POSTMORTEM PROTEOLYSIS IN LONGISSIMUS MUSCLE FROM BEEF, LAMB AND PORK CARCASSES

M. Koohmaraie[1], G. Whipple[1], D. H. Kretchmar[2], J. D. Crouse[1] and H. J. Mersmann[3]

U.S. Department of Agriculture[4], Clay Center, NE 68933

ABSTRACT

Postmortem proteolysis in skeletal muscle and factors affecting this process were examined in pork, lamb and beef longissimus muscles (LM) to determine the cause of differences in meat tenderness among these species. Fat thickness differed among species in the following order: pork > beef > lamb. The following patterns were observed for rate of temperature and pH decline: lamb > pork > beef and pork > beef > lamb, respectively. At 1 d postmortem, pork was the most tender, followed by beef and lamb, respectively. Between 1 and 14 d of postmortem storage, lamb LM was the most improved in tenderness, followed by beef and pork, respectively. Species did not differ ($P > .05$) in LM collagen solubility. Pork LM from fed pigs had the highest ($P < .05$) level of cathepsins B + L and cystatin(s) activities, whereas no differences ($P > .05$) were observed among the species for cathepsin B activity. The lowest ($P < .01$) Ca^{2+}-dependent protease (CDP)-II and CDP inhibitor activities were observed in pork LM. Beef LM had the highest CDP inhibitor activity ($P < .05$) but was intermediate in CDP-II activity. No differences were observed among species for CDP-I activity. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of myofibrils isolated at 0, 1 and 14 d postmortem indicated that by d 1, desmin hydrolysis was most extensive in pork muscle, followed by lamb and beef. This difference in the rate of myofibrillar protein hydrolysis may account for the differences observed in the rate of postmortem tenderization in LM among these species.

Key Words: Skeletal Muscle, Proteolysis, Protease, Tenderness, Species

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Introduction

Storage of carcasses at refrigerated temperatures improves meat tenderness. Although this improvement in tenderness is measurable both subjectively and objectively, the mechanism of this postmortem improvement in tenderness is not clearly defined (for review see Penny, 1980; Dutson, 1983; Goll et al., 1983; Koohmaraie, 1988). Proteolysis of myofibrillar proteins presumably plays a major role in postmortem meat tenderization. Numerous proteolytic enzymes are found in skeletal muscle; however, only Ca^{2+}-dependent proteases (CDP) and certain cathepsins have been shown, to date, to degrade myofibrillar proteins. Either CDP, cathepsins or their synergistic action are primarily responsible for postmortem changes leading to meat tenderization (Dutson, 1983; Pearson et al., 1983; Dutson and Pearson, 1985; Greaser, 1986; Asghar and Bhatti, 1987; Koohmaraier, 1988).

Rate of postmortem meat tenderization differs among species (Dransfield et al., 1981; Etherington et al., 1987). For example, although 80% of the tenderization is completed in about 5 d for pork, beef takes almost 2 wk

[1]USDA, ARS, Roman L. Hruska U.S. Meat Anim. Res. Center, Clay Center, NE 68933.

[2]Present address: Dept. of Surgery, Creighton Univ., Omaha, NE 68131.

[3]Present address: USDA, ARS, Children's Nutrition Res. Center, Houston, TX 77030.

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and lamb requires an intermediate amount of time to achieve the same degree of tenderization (Dransfield et al., 1981; Etherington et al., 1987). In chickens, tenderization is completed in about 2 d (Sayre, 1970; Yamamoto and Samejima, 1977).

The present experiment was undertaken to examine the relationship between CDP, CDP inhibitor, cathepsins B and L activities and postmortem tenderization in beef, lamb and pork longissimus muscle.

