meat Animal Research Center, ARS, USDA, Clay Center, NE 68933-0166 and <sup>†</sup>Department of Animal Science, University of Nebraska-Lincoln, Lincoln 68583

**ABSTRACT:** Restriction fragment length polymorphisms (RFLP) have been identified at the bovine calpastatin locus. The objective of the present study was to determine whether these polymorphisms are related to variations in calpastatin activity or beef tenderness in unrelated animals of mixed breeding. A sample of 83 crossbred steers from sires representing eight different breeds was examined to determine this relationship. A 2.2-kb cDNA coding for domains 2 through 4 plus a 3' untranslated region of bovine skeletal muscle calpastatin was used as a probe for calpastatin RFLP. Polymorphisms were found using the restriction enzymes *Bam*HI and *Eco*RI. Polymorphic restriction fragments for *Bam*HI were 9.0 and 5.0 kb and for *Eco*RI were 6.0 and 4.0 kb. Allelic frequencies for *Bam*HI restriction fragments were .53 for the 9.0-kb allele and .47 for the 5.0-kb allele. Allelic frequencies for *Eco*RI restriction fragments were .43 for the 6.0-kb allele and .57 for the 4.0-kb allele. No polymorphisms were identified using

the restriction enzymes *Bgl*II, *Dra*I, or *Pst*I. No associations between *Eco*RI and *Bam*HI RFLP and 24-h calpastatin activity or Warner-Bratzler shear force at 14 d postmortem were detected. Therefore, the polymorphic *Eco*RI and *Bam*HI restriction sites within the bovine calpastatin locus do not detect DNA sequence differences responsible for variation in calpastatin activity or tenderness of aged beef. Therefore, these polymorphisms cannot be used to predict tenderness of aged beef from unrelated animals of mixed breeding. These results do not exclude the possibility that other DNA sequences in or near the bovine calpastatin gene are responsible for variation in calpastatin activity or meat tenderness. The lack of a relationship between these calpastatin RFLP and meat tenderness must be distinguished from the well-documented relationship between calpastatin activity and meat tenderness. Therefore, further development of calpastatin-based methods for predicting beef tenderness in unrelated animals of mixed breeding should focus on basic factors influencing calpastatin activity at the molecular and cellular level.

Key Words: Calpastatin, RFLP, Beef, Tenderness

J. Anim. Sci. 1995. 73:3608-3612

## Introduction

<sup>1</sup>Names are necessary to report factually on 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 other products that might be suitable. The authors are grateful for the technical assistance of S. Hauver, K. Theer, P. Tammen, and L. Flathman and the secretarial assistance of M. Bierman. We also acknowledge P. Bures for photographic assistance.

<sup>2</sup>Published as paper no. 11117, journal series, Nebraska Agric. Res. Division.

<sup>3</sup>Present address: American Breeders Service, DeForest, WI 53532.

<sup>4</sup>To whom correspondence should be addressed: P.O. Box 166. Received April 20, 1995.

Accepted August 2, 1995.

The inconsistency in meat tenderness is one of the major problems facing the meat industry (Morgan et al., 1991; Morgan, 1992; Savell and Shackelford, 1992). This inconsistency is due to the inability to accurately classify carcasses based on ultimate meat tenderness. Current methodology (i.e., the USDA quality grading system) does not sufficiently predict meat tenderness (Wheeler et al., 1994). Thus, methods to classify beef based on tenderness need to be developed to either supplement or replace the current quality grading system.

It is now accepted that calpain-mediated degradation of myofibrillar proteins is responsible for postmortem meat tenderization that occurs during storage at refrigeration temperatures (for reviews see Koohmaraie, 1988, 1992a,b, 1994). Furthermore, it seems

that calpastatin, the endogenous and specific inhibitor of calpains, inhibits the calpains in postmortem tissue and thus regulates the rate and extent of postmortem tenderization (for review see Koohmaraie et al., 1995a).

