Acceleration of Postmortem Tenderization in Ovine Carcasses Through Activation of Ca²⁺-Dependent Proteases


ABSTRACT
Infusion of lamb carcasses with 0.3M CaCl₂ resulted in acceleration of postmortem tenderization process. Control and treated animals had similar cathepsin B, H, and L activities. Various control groups had similar CDP-I, -II and inhibitor activities, whereas these were all decreased in CaCl₂ infused animals. Hence, it was concluded that the activation of Ca²⁺-dependent proteases was responsible for the observed postmortem proteolysis and tenderization and, it seems unlikely that activity of these cathepsins is related to postmortem tenderization under conditions used in this experiment.

INTRODUCTION
TENDERNESS is the prominent quality determinant and probably the most important sensory characteristic of meat. Currently, postmortem aging appears to be one of the best methods for producing tender meat. Although the increase in meat tenderness is measurable both subjectively and objectively, the exact mechanism of postmortem tenderization still remains an unresolved issue.

Many studies have been carried out on the changes of myofibrillar proteins during postmortem aging, the mechanism of these changes, and the relationship between these changes and meat tenderization (Penny, 1980; Asghar and Bhatti, 1987). Proteolysis of muscle appear to be a major contributor to the tenderization process during postmortem aging. Proteolytic changes in collagen during postmortem storage comparable to those of myofibrillar proteins (Tarrant, 1987) have not been observed. Therefore, the principal mechanism of meat tenderization during postmortem storage may be the limited hydrolysis of myofibrillar proteins by endogenous muscle proteases (Goll et al., 1983a).

Of the proteases indigenous to skeletal muscle, two likely proteolytic systems include, (1) the Ca²⁺-dependent proteases (Goll et al., 1974a; Olson et al., 1977; Goll et al., 1983a; Koochmare et al., 1986; Zeece et al., 1986; Koochmare et al., 1987; Koochmare et al., 1988a,b) and (2) the cathepsins (Moeller et al., 1977; Dutson, 1983; Ouali et al., 1987). Some have suggested that cooperative action of these two protease systems also may occur (Penny and Ferguson-Pryce, 1979; Dutson, 1983; Asghar and Bhatti, 1987).

Incubation of bovine longissimus muscle slices at 12 hrs postmortem (Koochmare et al., 1988b) in a buffer solution containing CaCl₂ demonstrated that most of the postmortem changes occurred within the first 24 hrs of incubation. On the other hand, when muscle slices were incubated in the same buffer containing 10 mM EGTA or 10 mM EDTA, these changes were completely blocked (Koochmare et al., 1988b). Because none of the treatments affected lysosomal enzyme activities (cathepsin B, H and L) and because there was a decrease in calcium-dependent protease activity, Koochmare et al. (1988b) concluded that: (1) the postmortem tenderization events are Ca²⁺ mediated, and that (2) calcium-dependent proteases are responsible for postmortem tenderization. The present experiment was conducted to determine whether the tenderization process could be accelerated by infusion of carcasses with CaCl₂ immediately after death.

MATERIAL & METHODS
A TOTAL of 24 lambs (8-12 month old) were slaughtered in groups of four; one lamb for each of the four treatments: (1) control (animals slaughtered according to normal procedures); (2) electrically stimulated (6 cycles, each cycle consists of 20 sec on, 20 sec off, 21 volts, rectangular waves, 1 cycle/sec); (3) electrically stimulated and then infused (10% of the live weight) with water (distilled, deionized, glass distilled), and (4) electrically stimulated and then infused (10% of the live weight) with 0.3M CaCl₂. The above solutions were infused through the carotid artery (following electrical stimulation and prior to evisceration) with a ham pumping device (Presto Precision Product, Inc. Farmingdale, NY). After completion of the infusion process, the carcasses were transferred to a holding cooler (1-2°C). Twenty-four hr after slaughter, the entire loin and rib were removed from each carcass and divided into three sections. Each section was then randomly assigned to day 1, 3, or 6 for the following determinations. Day 1 shear force, Ca²⁺-dependent protease-I, II, and their inhibitor activities, cathepsin B, H and L activities, sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of isolated myofibrils, and Ca²⁺ content. Day 3 & SDS-PAGE of isolated myofibrils. Day 6: shear force, SDS-PAGE of isolated myofibrils.