Materials and Methods

Animals. Five steers and one heifer (Hereford × Angus, 16 to 18 mo of age, live weight 576 ± 85 kg), six wethers (1/2 Finn × 1/4 Dorset × 1/4 Rambouillet, 5 to 7 mo of age, live weight 54 ± 3.3 kg), and 12 barrows (1/4 Yorkshire × 1/4 Landrace × 1/4 Large White × 1/4 Chester White, 6 mo of age) were slaughtered. One-half of the barrows were fasted for 48 h prior to slaughter (live weight 93 ± 2.5 kg) to simulate commercial handling, and the other half were fed until time of slaughter (live weight 102 ± 4 kg). Cattle had access to water until 12 h before slaughter, and sheep were fed up to slaughter. Each species was fed grain diets similar in energy and protein content to those commercially fed. Animals were slaughtered in three groups by species. None of the carcasses used in this experiment was electrically stimulated. Carcasses were chilled at –1°C (no air movement) for 24 h; then, the *Longissimus* muscle (LM, sixth rib to the fifth lumbar region) was removed from alternating sides. Steaks or chops, 2.5 cm thick, were removed, vacuum-packaged and aged at 2°C for 3, 7 or 14 d.

PH and Temperature Determinations. Temperature and pH of the LM on the side opposite that used for shear determination were determined at 0, 3, 6, 9, 12 and 24 h postmortem. The procedure of Bendall (1973) was used to determine pH on 2.5 g of cored tissue obtained at the 11th to 12th rib region. Temperature of the LM was monitored using an electronic digital probe at the 11th-rib region. From the dorsal surface, three LM temperatures were recorded at approximately the center and the periphery of the muscle with one measurement between these two extremes; an average temperature was calculated.

Shear Force Determination. Steaks or chops frozen after either 1, 7 or 14 d of aging (frozen ≤ 6 wk) were thawed and cooked on Farberware "Open-Hearth" broilers[5] to an endpoint temperature of 70°C. Steaks/chops were tempered for approximately 24 h at refrigerator temperature. Three (chops) to six (steaks) 1.3-cm cores were removed parallel to the muscle fibers with a hand coring device, and shear force measurements were made using an Instron Universal testing machine[6] with a microprocessor.

Myofibril Fragmentation Index Determination. Samples were taken from LM steaks and chops that had been vacuum-aged for 3, 7 or 14 d at 2°C. Myofibril fragmentation indices (MFI) were determined on fresh muscle at 1, 3, 7 and 14 d postmortem by the method of Culler et al. (1978).

Determination of the Amount and Solubility of Collagen. Heat-soluble and insoluble collagen were extracted from LM that had been frozen at 24 h postmortem for subsequent analysis (Hill, 1966). Hydroxyproline contents (Bergman and Loxley, 1963) of the heat-soluble and insoluble portions were multiplied by factors of 7.52 and 7.25, respectively, according to Cross et al. (1973). Collagen values are reported per gram of wet tissue.

Preparation of Ca^{2+}-Dependent Proteases and Their Inhibitor. Low calcium-requiring and high calcium-requiring Ca^{2+}-dependent proteases (CDP-I and CDP-II, respectively) and their inhibitor were prepared from 100 g of LM taken from the 6th rib region immediately after slaughter, according to the procedure described by Koohmaraie (1990a), except that dialysis against elution buffer rather than addition of cold water was used to reduce the ionic strength of the muscle extract prior to ion-exchange chromatography.

Preparation of Cathepsins B and B + L. Samples from the LM, 6th rib region, were frozen in liquid N immediately after slaughter and stored at –70°C. Cathepsins B, B + L and cystatin(s) activities were determined using S-Carboxymethylated-papain-Sepharose affinity chromatography according to the procedure described by Koohmaraie and Kretchmar (1990).

Determination of Plasma Free-Fatty-Acid Concentration. To verify that the pigs held off feed were fasted, the concentration of porcine

[5]Model #450N, Farberware, Bronx, NY.
[6]Model #1132, Instron Corp., Caton, MA.

TABLE 1. EFFECT OF SPECIES ON CARCASS BACKFAT AND LONGISSIMUS MUSCLE COLLAGEN CHARACTERISITICS

	Beef	Lamb	Fed pig	Fasted pig	SE
Backfat[a], cm	1.48[b]	1.02[b]	3.40[c]	3.47[c]	.16
Soluble collagen, mg/g	.43	.43	.34	.35	.03
Insoluble collagen, mg/g	2.56[b]	2.17[c]	1.89[d]	2.10[cd]	.09
Total collagen, mg/g	2.99[b]	2.61[c]	2.25[d]	2.44[cd]	.11
Soluble Collagen, %	14.19	16.6	15.7	14.1	.86

[a]Backfat measurements were made at the 12th rib for beef and lamb carcasses and for pig carcasses an average of measurements taken at the first and last ribs and last lumbar vertebra is reported.