Recently, restriction fragment length polymorphisms (RFLP) have been defined for the bovine calpastatin locus (Bishop et al., 1993). The objective of the present experiment was to determine whether a relationship between the calpastatin RFLP and calpastatin activity or Warner-Bratzler shear force (WBS) could be used to predict beef tenderness in carcasses from unrelated animals of mixed breeding. Because there is considerable genetic variation in the U.S. beef slaughter population and the genetic makeup of most slaughter cattle is not known, a system must be capable of predicting meat tenderness among unrelated animals. Assuming a mutation within or near the calpastatin gene would affect calpastatin activity, the only way these RFLP would be useful in unrelated animals is if the RFLP detected that particular mutation.

## Materials and Methods

**Animals.** These experiments were conducted at the Roman L. Hruska U.S. Meat Animal Research Center and its Animal Care and Use Committee approved the use of animals in this study. Crossbred (F<sub>1</sub>) steers out of Angus (8 sires; 12 progeny), Hereford (9 sires; 12 progeny), Piedmontese (7 sires; 10 progeny), Belgian Blue (7 sires; 8 progeny), Tuli (5 sires; 10 progeny), Brahman (10 sires; 14 progeny), Boran (4 sires; 9 progeny), or Charolais (5 sires; 8 progeny) sires and Hereford, Angus, or MARC III (1/4 each Angus, Hereford, Red Poll, and Pinzgauer) dams were used for this study. Calves were weaned at 200 d, adjusted to a high-energy diet for 90 d, fed the same diet for 140 d, and slaughtered.

**DNA Isolation and Southern Blotting Analysis.** Within 30 min postmortem, liver tissue was removed, minced, frozen in liquid nitrogen, and stored at -70°C. Isolation of DNA was performed as described by Sambrook et al. (1989). Briefly, approximately 100 mg of tissue was pulverized and suspended in 10 mL of 100 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, pH 8.5, containing .2% SDS. After a 60-min incubation with RNase A (500 mg) at 37°C, the slurry was incubated with proteinase K at 55°C overnight. DNA was extracted with buffered phenol (pH 8.0) and chloroform.

Ten micrograms of DNA was digested with 40 units of *Bam*H1, *Eco*R1, *Pst*I, *Dra*I, or *Bg*III (Pharmacia Biotech, Piscataway, NJ) restriction enzymes at temperatures and conditions prescribed by the manufacturer for 8 h. Digested DNA was fractionated on .8% agarose gels and transferred to nylon mem-

branes (MSI, Westboro, MA) as described by Sambrook et al. (1989).

Probes labeled with <sup>32</sup>P-dATP were generated by random hexanucleotide priming (Amersham, Arlington Heights, IL) from a 2.2-kb cDNA coding for domains 2,3,4 and a 3' untranslated region of bovine calpastatin (Killefer and Koohmaraie, 1994) and a 1.2-kb cDNA coding for domains L and 1 of bovine calpastatin (Killefer and Koohmaraie, 1994). Blots were prehybridized in .5 M NaPO<sub>4</sub>(H<sub>3</sub>PO<sub>4</sub>), pH 7.4, 7% SDS, 1% BSA, and .65% PEG containing 100 mg/mL of salmon sperm DNA at 60°C for 2 h. Labeled probe (5.0 × 10<sup>6</sup> cpm) was added to 5 mL of hybridization buffer (same as above) and blots were hybridized at 60°C for 16 h. Membranes were rinsed with 2× SSC and washed with 1× SSC, .1% SDS for 15 min at 60°C. If background persisted, a final wash of .5× SSC, .1% SDS at 60°C was performed. Results were visualized by exposing blots to X-OMAT™ AR diagnostic film (Eastman Kodak, Rochester, NY).

**Calpastatin Activity.** Calpastatin activity in longissimus steaks was determined at 24 h postmortem as described by Koohmaraie et al. (1995b). Calpastatin activity was reported as units per gram of tissue. One unit of activity is defined as the amount of calpastatin necessary to inhibit one unit of m-calpain activity (Koohmaraie, 1990).

