Effect of Preslaughter Feed Withdrawal Period on Longissimus Tenderness and the Expression of Calpains in the Ovine

Mohammad A. Ilian,*,† James D. Morton,† Alaa El-Din Bekhit,† Noelle Roberts,† Barry Palmer,†,‡ Hiroyuki Sorimachi,§ and Roy Bickerstaffe†

Molecular Biotechnology Group, Animal and Food Sciences Division, P.O. Box 84, Lincoln University, Canterbury, New Zealand, and Laboratory of Biological Function, Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

The objective was to study the role of calpains in meat tenderness. Lambs were fasted for various periods of time to generate differences in meat tenderness and to determine in tandem the expression of calpain 1, calpain 2, calpain 3, and calpastatin. The assumption has been that increased calpain expression associated with an increase in tenderness indicates a role for calpain in the tenderization process and vice versa. Fasting lambs for 1 day caused a significant improvement in longissimus (LD) tenderness compared to the control. Correlations between the tenderness of the LD and the expression of the calpains and calpastatin were significant for calpains 1 and 3 but not for calpain 2 or calpastatin. Consequently, this study supports a role for calpains 1 and 3, but not for calpain 2, in the tenderization of the LD from fasted lambs during post-mortem aging.

Keywords: *Calpain; p94; fasting; lamb; longissimus; tenderness*

INTRODUCTION

Meat tenderization is a biochemical process involving the fracturing of proteins that contribute to the inter- (e.g., desmin and vinculin) and intramyofibril (e.g., titin, nebulin, and possibly troponin-T) linkages or proteins which link myofibrils to the sarcolemma (e.g., vinculin and dystrophin) by the calpain proteolytic system (*1*). Calpains (EC 3.4.22.17) are a family of intracellular Ca^{2+}-dependent neutral proteinases (*2*). In mammalian skeletal muscle, calpains comprise the ubiquitous calpains 1 and 2 and the tissue-specific calpain 3 (p94). The detailed catalytic role of these calpains in meat tenderization is not known.

Current literature on the role of calpains in meat tenderization refers almost exclusively to the ubiquitous calpains as it is these that can be routinely assayed. Koohmaraie et al. (*3*) examined the effect of post-mortem storage on the activity of the ubiquitous calpains and calpastatin, the endogeneous inhibitor of the ubiquitous calpains, in bovine skeletal muscle. They reported that, during post-mortem aging of meat, calpain 1 and calpastatin lost most of their activities, whereas calpain 2 activity remained relatively unchanged. On the basis of these results and the terminal activation theory of calpains (*4*), Koohmaraie et al. (*3*) suggested that calpain 1 is primarily responsible for post-mortem meat tenderization.

Until recently, it was not possible to study the potential role of calpain 3 in post-mortem meat tenderization because of a lack of the necessary probes to quantify the expression of calpain 3 in muscles from livestock animals. With the development of a nuclease protection assay (*5*) and antibodies against porcine calpain 3 (*6*), some information about the role of calpain 3 in meat tenderization has been obtained. Ilian et al. (*5*) reported a strong correlation between the intermuscular variation in tenderness and the expression of calpain 3 mRNA in cattle and sheep. On the other hand, Parr et al. (*6*) reported that in the porcine there was no correlation between calpain 3 level at slaughter and post-mortem tenderness of the longissimus dorsi (LD). The objective of this study was to investigate the role of the ubiquitous and muscle-specific calpains at the protein level in post-mortem tenderization of the LD from lambs subjected to various periods of feed withdrawal prior to slaughter as a biological model. This model was selected for two reasons: (1) Feed withdrawal prior to slaughter has been observed to increase the rate of myofibrillar protein breakdown and the expression of the ubiquitous calpains at the mRNA level in New Zealand White rabbits (*7*). (2) Feed withdrawal prior to slaughter has been reported to improve tenderness of thigh (*8*) and breast (*9*) meat in broilers. We hypothesize that variations in the activity/level of calpains underlie the variations in lamb meat tenderness due to fasting.

MATERIALS AND METHODS

Animals. The Lincoln University Animal Ethics Committee approved the experimental protocol. A total of 40 Perendale female lambs of similar age (≅5 months) and size were procured from a farm and transported for 5 h on a stock truck to the Lincoln University farm. On arrival, animals were tagged, weighed, and allocated to pasture for 2 days to recover from the transport stress. At the beginning of the feed withdrawal period all animals were moved to a holding pen with access to water only. Subsequently, 10 lambs were randomly picked and processed as the control animals. The

* Corresponding author (telephone 64-3-3253803; fax 64-3-3253851; e-mail alayanm@whio.lincoln.ac.nz).
† Lincoln University.
‡ Present address: Department of Medicine, Christchurch School of Medicine, P.O. Box 4345, Christchurch, New Zealand.
§ The University of Tokyo.

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remaining lambs were randomly divided into three treatments: 1, 3, and 7 days of fasting. Thus, the control animals were 1−7 days younger than the fasted animals. Prior to slaughter, blood was collected in heparin-containing tubes. The lambs were slaughtered in accordance with New Zealand industry-practice guidelines by captive bolt stunning followed by exsanguination. Each carcass was dressed and the liver dissected for weighing. The biceps femoris (BF), longissimus dorsi (LD), psoas major (PM), semimembranosus (SM), semitendinosus (ST), and supraspinatus (SS) from the right side of each carcass were exposed ~15 min post-mortem for sampling and probing. Samples from the LD (≅7 g) were taken at 0, 6, and 24 h post-mortem for measuring the activity of calpain 1, calpain 2, and calpastatin and the level of calpain 3. The carcasses were held at 15 °C for 4 h and then were moved to a 10 °C chiller for 12 h, after which time the temperature was lowered to 2 °C and maintained for an additional 8 h. A steak from the LD was cut for the determination of tenderness at 24 h post-mortem.