[b,c,d]Least square means within the same row with different superscripts differ ($P < .05$).

plasma free-fatty-acid was determined according to the procedure described by Mersmann (1986).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Myofibrils were isolated from LM after 0, 1, and 14 d of postmortem storage at 2°C (Olson et al, 1976). Discontinuous 7.5 to 15% gradient gels were run according to the procedures described by Hames and Rickwood (1981). The ratio of acrylamide to bisacrylamide was 75 to 1. The acrylamide solution (30%) contained 50% glycerol.

Statistical Analysis. Data were analyzed by one-way analysis of variance. When significant differences were detected, species means were compared using the least significant difference method. The comparison error rate was .05 (SAS, 1985).

Results

Carcass Characteristics and Tenderness Measurements. Free-fatty-acid analyses indicated that fasted pigs had a higher serum free-fatty-acid concentration (1,100 vs 432 μeq/liter), confirming that these six barrows were in a fasted state (Mersmann, 1986).

Pork carcasses had the greatest ($P < .01$) fat thickness and lamb had the least fat thickness (Table 1). The temperature and pH decline of postmortem muscle varied in the following order: lamb > pork > beef and pork > beef > lamb, respectively (Table 2). Factors such as postmortem metabolism, carcass size, fat thickness, muscle thickness and their interactions could contribute to these differences. Lundberg et al. (1987) reported that the rate of postmortem metabolism in LM varied among different species and that pork muscle had the highest rate.

Beef LM had a greater amount of total and of insoluble collagen than pork or lamb LM (Table 1). Lamb LM had more total and insoluble collagen than LM from fed pigs but not from fasted pigs. However, no differences ($P > .05$) were observed among the species for the percentage of heat-soluble collagen.

Shear force and MFI values as a function of postmortem time are reported in Table 3. At d 1, pork LM had the lowest and lamb LM had the highest shear force. Pork LM MFI values were highest at d 1, which agreed with the shear force data (most tender). However, based on MFI values, lamb LM was intermediate in degree of tenderness and beef was least tender. The reason for the discrepancy between the

TABLE 2. EFFECT OF SPECIES ON TEMPERATURE AND pH DECLINE IN LONGISSIMUS MUSCLE[a]

Item	Beef	Lamb	Pig	Fasted pig
Temperature, °C				
0 h	39.4	39.6	39.3	38.1
3 h	25.3	14.0	19.3	17.3
6 h	15.6	4.7	10.9	8.5
9 h	10.4	1.5	4.8	4.2
12 h	7.2	.7	1.6	1.2
24 h	1.5	−.8	−.4	−.5
pH				
0 h	6.86	6.89	6.40	6.49
3 h	6.47	6.66	6.44	6.06
6 h	6.12	6.42	6.00	6.01
9 h	5.87	6.28	5.78	5.84
12 h	5.77	6.04	5.51	5.62
24 h	5.63	5.65	5.42	5.46

[a]Least squares means differences greater than .82 for temperature and greater than .14 for pH are statistically significant ($P < .05$). Standard errors of least square means were .34 and .06 for temperature and pH measurements, respectively.

ranking of MFI and shear force for lamb vs beef is not apparent at this time. Because MFI is a measure of postmortem proteolysis (for review see Koohmaraie, 1988), presumably pork LM was at a more advanced stage of proteolysis at d 1 than either lamb or beef LM were. To determine whether this conclusion was correct, myofibrils were isolated from LM at different postmortem times and analyzed by SDS-PAGE. Results indicated that desmin was extensively hydrolyzed in pork muscle by d 1 postmortem, whereas in beef and lamb muscle, desmin degradation was minimal at d 1 (Figure 1). However, after 14 d of postmortem storage, no differences were observed among the species.