**Warner-Bratzler Shear.** At 24 h postmortem, steaks (2.54 cm thick) were removed from the longissimus, vacuum-packaged, and aged at 1°C until 14 d postmortem. After aging, steaks were blast-frozen and stored (-30°C) until subsequent analysis. Steaks were thawed until an internal temperature of 2 to 5°C was reached and then broiled on Farberware (Kidde, Bronx, NY) open hearth broilers to an internal temperature of 40°C, turned, and cooked to a final internal temperature of 70°C. For assessment of shear force, steaks were cooled for 24 h at 4°C before removal of six cores (1.27 cm diameter) parallel to the longitudinal orientation of the muscle fibers. Each core was sheared once with a Warner-Bratzler attachment using an Instron Universal testing machine (Canton, MA) with a 50-kg load cell and 5-cm/min crosshead speed.

**Statistical Analysis.** Steers were classified by calpastatin genotype. The main effect of calpastatin genotype was tested with a model that included effects of sire and dam breed. Data were analyzed by analysis of variance (Steel and Torrie, 1980) and least squares means were compared using Fisher's least significant difference test (SAS, 1988) with a comparison error rate of .05.

## Results

Polymorphisms at the bovine calpastatin locus were detected following digestion of genomic DNA with the

Table 1. Least squares means for calpastatin activity and Warner-Bratzler shear force separated by *Bam*HI RFLP genotype<sup>a</sup>

<i>Bam</i> HI genotype	n	Calpastatin activity <sup>b</sup>			Warner-Bratzler shear force <sup>c</sup>		
		LS means	SE	Range	LS means	SE	Range
AA	25	2.61	.32	1.8–3.3	5.45	.25	3.8–8.4
AB	38	2.30	.26	.9–3.1	5.05	.22	3.7–7.7
BB	20	2.19	.35	.7–2.9	5.12	.26	2.9–8.7

<sup>a</sup>No significant differences between defined genotypes for calpastatin activity ( $P > .10$ ) or Warner-Bratzler shear force ( $P > .20$ ) were observed.

<sup>b</sup>Calpastatin activity is reported as units of activity per gram of tissue.

<sup>c</sup>Warner-Bratzler shear force is reported in kilograms.

restriction enzymes *Bam*HI and *Eco*RI. Blots of these digests were probed with a radiolabeled cDNA encoding for domains 2, 3, 4, and a 3' untranslated region of bovine calpastatin (Killefer and Koohmaraie, 1994). Polymorphic *Bam*HI fragments were approximately 9.0 and 5.0 kb in length (Figure 1). A 15-kb monomorphic fragment was also detected. A band at approximately 6.0 kb was observed in some samples. This was determined to be an artifact because the appearance of the band was not reproducible within samples. The allelic frequencies for the *Bam*HI polymorphic alleles were .53 for the 9.0-kb allele and .47 for the 5.0-kb allele. A calpastatin genotype was assigned based on the absence or presence of the polymorphic alleles. The 9.0-kb allele was termed the A allele and the 5.0-kb allele was termed the B allele. Those animals with both alleles were assigned the AB genotype, whereas those possessing only the A or B allele were assigned the AA or BB genotypes, respectively (Figure 1).

Digestion with *Eco*RI revealed 6.0- and 4.0-kb polymorphic fragments and monomorphic fragments of

approximately 2.0 and .3 kb (Figure 2). The frequencies for these alleles were .43 for the 6.0-kb allele and .57 for the 4.0-kb allele. A calpastatin genotype was assigned based on the absence or presence of the polymorphic alleles. The 6.0-kb allele was termed the C allele and the 4.0-kb allele was termed the D allele. Those steers with both alleles were assigned the CD genotype. Steers with only the C or D allele were assigned the CC and DD genotypes, respectively (Figure 2). The detection of calpastatin RFLP with these restriction enzymes is consistent with the initial report of RFLP at the bovine calpastatin locus (Bishop et al., 1993).

No polymorphisms were detected in this 83-steer population using the restriction enzymes *Dra*I, *Pst*I, or *Bgl*II. In addition, no RFLP were detected using a probe generated from the cDNA encoding for domains L and 1 of bovine calpastatin (Killefer and Koohmaraie, 1994).