Shear force determination
Shear force was determined on unfrozen lamb chops according to standard procedures. Briefly, chops were broiled on a Farberware "open hearth" broiler and internal temperature was monitored with an iron constant thermocouple wires attached to a Honeywell 112 multi-point recorder. Chops were turned when internal temperature reached 40°C and removed from broiler when they reached 70°C. Chops were cooled for 24 hr at 2-4°C and six cores (1.27 cm diameter) were removed from each chop. Each core was sliced three times (total of 18 readings per chop) with a Warner-Bratzler device.

Preparation and assay of cathepsins B, H and L
Extraction and assay of catheptic enzymes were done according to the procedure described by Koochmare et al. (1988a).

Preparation and assay of Ca²⁺-dependent protease-I, -II, and their inhibitor
Ca²⁺-dependent protease-I (CDP-I), -II (CDP-II) and their inhibitor were prepared from 200g longissimus muscle. The muscles were trimmed of fat and connective tissue, cut into 2 x 2 cm pieces and stored at -70°C (not more than 14 days). The samples were removed from the freezer, allowed to stand at 2°C for 90 min and then homogenized in 2.5 vol 50 mM Tris-HCl, pH 8.3 containing 5 mM ethylene glycol-bis (β-aminoethylalcohol) (EGTA) and 10 mM 2-mercaptoethanol (MCE), in a Waring Blender. To prevent Ca²⁺-stimulated autolysis of CDP-I and CDP-II during extraction of muscle (Hallaway et al., 1982; McCullogh et al., 1982; Goll et al., 1983b), from lambs infused with CaCl₂, the samples were homogenized in 2.5 vol 150 mM Tris-HCl, pH 8.3 containing 50 mM EGTA, and 10 mM MCE. Homogenization was done twice for 30 sec at the low speed setting and twice for 30 sec at the high

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speed setting, with a 30 scc cooling period between bursts. The homogenate was centrifuged at 30,000 × g for 60 min. The supernate was filtered through two layers of cheese cloth and pH was adjusted to 7.5. The pH-adjusted supernate was then passed through glass wool and dialyzed against 20 mM Tris-HCl, pH 7.5 containing 5 mM EGTA and 10 mM MCE, two changes for 18–24 hr. After dialysis, the extracts were centrifuged at 30,000 × g for 60 min, chromatographed on DEAE-Sephadex column that had been equilibrated with 20 mM Tris-HCl, pH 7.5 containing 10 mM NaCl, 0.1 mM EGTA and 10 mM MCE. Columns were then washed with the same buffer to remove unbound protein, until the absorbance of the outflow at 278 nm was between 0.1 to 0.4. The bound proteins were then eluted with a continuous gradient (total volume of 700 mL) of 10–500 mM NaCl in the elution buffer.

Activities of CDP-I and CDP-II were determined by using casein as substrate. The reaction mixture contained 5 mg/mL casein in 100 mM Tris-acetate, pH 7.5 containing 10 mM MCE, 1 mM NaN3, and 5 mM CaCl2 in a total volume of 2.0 mL. To determine Ca2+-independent activity for each fraction assayed, 5 mM CaCl2 was replaced with 10 mM EDTA. After 50 min incubation at 25°C, the reaction was stopped by adding 2 mL 5% trichloroacetic acid. After centrifugation at 3000 × g for 30 min, the ANS fluorescence of the supernatant was measured. The inhibitor activities were determined as described by Koohmaraie et al. (1988a).

**Myofibril isolation**

Myofibrils were isolated according to the procedure as described by Goll et al. (1974b).

**Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was conducted according to Laemmli (1970) on 10% polyacrylamide slab gels.