As postmortem storage time increased, shear-force values decreased for all species; however, the extent of decline varied considerably among species (Table 3). For all species, the majority of the improvement in tenderness occurred within 7 d of aging. At 14 d of aging, lamb LM had the greatest decline in shear force (4.10 kg), followed by beef (1.77 kg), fasted pig (1.20 kg) and fed pig (.76 kg). These results are in agreement with those of Dransfield et al. (1981) in which lamb LM had the largest decline in shear force, followed by beef and pork, respectively. As indicated by shear force and MFI values, meat from fasted pigs tended to be more tender than that from fed pigs at all postmortem times.

TABLE 3. EFFECT OF SPECIES ON SHEAR FORCE AND MYOFIBRIL FRAGMENTATION VALUES OF LONGISSIMUS MUSCLE AT DIFFERENT POSTMORTEM TIMES

Trait[a]	Beef	Lamb	Pig	Fasted pig
Shear force, kg				
Day 1	6.30	7.31	4.99	4.79
Day 7	4.61	3.86	4.77	3.37
Day 14	4.53	3.21	4.23	3.59
Myofibril fragmentation index[b]				
Day 1	43.8	51.9	64.6	63.1
Day 3	49.6	60.2	74.7	75.2
Day 7	69.2	73.0	78.1	90.7
Day 14	77.4	95.7	85.8	90.2

[a]Least squares means differences greater than .62 for shear force and greater than 7.79 for myofibril fragmentation index are statistically significant ($P < .05$). Standard errors of least squares means were .15 and 1.65 for shear force and myofibril fragmentation index, respectively.

[b]Absorbance at 540 nm of a .5 mg protein/ml myofibril solution × 200.

Lysosomal Enzymes. Of the lysosomal enzymes, cathepsins B and L are thought to have a major role in postmortem tenderization of meat (Ouali et al., 1987). Both of these enzymes are cysteine proteases and can hydrolyze Z-Phe-Arg-NMec. Additionally, cathepsin B, but not L, can be assayed with Z-Arg-Arg-NMec (Barrett and Kirschke, 1981). Thus, activities of these proteases are reported as B and B + L (Table 4).

A crude muscle extract was prepared and, after centrifugation, the activities of these proteases were determined in the supernatant. In addition to these proteases, the supernatant contains the endogenous inhibitor (cystatin) of these proteases. Whereas cathepsins are located in the lysosome, cystatin(s) is localized in the cytosol. However, this cellular compartmentalization is destroyed during homogenization of muscle samples, which allows for the reaction between cystatin(s) and cathepsins to occur. Cystatin(s) has been shown to be more specific for cathepsin L than B (Barrett, 1985). Therefore, the activity of cathepsins, especially cathepsin L, in the muscle extract is greatly underestimated. To correct for this, an aliquot of the crude muscle extract was passed through a S-Carboxymethylated (cm)-papain-Sepharose

TABLE 4. EFFECT OF SPECIES ON THE ENZYME ACTIVITY FOR LONGISSIMUS MUSCLE

	Beef	Lamb	Fed pig	Fasted pig	SE
CDP-I[a]	109.3	123.4	118.3	112.6	6.3
CDP-II[b]	112.2^{g}	139.9^{f}	93.2^{h}	78.8^{i}	4.7
CDP inhibitor[c]	413.4^{f}	243.9^{g}	149.5^{h}	117.0^{h}	24.6
Cathepsin B					
Specific activity[d]	1.16	1.03	1.13	1.07	.10
Total activity[e]	56.1	48.8	55.0	54.8	3.8
Cathepsins B + L					
Specific activity[d]	2.19^{g}	2.24^{g}	3.24^{f}	2.76^{fg}	.22
Total activity[e]	105.2^{g}	106.0^{g}	156.7^{f}	142.4^{f}	9.51
Cystatin(s)	1.5^{g}	1.6^{g}	2.8^{f}	2.7^{f}	.2

[a]Low Ca^{2+}-requiring Ca^{2+}-dependent protease total activity/100 g muscle (caseinolytic activity).