Meat Quality Measurements. The pH was determined on the intact muscles at 0, 1, 3, 6, 12, and 24 h post-mortem using an Orion 8163 glass electrode attached to a Hanna HI 9025 portable pH meter. The tenderness was measured as described earlier (*10*). Meat color was measured at day 1 post-mortem after slicing across the fibers and exposing the surface to the air at 4 °C for 20 min. A Minolta chromameter Model CR-210 set on the L^*, a^*, b^* system (where L^* measures relative lightness, a^* relative redness, and b^* relative yellowness) was used. The chromameter was operated using illuminant 65, observer 2°, and white tile standard ($L^* = 98.14$, $a^* = -0.23$, and $b^* = 1.89$).

Enzyme Assay. Analysis of calpain activity was performed following DEAE ion-exchange chromatography as described earlier (*11*). One unit of calpain activity was defined as the amount of enzyme that produced an increase of one absorbance unit at 278 nm after incubation for 1 h at 25 °C, corrected by subtracting the activity found in the presence of 1 mM EDTA. One unit of calpastatin inhibited one unit of bovine lung calpain 2.

Cloning of Bovine and Ovine Calpain 3 cDNA. Total RNA was extracted from bovine and ovine LD muscle using the method of Chomczynski and Sacchi (*12*). Poly (A^+) RNA was purified by using an oligo (dT)−cellulose column chromatography kit (Life Technologies). Double-stranded cDNA libraries of bovine and ovine skeletal muscle were synthesized using the Marathon cDNA kit (Clonetech). To amplify calpain 3 cDNA, two primers were designed, namely, 5′ RACE-p94, CTCCTTGTTGCTGTTTGCTCTGTC; and 3′ RACE-p94, TCCTTCTGGTCTGAACATGGGGGA. The 5′ RACE-p94 and 3′ RACE-p94 primers were derived from the IS1 and IS2 regions, respectively, and are conserved among human, mouse, rat, and porcine. PCR was carried out using a protocol that consisted of 94 °C for 45 s; 35 cycles, 94 °C for 15 s, 68 °C for 4 min; hold at 4 °C. The amplicons were gel purified, cloned in pGEMT-Easy according to the supplier's instructions (Promega), and sequenced using the dideoxy chain termination method (*13*).

Generation of Anti-Calpain 3 Polyclonal Antibody. On the basis of the calpain 3 cDNA sequences for the bovine (GenBank accession number AF115745) and ovine (GenBank accession number AF115744) a mixture of two polypeptides was synthesized. These two polypeptides are located in the NH_2 side of the IS2 region with the sequence NTISVDRPVKKKKXKPIIF, where X is asparagine (as in the bovine) and proline (as in the ovine), and were synthesized with an extra COOH-terminal cysteine for coupling to hemocyanin. The synthetic peptides were coupled to keyhole limpet hemocyanin (KLH). Two hens were immunized. The yolks were IgY purified and then affinity purified against the polypeptide. The antibody, designated Calp3IS2:b/o, was eluted with low pH (Research Genetics).

Determination of Calpain 3 Level in LD Muscle. Preliminary studies indicated that calpain 3 was associated with the myofibrillar proteins. Consequently, we separated the LD proteins from samples at-death, 6 h post-mortem, and 24 h post-mortem into sarcoplasmic and myofibrillar fractions according to the procedure described by Boehm et al. (*14*) with some modifications. Muscle tissue (0.2 g) free from fat and connective tissue was cut into small pieces and homogenized in 3 mL of ice0chilled homogenization buffer (HB) using a Polytron at maximum speed for 30 s. The composition of HB is 20 mM Tris-HCl, pH 8, 5 mM EDTA, 1% 2-mercaptoethanol (MCE), 100 mg/L trypsin inhibitor, 2.5 μM E-64, and 2 mM phenymethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at 12500*g* for 15 min at 4 °C. The supernatant was decanted into a tube labeled sarcoplasmic proteins fraction and was kept at 4 °C prior to the determination of calpain 3 level. The pellet was washed with 3 mL of HB and recovered by centrifugation as before. This step was repeated three times. The washed pellet was then dissolved in 3 mL of 50 mM Tris-HCl, pH 6.8, 1% SDS, 2% MCE, and 3% glycerol and designated the myofibrillar protein fraction. This step required (1) suspending the pellet into solution, (2) heating in an 80 °C water bath for 3 min, (3) vortexing the hot sample, and (4) filtering it through Whatman No. 1 filter paper. The protein concentration of the various fractions was determined according to the method of Karlsson et al. (*15*). The calpain 3 level in LD was determined by western blotting. Initially, we validated the utility of Calp3IS2:b/o Ab for the analysis of calpain 3 and produced a calibration curve using 0, 10, 20, 30, and 40 μg of myofibrillar proteins to establish the linearity of the signal response to gradation of protein concentration. Afterward, portions of 20 μg of protein together with biotinylated HRP broad range SDS-PAGE standards (Bio-Rad) were loaded onto discontinuous gels for SDS-PAGE (stacking gel, 3.5% polyacrylamide; separating gel, 8% polyacrylamide) using minigels according to the method of Laemmli (*16*). Proteins on the gels were electroblotted on poly(vinylidene difluoride) (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) at 4 °C overnight and 30 V using a trans-blot unit (Bio-Rad) according to the method of Towbin et al. (*17*). The transfer buffer was 25 mM Tris, pH 8.3, 192 mM glycine, 15% methanol, and 0.5% SDS. Gels after transfer were stained with Coomassie Blue to examine the efficiency of transfer. Immunoprobing of the immobilized proteins was carried out using Calp3IS2:b/o diluted 1:500 and anti-chick IgY (HRP) diluted 1:5000 (Promega) in blocking buffer (100 mM Tris-Cl, pH 7.5, 0.9% NaCl, 0.1% Tween 20, and 5% nonfat dry milk) according to the method of Towbin et al. (*17*). After washing, detection of bound antibodies was performed with a chemiluminescence kit (Pierce), and the intensity of the signal was determined using UVP gel documentation systems (UVP Inc., Upland, CA).