**Protein concentration**

Protein concentration were determined by the biuret method (Gornall et al., 1949).

**Calcium determination**

The total calcium contents of the longissimus muscle was measured using standard techniques, with an atomic absorption spectrophotometer (model D 951, Instrument Lab. Inc., Lexington, MA). Briefly, 5–7 g muscle (at 24 hr postmortem) were ashed at 540°C followed by HNO3-HClO4 digestion. Upon completion of the digestion process, the amount of Ca2+ was measured by atomic absorption spectrometry.

**Statistical analysis**

Animals were randomly assigned to treatments. Data were analyzed by one way analysis of variance. When significant differences among treatments were detected, treatment means were compared using the least significant difference method. The comparisonwise error rate was 0.05 (SAS, 1982).

**RESULTS**

THE CALCIUM CONTENT (μg/g wet tissue) of the carcasses infused with CaCl2 was increased about 40-fold as compared to the other treatments (Table 1). Infusion of carcasses with CaCl2 significantly (P<0.05) lowered longissimus muscle shea force values by 24 hr and these values did not change with further postmortem storage time. To avoid the Ca2+-induced muscle shortening, low frequency electrical stimulation (LFES) was used to diminish the ATP supplier in the prerigor muscle. LFES improved tenderness (shear value) by about 17%. To ensure that the observed effects were not due to infused water lamb carcasses were infused with water only after LFES. There was no affect on shear value. Catheptic enzyme activities were not affected by treatment. CDP-I and CDP-II and their inhibitor had similar activities for the CON, LFES, and LFES + H2O treatments while they were significantly lowered in carcasses infused with CaCl2.

The effect of these treatments on myofibrillar proteins were studied on myofibrils purified from the longissimus muscle after 1, 3, and 6 days of postmortem storage and subjected to SDS-PAGE (Fig. 1). The 30,000 dalton component was already present in longissimus muscle of CaCl2 infused lamb 24 hrs postmortem and its intensity increased only slightly with further postmortem storage. In contrast, the 30,000 dalton component appeared gradually with postmortem storage time for muscle from CON lambs but it was readily apparent after 6 days of storage. It appears that LFES accelerated the appearance of the 30,000 dalton component because this proteolytic fragment was present in LFES and in LFES + H2O groups after 3 days of postmortem storage. Appearance of the 30,000 dalton component has been reported to be the most consistent and noticeable change that occurs in myofibrillar proteins during postmortem storage (MacBride and Parrish, 1977; Olson and Parrish, 1977; Olson et al., 1977; Parrish et al., 1981; Penny, 1980; Koohmaraie et al., 1984a,b,c).

**DISCUSSION**

INFUSION of carcasses with CaCl2, eliminated the requirement for extended postmortem storage as indicated by shear force measurements at 24 hrs postmortem (Table 1). SDS-PAGE results indicated that changes in the myofibrillar proteins were completed within 24 hr as opposed to 3–7 days required for the same changes to occur in the other treatment groups. Because these treatments had no effect on the lysosomal enzymes and a clear and definable decrease in activities (autolysis) of CDP-I, CDP-II and their inhibitor, we conclude that: (1) postmortem tenderization is a Ca2+-mediated process and that (2) there is sufficient evidence to conclude that Ca2+-functions through activation of the CDPs.