[b]High Ca^{2+}-requiring Ca^{2+}-dependent protease total activity/100 g muscle (caseinolytic activity).

[c]Inhibitor of CDP-I and CDP-II, A_{278}/100g muscle (inhibition of casein hydrolysis by CDP-II).

[d]nmol of NMec (amido-methylcoumarin) released·min^{-1}·mg $protein^{-1}$.

[e]nmol of NMec (amido-methylcoumarin) released·min^{-1}·g $muscle^{-1}$.

[f,g,h,i]Least squares means within the same row with different superscripts differ ($P < .05$).

affinity column to remove cystatin(s). Because the K_i of cystatin(s) for papain is much lower than that of cathepsins B and L (Barrett, 1987), the eluent of the affinity column should be free of cystatin(s). Therefore, the activities of these proteases were measured before and after cm-papain-Sepharose chromatography (Koohmaraie and Kretchmar, 1990). Theoretically, the difference obtained should be an indication of the level of cystatin activity present in the muscle extract. In this experiment the cystatin(s) activity was defined as the ratio of cathepsins B + L activity after affinity chromatography to cathepsins B + L activity before affinity chromatography. Results indicated that beef and lamb had similar cathepsins B + L activity; this activity was significantly lower than the activity in muscle from fed pigs (Table 4). However, no differences were observed for cathepsin B activity. Results also indicated that pork muscle contains more cystatin(s) than either lamb or beef. These results agree with those of Etherington et al. (1987).

Ca^{2+}-Dependent Protease-I, -II and Inhibitor. The Ca^{2+}-dependent proteases and their inhibitor were extracted from LM of all species immediately after death. Elution profiles obtained for all species were similar to each other and to those reported by Koohmaraie (1990a). There were no significant differences in the activity of CDP-I among species (Table 4). However, lamb had the highest CDP-II activity, followed by beef and pork ($P < .05$), respectively. The following pattern was observed for CDP inhibitor activity among species: beef > lamb > pork ($P < .05$).

Discussion

The structural changes occurring in skeletal muscle during postmortem storage of carcasses at refrigerated temperature that lead to meat tenderization are thought to be due to the proteolytic action of endogenous proteases (Penny, 1980; Dutson, 1983; Goll et al., 1983;