Blood Analysis. Plasma creatinine concentrations at various times after feed withdrawal were analyzed using a commercial kit (555A, Sima Chemical, St. Louis, MO).

Analyses of Data. Statistical analysis was carried out using the Minitab version 8 computer package (State College, PA). Curves were fitted to the data using the Sigma Plot computer package (San Rafael, CA).

RESULTS AND DISCUSSION

Development of Western Assay for Bovine and Ovine Calpain 3. To determine the role of calpain 3 in meat tenderization during post-mortem aging, quantitative methods for the determination of calpain 3 expression at the protein level are needed. Currently, enzyme activity measurements and chromatography purification methods routinely used to study the ubiquitous calpains are not applicable to calpain 3. This is because calpain 3 is associated with titin (*18*). Upon solubilization of titin, calpain 3 undergoes rapid autolysis (*19*). However, the determination of the level of calpain 3 in skeletal muscle immunochemically has been reported for mouse (*20*), human (*21*), and porcine (*6*). A drawback to determining the level of calpain 3 immunochemically is that it may not reflect the in vivo activity of the enzyme.

Table 1. Calpain 3 (p94) Amino Acid Sequence Identity among Six Mammalian Species and Chicken (Percent)

	human	porcine	ovine	bovine	rat	mouse
porcine	93.8					
ovine	93.2	97.5				
bovine	92.9	97.8	98.8			
rat	95.0	95.3	95.0	95.0		
mouse	95.0	95.3	95.0	95.0	99.4	
chicken	79.8	78.9	79.2	78.9	79.8	80.4

To determine calpain 3 level in bovine and ovine skeletal muscle, we produced a polyclonal antibody specific against the IS2 region of bovine and ovine calpain 3. The IS2 region was chosen because it is unique to calpain 3 and not present in the ubiquitous calpains (*22*). We cloned and sequenced the cDNA of bovine (GenBank accession number AF115745) and ovine (GenBank accession number AF115744) calpain 3 as a prerequisite for designing the antigenic polypeptide. From the cDNA sequences, a string of 19 amino acids from the NH_2-terminal region of IS2 was selected as the antigenic site. Because there was one amino acid difference between bovine and ovine calpain 3 in the sequence of the antigenic polypeptide (asparagine for bovine versus proline for ovine), we designed the antigen to be a mixture of both sequences to maximize the sensitivity of the Ab for both species.

To select a host for eliciting the antibody, we conducted a full comparison of calpain 3 amino acid sequence identity among bovine, ovine, human, pig, mouse, rat, and chicken (Table 1). The results revealed that the bovine and ovine calpain 3 have 98.8% amino acid identity. This result supports the hypothesis of extensive conservation of the genes between cattle and sheep (*23*). Furthermore, the bovine and ovine calpain 3 have very high amino acid sequence identity with mammalian species such as pig (97.5), mouse (95%), rat (95%), and human (93%). When comparing the sequence identity of bovine and ovine calpain 3 with that of chicken calpain 3, we obtained much lower values (~79%). To enhance the antigenicity of the polypeptides, we selected chicken as the animal host for eliciting Calp3IS2:b/o. The Calp3IS2:b/o had an anti-peptide titer (expressed as the reciprocal of the serum dilution that results in an absorbency reading of 0.2 at 405 nm) above 204800.

The specificity and the utility of Calp3IS2:b/o for the quantitative determination of ovine calpain 3 level by western assay were established by reacting it with gradations of LD myofibrillar proteins, with solubilized whole liver, heart, and lung as representatives of nonskeletal muscle tissues, and with a recombinant inactive calpain 3 protein, p94:C129S, as a positive control (*31*). The p94:C129S is a mutated calpain 3 in which the active site (Cys-129) had been changed to serine to eliminate the protease activity. The results (Figure 1) revealed that Calp3IS2:b/o was specific for a protein at 94 kDa in the LD (lanes 4−7) but not for the liver, heart, and lung (lanes 1−3). Results of the calibration curve using 0, 10, 20, 30, and 40 μg of myofibrillar proteins showed a linear response of image density to gradation in the myofibrillar protein concentration (Figure 2). Consequently, 20 μg of proteins was used in the analysis of calpain 3 in the muscle samples of the study. The specificity of Calp3IS2:b/o was tested using p94:C129S (Figure 3). As shown in lane 4 of Figure 3, Calp3IS2:b/o reacted with p94:C129S very strongly. Essentially the same signal was obtained with

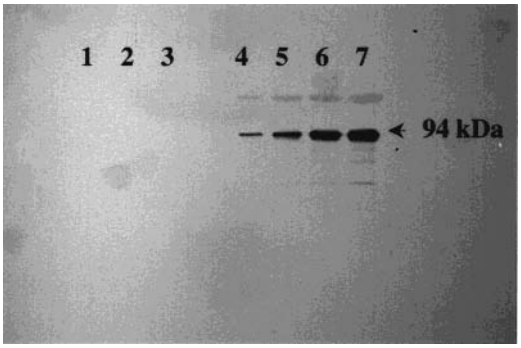

Figure 1. Validation of Calp3IS2:b/o for western analysis of ovine calpain 3. Lanes 1, 2, and 3 had 20 μg of protein of whole liver, heart, and lung, respectively, as nonskeletal muscle negative control. Lanes 4, 5, 6, and 7 had 10, 20, 30, and 40 μg of myofibrillar proteins, respectively, from the LD.