There was no evidence that allelic frequencies differed among breed groups. Thus, breed did not significantly affect the frequency of calpastatin genotype. Genotypes based on *Bam*HI calpastatin RFLP did not explain variation in calpastatin activity at 24

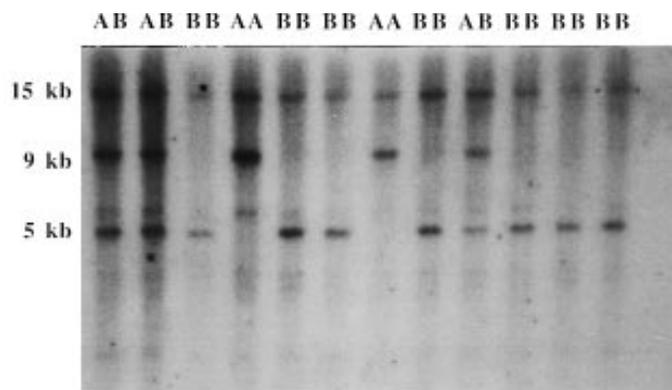


Figure 1. Restriction fragment patterns of 12 unrelated steers after genomic DNA was digested with *Bam*HI and probed with a radiolabeled cDNA encoding for domains 2, 3, 4, and a 3' untranslated region of bovine calpastatin. The 9.0-kb fragment was termed the A allele and the 5.0-kb fragment was termed the B allele. Assigned genotypes label each lane.

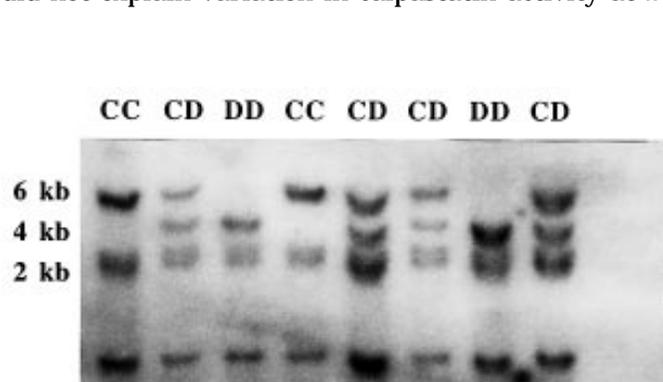


Figure 2. Restriction fragment patterns of eight unrelated steers after genomic DNA was digested with *Eco*RI and probed with a radiolabeled cDNA encoding for domains 2, 3, 4, and a 3' untranslated region of bovine calpastatin. The 6.0-kb fragment was termed the C allele and the 4.0-kb fragment was termed the D allele. Assigned genotypes label each lane.

Table 2. Least squares means for calpastatin activity and Warner-Bratzler shear force separated by *Eco*RI RFLP genotype<sup>a</sup>

<i>Eco</i> RI genotype	n	Calpastatin activity <sup>b</sup>			Warner-Bratzler shear force <sup>c</sup>		
		LS means	SE	Range	LS means	SE	Range
CC	25	2.16	.35	1.3–3.2	5.21	.26	2.9–7.8
CD	44	2.38	.25	.7–3.1	5.04	.19	3.5–8.7
DD	14	2.61	.47	.9–3.3	5.43	.33	2.9–8.4

<sup>a</sup>No significant differences between defined genotypes for calpastatin activity ( $P > .10$ ) or Warner-Bratzler shear force ( $P > .20$ ) were observed.

<sup>b</sup>Calpastatin activity is reported as units of activity per gram of tissue.

<sup>c</sup>Warner-Bratzler shear force is reported in kilograms.

h postmortem ( $P > .10$ ) or WBS at 14 d postmortem ( $P > .20$ ) (Table 1). Similarly, calpastatin activity ( $P > .12$ ) and WBS ( $P > .20$ ) were not different when classified by *Eco*RI RFLP genotypes (Table 2). In addition, calpastatin haplotypes defined by combining the data for both RFLP did not account for variation in calpastatin activity or WBS.