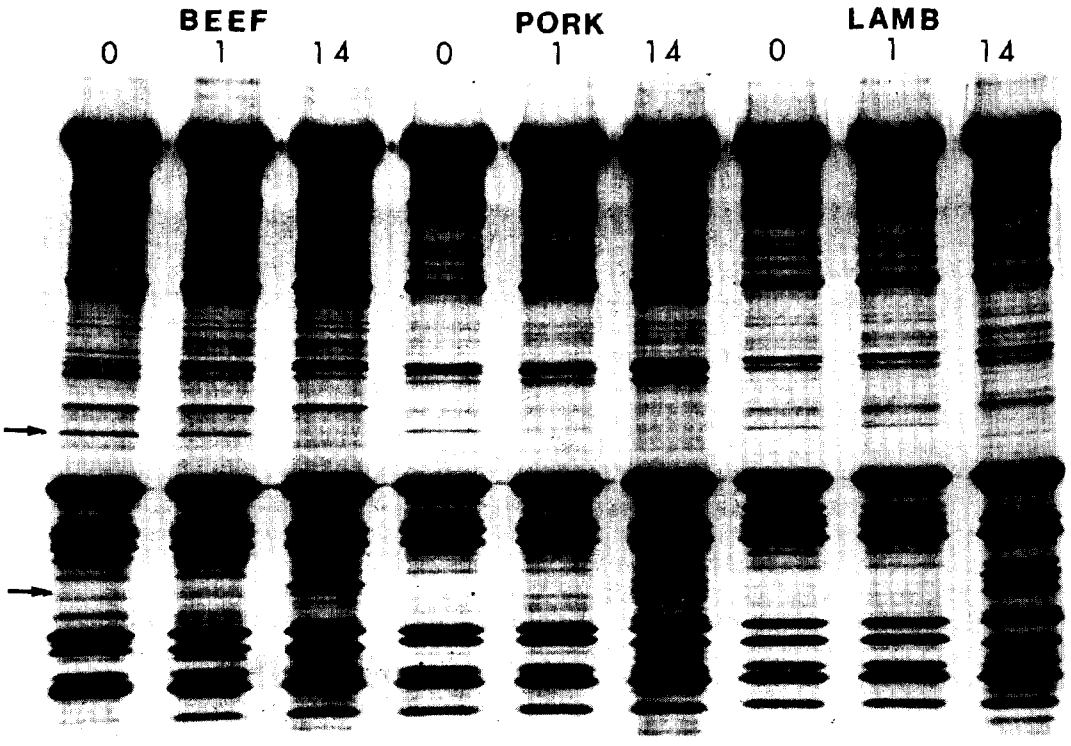

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of myofibrils isolated from longissimus muscle of beef, pork and lamb after 0, 1 and 14 d of postmortem storage. Top and bottom arrows correspond to the positions of desmin and 30,000-Da fragment, respectively.

Koohmaraie, 1988). Because CDP and certain cathepsins are capable of hydrolyzing myofibrillar proteins, action of one or more of these classes of proteases presumably is responsible for postmortem changes in skeletal muscle. The class of protease responsible for postmortem changes should have greater activity in the carcasses with a greater aging response, and vice versa. The aging response is defined as the extent of postmortem tenderization. Indeed, there is evidence within a species to support this hypothesis. For example, the LM, which has a high aging response, also has high CDP activity compared with the psoas major muscle, which has low CDP activity and a low aging response (Olson et al., 1976; Koohmaraie et al., 1988).

The rate of postmortem tenderization differs among species (Sayre, 1970; Yamamoto and Samejima, 1977; Dransfield et al., 1981; Etherington et al., 1987); pork requires the least amount of time for tenderization, beef needs the most and lamb needs an intermediate amount of time to achieve the same degree of tenderization. The objectives of the present study were to measure the activity of proteases proposed to be involved in postmortem tenderization in pork, lamb and beef, and to determine to what extent the variation in the protease(s) activity may account for the differences observed in the tenderization among these species. Under normal slaughter conditions, animals usually are in a fasted state, and because fasting may affect these proteolytic systems, we included an additional six pigs that were fasted for 48 h prior to slaughter. We fasted only this species because a relatively short fasting period is sufficient to produce changes in pigs (nonruminants) but not in cattle or sheep (ruminants). For example, plasma free-fatty-acid concentrations do not reach a plateau in cattle until about 8 d of fasting (Dimarco et al., 1981), whereas a plateau is reached at approximately 24 h in the pig (Mersmann and MacNeil, 1985).

The results of this experiment indicate that the differences in rates of proteolysis of myofibrillar proteins may account for the differences observed in the rates of postmortem tenderization in these species. This conclusion was derived from the MFI and SDS-PAGE results. At 1 d postmortem, the MFI value for pork samples was about 65.0. The same MFI values were achieved only after 3 and 7 d in lamb and beef samples, respectively. These results were supported by SDS-PAGE results, in that desmin was extensively hydrolyzed in myofibrils prepared for pork samples at 1 d postmortem, but desmin degradation was minimal in beef and lamb samples at the same postmortem time (Figure 1). Therefore, this accelerated rate of postmortem proteolysis seems responsible for the rapid tenderization in pork muscle. The question at this point is why pork muscle undergoes proteolysis faster than beef or lamb muscle. To answer this question, we determined the activity of two classes of endogenous proteases proposed to be involved in postmortem proteolysis of myofibrillar proteins, namely lysosomal enzymes and CDP. The same approach was taken by Etherington et al. (1987). However, the major difference between this study and that of Etherington et al. (1987) is that we also quantified the activities of the endogenous inhibitors of these two proteolytic systems.