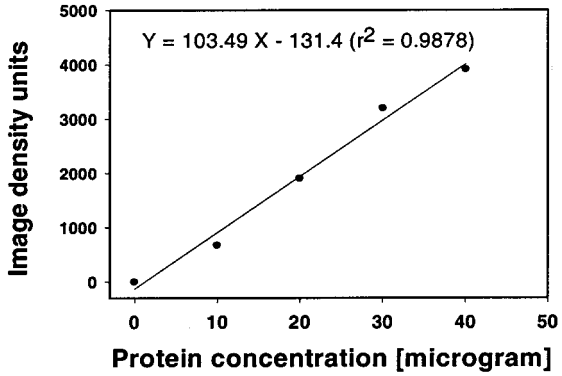

Figure 2. Calibration curve for Calp3IS2:b/o against gradations of LD myofibrillar proteins.

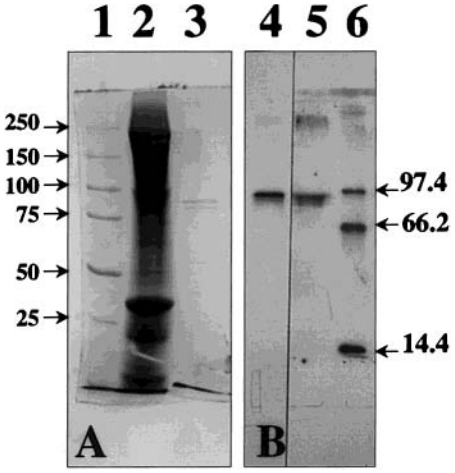

Figure 3. Specificity of Calp3IS2:b/o antibody. (A) Coomassie Brilliant Blue-stained SDS gel: (lane 1) Bio-Rad broad-range precision protein standards; (lane 2) 50 μg of LD myofibrillar proteins fraction; (lane 3) 1.5 μg of p94:C129S protein. (B) Western blot with Calp3IS2:b/o: (lane 4) 500 ng of p94:C129S; (lane 5) same as lane 2; (lane 6) Bio-Rad low-range biotinylated precision protein standards.

the myofibrillar fraction of the LD (Figure 3, lane 5). The fact that Calp3IS2:b/o reacted with p94:C129S and with a 94-kDa protein in the LD but not in the liver, heart, and lung is strong evidence that Calp3IS2:b/o is specific for bovine/ovine calpain 3.

Characterization of the Animal Model Used. To provide a frame of reference for our work, the effects of feed withdrawal time prior to slaughter on some body measurements, blood creatinine level, and LD attributes

Table 2. Effect of Feed Withdrawal Period before Slaughter on Some Body Measurements, Blood Creatinine Level, and LD Attributes[a]

parameter	control	1 day of fasting	3 days of fasting	7 days of fasting
initial wt (kg)	28.05 ± 1.84[a]	27.11 ± 1.29[a]	27.25 ± 0.83[a]	27.11 ± 0.69[a]
wt at slaughter (kg)	28.47 ± 1.74[d]	26.74± 1.31[c]	23.13 ± 1.08[b]	21.38 ± 0.97 [a]
hot carcass wt (kg)	11.29 ± 0.94[c]	11.09± 0.73[bc]	10.35 ± 0.38[ab]	9.78 ± 0.69[a]
skeletal muscles wt (g)				
biceps femoris	107 ± 13[b]	104 ± 15[b]	79 ± 12[a]	80 ± 14[a]
psoas major	23 ± 9[a]	30 ± 6[a]	27 ± 8[a]	27 ± 7[a]
semimembranosus	51 ± 8[b]	47 ± 7[a,b]	44 ± 4[a,b]	42 ± 6[a]
semitendinosus	16 ± 3[a]	16 ± 2[a]	19 ± 3[a]	17 ± 3[a]
supraspinatus	56 ± 11[ab]	63 ± 8[b]	53 ± 5[a,b]	48 ± 10[a]
liver wt (g)	576 ± 54[d]	484 ± 31[c]	413 ± 20[b]	349 ± 28[a]
blood creatinine (mg/dL)	1.34± 0.14[b]	1.46 ± 0.14[b]	1.39 ± 0.25[b]	0.97 ± 0.32[a]
LD attributes (at 24 h post-mortem)				
shear force (kg)	6.61 ± 1.31[b]	3.84 ± 0.65[a]	5.48 ± 1.27[b]	6.19 ± 0.9[b]
pH	5.94 ± 0.22[a]	5.84 ± 0.08[a]	5.88 ± 0.15[a]	5.93 ± 0.13[a]
*L** (relative lightness)	51.75 ± 1.33[b]	52.26 ± 2.11[b]	49.85 ± 1.33[a]	52.00 ± 1.78[b]
*a** (relative redness)	14.82 ± 1.63[a]	14.78 ± 1.73[a]	13.94 ± 1.20[a]	16.64 ± 1.00[b]
*b** (relative yellowness)	3.29 ± 0.86[a]	4.43 ± 1.09[b]	3.93 ± 0.86[ab]	3.66 ± 0.71[ab]

[a] Means ± standard deviation (average of 10 observations) within each row followed by different superscripts are significantly different at $P \leq 0.05$.

are listed in Table 2. Analysis of variance of the initial live weights of the lambs allocated to the various treatments revealed there were no significant differences. It is well-known that fasting causes live weight reduction in animals (*7*, *24*, *25*). In this study, the feed withdrawal time prior to slaughter caused a significant decrease in the live weight of the lambs. Compared to the control, the lambs lost 1.73 kg per day during the first 3 days of fast and 0.44 kg per day during the last 4 days of fast. The body weight loss in the fasted lambs was mainly due to the emptying of the alimentary tract after 1 day of fasting and by the loss of edible parts after 3 and 7 days of fasting. This is clear from the changes in the weights of the hot carcasses and of some of the skeletal muscles and the plasma creatinine levels. Creatinine is a product of muscle creatine metabolism and is an index for muscle wasting (*26*). Urinary creatinine excretion has traditionally been used as an index of total muscle mass (*7*, *27*, *28*). The lambs subjected to 7 days of fasting had significantly lower blood creatinine levels and lower hot carcass weights compared to the control animals. The responses of various muscles to fasting, in terms of weight reduction, did not follow the same pattern. This may be related to the differences in the type of fibers within a muscle. For example, neither the PM nor ST muscles were affected by the fasting treatments. However, the BF weight was significantly lower after a 3 day fast compared to that of the control animals. After a 7 day fast, there was no further change in BF weight over that caused by a 3 day fast. The SM gradually lost weight as fasting progressed and was significantly smaller than the control after a 7 day fast. The SS was significantly smaller after a 7 day fast compared to the 1 day fast. The response of the liver to fasting was more marked than that of the skeletal muscle. The reduction in liver mass was 16, 29, and 40% of the control after 1, 3, and 7 days of fasting, respectively.