## Discussion

A major problem facing the beef industry is inconsistency in meat tenderness at the consumer level. This inconsistency is caused by an inability to consistently produce tender beef as well as the inability to accurately classify carcasses based on ultimate tenderness. Determination of genetic polymorphisms that may relate to beef tenderness could benefit the industry in two ways. First, identification of a particular genotype could be used as a predictor of beef tenderness, allowing breeding decisions that would enhance the trait. This information could be used along with conventional performance records to decrease the variation in the quality of beef produced (Seiler, 1994). Second, such a marker could be used to predict meat tenderness before slaughter in groups of unrelated animals if the marker detects sequence differences responsible for genetic variation in tenderness. This predictor could be used in the industry as a tool to more accurately classify carcasses based on ultimate eating quality by genotyping animals before slaughter.

To evaluate the value of the calpastatin RFLP as a predictor of meat tenderness, a sample of unrelated cattle representing eight sire breeds was used. The polymorphisms reported here do not have the potential to be used as tenderness predictors because they are not related to Warner-Bratzler shear force at 14 d postmortem. Furthermore, the calpastatin genotypes do not explain variation in calpastatin activity at 24 h postmortem. It should be noted that although the calpastatin RFLP were not related to WBS values, calpastatin activity was correlated to WBS for the animals used in this study ( $r = .31$ ). This correlation

is lower than has been previously reported. Calpastatin activity at 24 h postmortem, on average, explains about 40% of the variation in beef tenderness (for review see Koochmaraie et al., 1995a). Thus, differences in calpastatin activity are related to meat tenderness, although calpastatin RFLP are not.

It is possible that calpastatin mRNA processing may affect calpastatin expression and activity. Emori et al. (1987) detected rabbit heart calpastatin mRNA isoforms of 3.8, 3.0, and 2.5 kb. The variation in length of the calpastatin mRNA was shown to be due to different lengths of the 3'-noncoding sequence (Emori et al., 1987). Killefer and Koochmaraie (1994) reported that bovine longissimus muscle mRNA contained calpastatin mRNA isoforms of 3.8, 3.0, and 1.5 kb. The level of these mRNA isoforms seems to be differentially regulated; treatment with b-adrenergic agonists increased the ratio of 3.0-kb:3.8-kb calpastatin mRNA isoforms (Killefer and Koochmaraie, 1994). Apparent deletions in domain L of calpastatin cDNAs have been shown to be due to exon skipping by alternative splicing in rat (Lee et al., 1992a) and human (Lee et al., 1992b). The molecular diversity of calpastatin at the amino-terminal domain or the 3'-untranslated region may have an affect on post-transcriptional and(or) post-translational modifications of the message or protein (Emori et al. 1987; Lee et al., 1992b). Thus, it is possible that it is the processing of the mRNA isoforms or the rate of translation of different mRNA isoforms that regulates the expression of calpastatin in muscle. If a genetic component(s) controls the variation in calpastatin activity, it has not yet been defined. Therefore, further attempts to develop methods to predict meat tenderness in unrelated animals should focus either on variations in calpastatin at the protein level or on identifying the source of genetic variation in meat tenderness rather than on calpastatin RFLP.

The present study was designed to evaluate the potential application of calpastatin RFLP in the prediction of meat tenderness of unrelated animals of mixed breeding. Therefore, a sample of unrelated animals was chosen to represent the diversity of beef breeding in the industry. Evaluations of defined

populations (i.e., family studies) would further characterize the calpastatin gene. Such an analysis would increase the understanding of the inheritance patterns of calpastatin and may explain differences in calpastatin activity within that population. Using this approach, one may be able to identify calpastatin RFLP that are related to meat tenderness. Such RFLP could then be used to predict meat tenderness within that family. However, such polymorphisms cannot aid the industry in efforts to predict meat tenderness because the genetic makeup of most cattle is not known at the feedlot or packer level.

### Implications

The restriction fragment length polymorphisms identified at the bovine calpastatin locus were not useful for the prediction of calpastatin activity or meat tenderness in unrelated animals of mixed breeding. This indicates that we have not yet identified a DNA sequence difference responsible for the genetic variation in calpastatin or tenderness of aged beef. The lack of a relationship between these calpastatin restriction fragment length polymorphisms and meat tenderness must be distinguished from the documented relationship between calpastatin activity and meat tenderness. Further molecular approaches for predicting the ultimate tenderness of aged beef of unrelated animals of mixed breeding should focus more on variation of calpastatin expression and activity.