The LM obtained immediately after slaughter from the different species had similar cathepsin B activities ($P > .05$); however, they differed (Table 4) in cathepsins B + L and their inhibitor (cystatin) activities ($P < .05$). Pork LM, which had the highest tenderization rate, had the highest cathepsins B + L activity. Based on the differences in the cathepsins B + L activities, one might conclude that the higher cathepsins B + L activity is the reason for faster rate of postmortem tenderization in pork LM. However, the problem with such a conclusion is that pork LM also had the highest cystatin activity (Table 4). Cystatins are located in the cytosol; presumably their function is to prevent inappropriate proteolysis in the cytosol by lysosomal enzymes (for review see Bond and Butler, 1987). Therefore, although pork LM had the highest cathepsins B + L activity, it may not be the reason for the accelerated proteolysis of myofibrillar proteins in pork LM. In addition, SDS-PAGE results do not support a role for lysosomal enzyme effect on myofibrils because 1) there was no evidence of myosin or actin degradation, which are the primary myofibril protein substrates for cathepsins (Goll et al, 1983; Koohmaraie, 1988) and 2) desmin, which clearly was degraded, is not degraded by these lysosomal enzymes (Whipple and Koohmaraie, unpublished results). Based on these results, the differences in cathepsins B + L activities cannot account for the differences observed in

the rate of postmortem proteolysis of myofibrillar proteins for these species. Certain lysosomal enzymes have the ability to hydrolyze the collagen component of the connective tissue (Maciewicz et al., 1987; Maciewicz and Etherington, 1988). Although the results of this study indicated that collagen solubility was not different among the species, perhaps the method used in its determination was not sensitive enough to detect small but significant differences. Therefore, in light of evidence presented by Stanton and Light (1987, 1988, 1990a,b) we believe that lysosomal enzymes are likely to be involved in the degradation of the extracellular matrix. However, based on current available information, it seems very unlikely that these cathepsins play a significant role in the proteolysis of myofibrillar proteins.

The LM muscle obtained immediately after death from different species had the same CDP-I activity ($P > .05$), but species differed in CDP-II and CDP inhibitor activity ($P < .05$). The dramatic differences observed in the activity of CDP inhibitor among species have the most significant biological implications (Table 4). The CDP inhibitor activity in pork (fed) LM was 62 and 36% of that of lamb and beef, respectively. We believe that the differences in the CDP inhibitor activity are significant and may be the reason for differences observed in rates of postmortem tenderization in the species studied. Additional evidence supporting this conclusion is as follows: 1) infusion of lamb carcasses with $ZnCl_2$, which prevents postmortem proteolysis and tenderization, also prevents the loss in CDP inhibitor activity that occurs in noninfused carcasses (Koohmaraie, 1990b; Kouhmaraie and Shackelford, 1991); 2) feeding a β-adrenergic agonist to lambs results in a reduced rate of myofibrillar protein degradation during postmortem storage and also significantly elevated the activity of CDP inhibitor (Kretchmar et al., 1990); and 3) a reduced rate of myofibrillar protein degradation during postmortem storage is reported to be one major reason for reduced tenderness of meat from *Bos indicus* compared to *Bos taurus* breeds of beef (Wheeler et al., 1990; Whipple et al., 1990a,b; Shackelford et al., 1991). All these investigators concluded that elevated CDP inhibitor activity probably was the cause of reduced proteolysis and tenderness of the *Bos indicus* breed. Based on the results of the current study, we conclude that accelerated proteolysis of key structural myofibrillar proteins probably is responsible for the differences observed in the tenderization rate among the species studied and that CDP inhibitor apparently influences this process.

Implications

This study was undertaken in an attempt to determine the reason for the differences observed in the tenderness of meat obtained from pork, beef and lamb carcasses. Differences in the rate of proteolysis of myofibrillar proteins during postmortem storage probably are responsible for the tenderness differences observed. Because pork muscle, the most tender muscle, contains considerably less Ca^{2+}-dependent protease inhibitor than beef muscle, the least tender muscle, we concluded that Ca^{2+}-dependent protease inhibitor is involved in the regulation of postmortem proteolysis of myofibrillar proteins. These results indicate that acceleration of postmortem proteolysis by manipulation of live animals through diet, sex or selection to reduce Ca^{2+}-dependent protease inhibitor activity probably will improve the tenderness of both beef and lamb, particularly of beef.

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