With regard to the effect of fasting on meat quality, the LDs of lambs fasted for 1 day were significantly more tender than the LD from the control anmials or animals fasted for 3 or 7 days. The shear force values of the LD from the 1 day fasted lambs were 41.9% lower than those for the LD from the control animals. These observed differences in tenderness were not due to differences in the rate or magnitude of post-mortem pH decline (Figure 4 and Table 2). This is important because it means that fasting can be used to generate a biological model for studying the role of calpains in post-mortem tenderization of meat, within the same muscle, without the complication of pH variations.

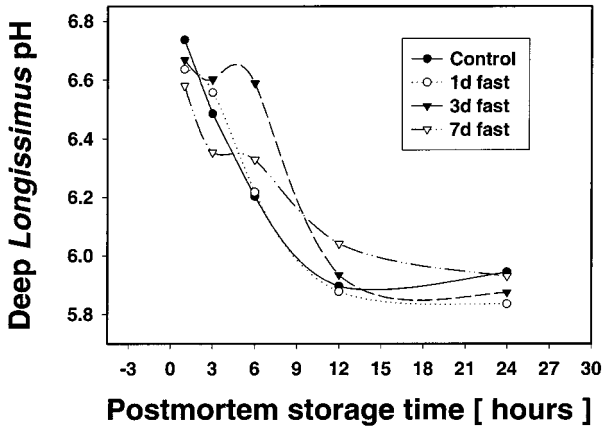

Figure 4. Temporal changes in the pH of various treatments during the 24 h post-mortem storage period.

The results in Table 2 also provided some information on the impact of preslaughter feed withdrawal on the quality of lamb meat. Meat animals are usually subjected to a period of fasting between removal from the farm and slaughter. This is mainly due to transportation, lack of pastures at the processing plants, and processing load. Most of the published research in this area has been on nonruminants such as pig (*29*) and poultry (*24*). Poultry scientists have reported that if fasting is administered properly, it results in significant benefits to the poultry industry. For example, withdrawal periods of feed for 8−12 h, while water was available throughout the fasting period, resulted in the highest eviscerated yields and minimized the potential for fecal contamination. In this study, feed withdrawal for 1 day prior to slaughtering of lambs caused a significant improvement in the LD tenderness without any adverse effects on carcass weight or meat color attributes such as lightness (*L**) or redness (*a**) compared to the control. However, there was a significant increase in the yellowness (*b**) of the LD after a 1 day fast compared to the control animals. Therefore, a 1 day fast prior to slaughtering of lambs may be recommended on the basis of the above results. Another advantage of a 1 day fast is that it will minimize the potential for fecal contamination of the carcass during processing.

Table 3. Effect of Feed Withdrawal Period before Slaughter on the At-Death Activity of the Ubiquitous Calpain System in the LD[a]

parameter	control	1 day of fasting	3 days of fasting	7 days of fasting
calpain 1	0.78 ± 0.17^{a}	1.09 ± 0.35^{ab}	0.91 ± 0.26^{ab}	1.49 ± 0.5^{b}
calpain 2	1.16 ± 0.35^{a}	1.24 ± 0.28^{a}	0.87 ± 0.29^{a}	0.99 ± 0.36^{a}
calpastatin	1.86 ± 0.57^{b}	2.28 ± 0.83^{b}	2.45 ± 0.42^{b}	1.20 ± 0.25^{a}
calpain 1/calpastatin	0.42 ± 0.21^{a}	0.48 ± 0.21^{a}	0.37 ± 0.08^{a}	1.23 ± 0.57^{b}

[a] Means ± standard deviation (average of 10 observations) within each raw followed by different superscripts are significantly different at $P \leq 0.05$.

Fasting for 3 or 7 days should be avoided because significant losses in hot carcass weight occurred without any significant improvement in meat quality attributes.

Effect of Fasting on the Ubiquitous Calpain System. The results of feed withdrawal prior to slaughter on the at-death activity of calpain 1, calpain 2, and calpastatin in the LD sarcoplasmic fraction are in Table 3. Calpain 1 activity gradually increased as the fasting period increased. However, a significant increase in calpain 1 activity over that in control animals was observed only after 7 days of fasting. Calpain 2 activity was not significantly altered by the feed withdrawal treatments. With regard to the activity of calpastatin (the endogenous inhibitor of the ubiquitous calpains), feed withdrawal for 1 and 3 days had no significant effect compared to the control. However, feed withdrawal for 7 days caused a significant decrease in calpastatin activity compared to the control. The effect of a feed withdrawal period prior to slaughter on the proteolytic potential of calpain 1 measured as the ratio of calpain 1 to calpastatin is also presented in Table 3. Feed withdrawal for 1 and 3 days prior to slaughter did not alter the calpain 1 proteolytic potential. However, fasting for 7 days prior to slaughter resulted in a significant increase in the proteolytic potential of calpain 1. The proteolytic potential of calpain 1 was 200% higher after a 7 day fast compared to the control, 1 day of fasting, and 3 days of fasting.

Ertbjerg et al. (*30*) examined the effect of depleting the energy stores in porcine LD on the activities of the ubiquitous calpain system. They observed a significant increase in calpain 1 activity and its proteolytic potential as body energy stores were depleted. There was no change in calpain 2 activity with depleted body energy stores. These observations are similar to the results obtained in this study. However, our results differ from those of Ertbjerg et al. (*30*) in that they did not observe any changes in calpastatin activity when the energy stores in the porcine LD were depleted by epinephrine and exercise treatments.