### Literature Cited

- Bishop, M. D., M. Koohmaraie, J. Killefer, and S. Kappes. 1993. Rapid communication: Restriction fragment length polymorphisms in the bovine *calpastatin* gene. *J. Anim. Sci.* 71:2277.
- Emori, Y., H. Kawasaki, S. Imajoh, K. Imahori, and K. Suzuki. 1987. Endogenous inhibitor for calcium-dependent cysteine proteinase contains four internal repeats that could be responsible for its multiple reactive sites. *Proc. Natl. Acad. Sci. USA* 84:3590.
- Killefer, J., and M. Koohmaraie. 1994. Bovine skeletal muscle calpastatin: Cloning, sequence analysis, and steady-state mRNA expression. *J. Anim. Sci.* 72:606.
- Koohmaraie, M. 1988. The role of endogenous proteases in meat tenderness. *Proc. Recip. Meat. Conf.* 41:89.
- Koohmaraie, M. 1990. Quantification of  $Ca^{2+}$ -dependent protease activities by hydrophobic and ion-exchange chromatography. *J. Anim. Sci.* 68:659.
- Koohmaraie, M. 1992a. Role of neutral proteinases in postmortem muscle degradation and meat tenderness. *Proc. Recip. Meat Conf.* 45:63.
- Koohmaraie, M. 1992b. The role of  $Ca^{2+}$ -dependent proteases (calpains) in post mortem proteolysis and meat tenderness. *Biochimie* 74:239.
- Koohmaraie, M. 1994. Muscle proteinases and meat aging. *Meat Sci.* 36:93.
- Koohmaraie, M., J. Killefer, M. D. Bishop, S. D. Shackelford, T. L. Wheeler, and J. R. Arbona. 1995a. Calpastatin-based methods for predicting meat tenderness. In: A. Ouali, D. Demeyer, and F. Smulders (Ed.) *Expression, Regulation and Role of Proteinases in Muscle Development and Meat Quality*. pp 395–412. ECCEAMST (European Consortium for Continuing Education in Advanced Meat Science and Technology), Utrecht, The Netherlands.
- Koohmaraie, M., S. D. Shackelford, T. L. Wheeler, S. M. Longergan, and M. E. Doumit. 1995b. A muscle hypertrophy condition in lamb (callipyge): Characterization of effects on muscle growth and meat quality traits. *J. Anim. Sci.* 73:3596.
- Lee, W. J., M. Hatanaka, and M. Maki. 1992a. Multiple forms of rat calpastatin cDNA in the coding region of functionally unknown amino-terminal domain. *Biochim. Biophys. Acta* 1129:251.
- Lee, W. J., H. Ma, E. Takano, H. Q. Yang, M. Hatanaka, and M. Maki. 1992b. Molecular diversity in amino-terminal domains of human calpastatin by exon skipping. *J. Biol. Chem.* 267:8437.
- Morgan, J. B. 1992. Tenderness problems and potential solutions. In: *The Final Report of the National Beef Quality Audit—1991*.
- Morgan, J. B., J. W. Savell, D. S. Hale, R. K. Miller, D. B. Griffin, H. R. Cross, and S. D. Shackelford. 1991. National beef tenderness survey. *J. Anim. Sci.* 69:3274.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual* (2nd Ed.). Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY.
- SAS. 1988. *SAS User's Guide: Statistics*. SAS Inst. Inc., Cary, NC.
- Savell, J. W., and S. D. Shackelford. 1992. Significance of tenderness to the meat industry. *Proc. Recip. Meat Conf.* 45:43.
- Seiler, J. 1994. The future role of molecular genetics in the control of meat production and meat quality. *Meat Sci.* 36:29.
- Steel, R.G.D., and J. H. Torrie. 1980. *Principles and procedures of statistics: A biometrical approach* (2nd Ed.). McGraw-Hill Publishing Co., New York.
- Wheeler, T. L., L. V. Cundiff, and R. M. Koch. 1994. Effect of marbling degree on beef palatability in *Bos taurus* and *Bos indicus* cattle. *J. Anim. Sci.* 72:3145.