Effect of Fasting on Calpain 3 Level. Previous studies on the subcellular distribution of calpain 3 indicated that it was associated with the myofibrillar protein titin (*18*). Therefore, we fractionated the LD samples taken at slaughter and 6 and 24 h post-mortem into sarcoplasmic and myofibrillar fractions and determined the level of calpain 3 in the two fractions. Representative western blots of calpain 3 in the myofibrillar and sarcoplasmic fractions of the LD samples taken at slaughter from lambs subjected to the various treatments are presented in Figure 5. In the myofibrillar fraction from all treatments, the anti-calpain 3 Ab labeled a protein with a molecular weight of 94 kDa. In the sarcoplasmic fraction, however, the major immunoreactive proteins were 94 and 52.5 kDa regardless of the treatment. The 94 kDa is the native calpain 3, and the 52.5 kDa is presumably the C-terminal-IS2-containing fragment of calpain 3 after autolysis. These results

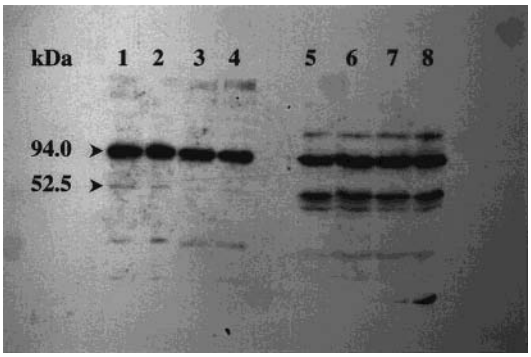

Figure 5. Representative western of calpain 3 in the myofibrillar (lanes 1−4) and sarcoplasmic (lanes 5−8) fractions of the LD samples (20 μg of protein) taken at slaughter from lambs of control (lanes 1 and 5) and after 1 day of fasting (lanes 2 and 6), 3 days of fasting (lanes 3 and 7), and 7 days of fasting (lanes 4 and 8).

indicate that calpain 3 in the sarcoplasm is less stable than at the myofibril. Kinbara et al. (*31*) suggested that protein−protein interactions are necessary for calpain 3 stability.

Although it has been known for more than a decade that calpain 3 mRNA is at least 10 times more abundant in skeletal muscle than the mRNAs for the ubiquitous calpains (*22*), the calpain 3 protein level has until recently proved difficult to determine (*19*). Recently, however, this difficulty was solved by the use of low ionic strength buffers containing a battery of protease inhibitors and SDS in the homogenization step (*6*, *22*). Now the calpain 3 level has been determined by immunochemical methods in whole skeletal muscle preparations for human (*22*) and porcine (*6*) and in the sarcoplasm of human muscle sections (*32*).

To determine the effect of feed withdrawal time prior to slaughter on the expression of calpain 3 protein in the LD, we measured the level of calpain 3 in the myofibrillar and sarcoplasmic fractions of the LD of various fasting treatments (10 animals per treatment) by western blotting (Figure 5). In the sarcoplasmic fraction, fasting caused a general increase in LD calpain 3 level. The steady state level [measured as the average mean ($n = 10$) of the intensity of the 94 kDa band at slaughter per milligram of tissue] of calpain 3 was significantly higher in the LD sarcoplasm ($P \leq 0.003$) at 1, 3, and 7 days of fasting compared to control. However, the effect of fasting on the level of calpain 3 in the LD myofibrillar fraction differed with time. Compared to the control, calpain 3 at 1, 3, and 7 days of fasting was significantly ($P \leq 0.001$) higher, not different, and significantly lower, respectively. Consequently, fasting caused a shift in the subcellular distribution of calpain 3 between the sarcoplasmic and the myofibrillar fractions in the LD. At 0, 1, 3, and 7 days of fasting, the sarcoplasm contained 9.2, 10.2, 13.0, and 16.3% of total LD calpain 3, respectively. These results indicate that differential regulation of the expression

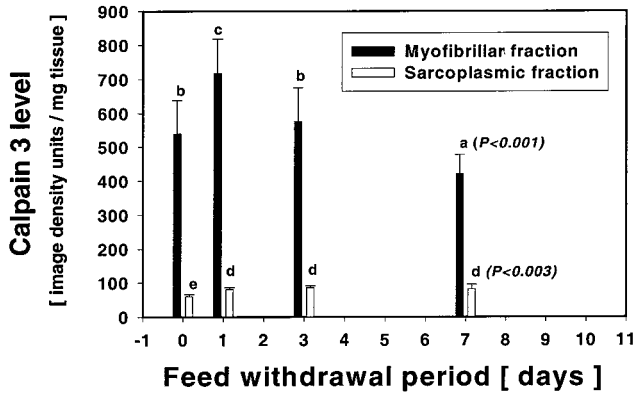

Figure 6. Effect of feed withdrawal time prior to slaughter on the level of calpain 3 in the LD of lamb. Error bars (standard deviation) with different letters represent significant differences.

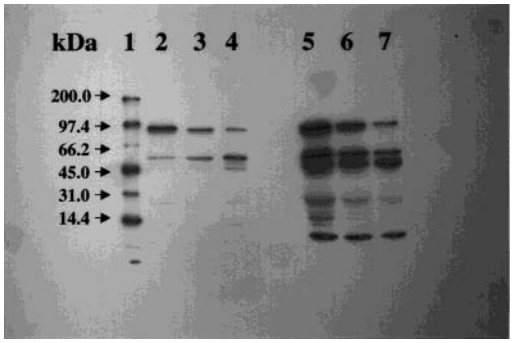

Figure 7. Representative western of temporal changes in calpain 3 in the myofibrillar (lanes 2−4) and sarcoplasmic (lanes 5−7) fractions of the LD samples (20 μg of protein) at 0, 6, and 24 h post-mortem storage.

of calpain 3 occurs in the LD in response to the fasting period. To our knowledge, this is the first study to show differential regulation and a shift in the subcellular distribution of calpain 3 protein in response to an experimental treatment. In 1997, Van den Hemel-Grooten et al. (*33*) studied the mRNA level of calpain 3 in the LD of young pigs during prolonged feeding of a protein-free diet. They observed a significant decrease in the mRNA level of calpain 3 for the protein-free group on day 14 compared to the control. Mutations in the calpain 3 gene have also been shown to be associated with limb-girdle muscular dystrophy type 2A in humans (*34*). Currently, the role of calpain 3 in muscle health and disease is a topic of intensive investigation. On the basis of the results of this study and those of Van den Hemel-Grooten et al. (*33*), fasting may provide a good biological model to study the physiological role of calpain 3 in skeletal muscle.

Temporal Changes in Calpain 3 during Postmortem Storage. To examine the effect of post-mortem storage on calpain 3 level and whether, like calpain 1, it is terminally activated, we studied the temporal changes in calpain 3 level in the sarcoplasmic and myofibrillar fractions of the LD from control animals during 24 h of post-mortem aging. A representative western blot of calpain 3 in the myofibrillar and sarcoplasmic fractions during post-mortem aging for 0, 6, and 24 h is shown in Figure 7. Quantifications of the western data ($n = 4$) of the temporal changes of calpain 3 and its 52.5 kDa fragment during post-mortem aging are presented in Figures 8 and 9, respectively.

Post-mortem aging caused a significant decline in

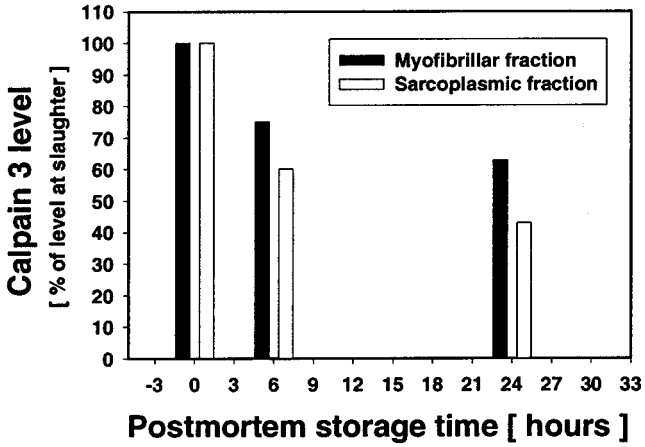

Figure 8. Temporal changes in calpain 3 level, expressed as percent of level at slaughter, in the sarcoplasmic and myofibrillar fractions at 0, 6, and 24 h post-mortem.

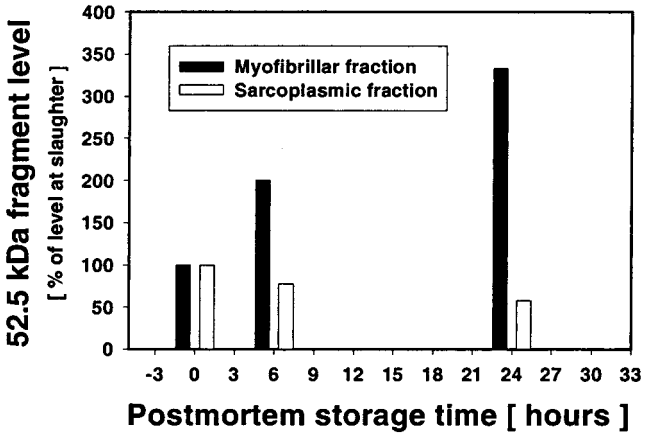

Figure 9. Temporal changes in calpain 3 IS2 fragment level, expressed as percent of level at slaughter, in the sarcoplasmic and myofibrillar fractions at 0, 6, and 24 h post-mortem.

calpain 3 level (Figure 7) by autolysis as judged by the appearance of the 52.5 kDa fragment (*31*). The kinetics of the native calpain 3 during post-mortem storage was similar in both the sarcoplasmic and myofibrillar fractions (Figure 8). At 6 h postslaughter, the sarcoplasmic and myofibrillar calpain 3 levels were about 60 and 75% of the steady state level, respectively. After 24 h of post-mortem aging, the sarcoplasmic and myofibrillar calpain 3 levels dropped to 43 and 63% of the steady state level, respectively (Figure 8). In contrast, the kinetics of the 52.5 kDa fragment during post-mortem aging were different in the sarcoplasmic and myofibrillar fractions (Figure 9). In the sarcoplasmic fraction, the level of the 52.5 kDa fragment declined to 78 and 58.2% of the post-slaughter level after 6 and 24 h of post-mortem aging, respectively. In the myofibrillar fraction, the level of the 52.5 kDa fragment increased to 200 and 333% of the post-slaughter level at 6 and 24 h post-mortem, respectively. These results indicate that the 52.5 kDa fragment is stable in the myofibrillar fraction but not in the sarcoplasmic fraction. Sorimachi et al. (*18*) reported that calpain 3 binds to titin at the IS2 region. It may be possible that the stability of the 52.5 kDa fragment in the myofibrillar fraction is related to the interaction of the IS2 domain of the 52.5 kDa fragment with titin.

Parr et al. (*6*) investigated the stability of calpain 3 in myofibrillar fractions of "tough" and "tender" porcine LD during 0−24 h of post-mortem aging. Their study revealed an exponential decline in the intensity of the

94 kDa band with post-mortem aging in both tough and tender porcine LD. These observations of Parr et al. (*6*) are similar to the results reported in this study. However, Parr et al. (*6*) did not observe the presence of a 52.5 kDa fragment, possibly because their Ab recognized an epitope that is not located in the C-terminal IS2 containing fragment.

Calpains and Tenderization of Meat. Studies on the role of calpains in meat tenderization apply mostly to the ubiquitous calpains 1 and 2 sometimes known as μ and m calpains, respectively. This is because analytical methods for the ubiquitous calpains are well established (*35*). A very recent review (*36*) on calpains in meat tenderization concluded the following. (1) Post-mortem tenderization is primarily caused by calpain 1-mediated degradation of key muscle structural proteins. (2) Calpain 2 appears not to be involved in post-mortem tenderization of meat unless meat is treated with calcium. (3) The role of calpain 3 in tenderization remains to be determined.

With the development of analytical methods for the determination of calpain 3 expression at the mRNA and protein levels, three reports on the role of calpain 3 in meat tenderization have been published (*5*, *6*, *37*). A common theme in two paperss (*6*, *37*) is that calpain 3, like calpain 1, is terminally activated in meat during post-mortem storage. This observation supports a role for calpain 3 in meat tenderization. With regard to the relationship between the abundance of calpain 3 and associated variations in meat tenderness, the available literature is contradictory. Parr et al. (*6*) did not find a correlation between variations in porcine LD tenderness and the abundance of calpain 3 level. However, Ilian et al. (*5*) observed a significant correlation between the rate of tenderization of different muscles and the steady state mRNA levels for calpain 3 in both the bovine and ovine.

To gain further insight into the role of calpains in meat tenderization, lambs were fasted for various periods of time to generate significant differences in the tenderness of a specific muscle and to determine in tandem the expression of calpain 1, calpain 2, calpain 3, and calpastatin. The general assumption has been that increased calpain expression or decreased calpastatin expression accompanied with an increase in the tenderness indicates a role for calpain in the tenderization process and vice versa. To our knowledge, this is the first study to investigate the role of the ubiquitous and the muscle-specific calpains in the meat tenderization process at the protein level simultaneously.

As indicated earlier, fasting lambs for 1 day caused a significant improvement in the tenderness of the LD compared to control lambs. To determine the underlying causes for this observation, we calculated the correlation coefficients between the tenderness of the LD at 24 h post-mortem storage and the expression of the calpain system for the control and 1 day fasted lambs (Table 4). The results indicated there was no significant correlation between tenderness and the activity of calpain 2 or calpastatin. However, the tenderness of the LD was significantly correlated with the activity of calpain 1 ($r = 0.467$, $P < 0.05$) and the levels of calpain 3 in the myofibrillar ($r = 0.454$, $P < 0.05$) and sarcoplasmic ($r = 0.583$, $P < 0.01$) fractions. These results and the observation that calpain 3, like calpain 1, is proteolytically active during 24 h post-mortem of storage (Figure 7) support a role for calpain 3 and calpain 1 in the post-mortem tenderization of meat. A comparison between the LD tenderness of animals fasted for 0, 1, and 3 days and the associated levels of the various calpains indicated that variations in the myofibrillar-bound calpain 3 contributed to variations in tenderness among these treatments. Comparison between the LD tenderness of animals fasted for 0 and 7 days and the associated levels of various calpains is interesting. The LD tenderness of lambs fasted for 7 days was not different from the control LD despite a significant reduction (30%) in the myofibrillar-bound calpain 3 level. This was expected to cause a significant reduction in the LD tenderness of lambs fasted for 7 days compared to the control. A possible explanation is that the expected effect on tenderness due to the reduction in the myofibrillar-bound calpain 3 level was counteracted by a significant increase (200%) in the proteolytic potential of calpain 1. In other words, the effect of fasting for 7 days on the LD tenderness has to take into account the condition of calpain 1 and the myofibrillar-bound calpain 3. Taken together, the results of the LD tenderness of animals fasted for 0, 1, 3, and 7 days and the associated levels of various calpains indicate that the myofibrillar-bound calpain 3 and calpain 1 are the key enzymes involved in meat tenderization during post-mortem storage.

Table 4. Correlation Coefficients between the Tenderness, at 24 h Post-mortem, and the Expression of the Calpains, at 0 h Post-mortem, in the LD Based on Individual Observations of These Parameters for Control and 1 Day of Fasting Samples

parameter	shear force, kg	significance level
calpain 1 activity	−0.467	$P \leq 0.05$
calpain 2 activity	−0.064	not significant
calpain 3 level		
myofibrillar fraction	−0.454	$P \leq 0.05$
sarcoplasmic fraction	−0.583	$P \leq 0.01$
calpastatin activity	−0.033	not significant

To close, the results in this study support a role for calpains 1 and 3, but not for calpain 2, in the tenderization of ovine LD during post-mortem storage. The conclusion on the role of the ubiquitous calpains in meat tenderness is in accord with several studies which have suggested that calpain 1 and not calpain 2 plays a role in the tenderization of meat (*3*, *30*, *38*). With regard to calpain 3, this study is in accord with our previous findings that showed there was a strong correlation between the intermuscular variation in tenderness and the expression of calpain 3 mRNA in the bovine and the ovine (*5*). These findings and the observation that calpain 3 was found terminally activated, like calpain 1, during post-mortem storage support a role for calpain 3 in post-mortem tenderization of meat. In contrast, Parr et al. (*6*) did not find a link between calpain 3 and post-mortem tenderization in the porcine LD. The discrepancy between our results and those of Parr et al. (*6*) may be related to the methodology or animal species studied.

Finally, meat tenderization is a complex biochemical process. Our hypothesis for the role of calpains in meat tenderization based on the findings in this study may be expressed as

$$\underset{\text{(less tender meat)}}{\text{muscle structural proteins (MSP)}} \xrightarrow[[Ca^{2+}]]{\text{calpains 1 and } 3_M} \underset{\text{(tender meat)}}{\text{fractured MSP}}$$

Additional experimental information is needed to determine the relative roles of calpain 1 and the myofibrillar-bound calpain 3 (calpain 3_M) in post-mortem tenderization.

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