These results clearly demonstrate that infusion of lamb carcasses with CaCl2 resulted in loss of CDP-I and inhibitor activities and a significant decrease in the activity of CDP-II within the first 24 hr (Table 1). Without further investigations, it would be impossible to explain the effects of CaCl2 infusion treatment on this proteolytic system. However, it has been clearly demonstrated that calcium-dependent proteases autoxylze in the presence of calcium (Hathaway et al., 1982; Mellgren et al., 1982; Goll et al., 1983b; Parkes et al., 1985; Crawford et al., 1987) and continued incubation in the presence of calcium results in loss of enzymatic activity (Mellgren et al., 1982; Parkes et al., 1985; Crawford et al., 1987). Therefore, the loss of CDP-I activity and significant decrease in CDP-II activity could be due to autoxylze of these proteases in the presence of calcium (Table 1). Studies conducted to examine the effects of postmortem storage on CDPs in bovine skeletal muscle (Ducasting et al., 1985; Koohmaraie et al., 1987) have demonstrated that conventional postmortem storage had no effect on CDP-II activity. Based on these observations (Ducasting et al., 1985; Koohmaraie et al., 1986) and the results of present study (Table 1) we have concluded that autolysis in the presence of calcium is the reason for loss of CDPs activities. Whether the process of autolysis and loss of CDP activity occurred before myofibrillar proteins degradations (Fig. 1) and tenderization (Table 1) cannot be determined without further studies. However, Crawford et al. (1987) reported that the loss of enzymatic activity due to autolysis in the presence of calcium is a slow process. For chicken CDP-II the large subunit is degraded, over 2 hr at 22°C, to active-site-containing fragments of 54,000 and 37,000 daltons, after which no further significant autolysis occurred (Parkes et al. 1985). However, the loss of enzyme activity due to autolysis in CDP-II from chicken muscles proceeds much more slowly such that even after 3 hr of exposure to 5 mM CaCl2 at 22°C, it retained over 50% of its original activity (before autolysis). Therefore, it seems reasonable to suggest that myofibrillar protein degra-
### Table 1—Effect of treatments on calcium content, shear forces and enzyme activities in ovine longissimus muscle

<table>
<thead>
<tr>
<th>Traits</th>
<th>Control</th>
<th>Electrically stimulated</th>
<th>Electrically stimulated, then infused with H₂O</th>
<th>Electrically stimulated, then infused with CaCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (μg/g tissue)</td>
<td>53.0 ± 4.1*</td>
<td>57.0 ± 4.8*</td>
<td>56.9 ± 4.8*</td>
<td>157.0 ± 186.4*</td>
</tr>
<tr>
<td>Shear force at day 1 (kg)*</td>
<td>7.60 ± 0.40*</td>
<td>6.32 ± 0.27*</td>
<td>6.23 ± 0.30*</td>
<td>3.56 ± 0.16*</td>
</tr>
<tr>
<td>Shear force at day 6 (kg)*</td>
<td>4.61 ± 0.99*</td>
<td>3.60 ± 0.31*</td>
<td>3.03 ± 0.29*</td>
<td>2.77 ± 0.29*</td>
</tr>
<tr>
<td>Cathepsin B'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsedimentable</td>
<td>25.6 ± 3.7*</td>
<td>31.4 ± 2.4*</td>
<td>41.1 ± 6.6*</td>
<td>51.1 ± 7.7*</td>
</tr>
<tr>
<td>Sedimentable</td>
<td>16.9 ± 2.2</td>
<td>20.6 ± 1.9</td>
<td>27.2 ± 7.5</td>
<td>17.6 ± 3.7</td>
</tr>
<tr>
<td>Total</td>
<td>42.9 ± 5.8</td>
<td>52.1 ± 3.7</td>
<td>68.3 ± 13.9</td>
<td>68.7 ± 7.5</td>
</tr>
<tr>
<td>Cathepsin H'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsedimentable</td>
<td>153.2 ± 21.8</td>
<td>163.5 ± 19.5</td>
<td>185.7 ± 20.5</td>
<td>204.7 ± 21.2</td>
</tr>
<tr>
<td>Sedimentable</td>
<td>20.1 ± 1.5</td>
<td>21.0 ± 2.2</td>
<td>28.4 ± 3.2</td>
<td>25.1 ± 2.9</td>
</tr>
<tr>
<td>Total</td>
<td>173.3 ± 22.5</td>
<td>184.5 ± 21.2</td>
<td>214.1 ± 21.8</td>
<td>229.9 ± 33.0</td>
</tr>
<tr>
<td>Cathepsin L&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsedimentable</td>
<td>25.1 ± 3.2*</td>
<td>29.5 ± 3.4*</td>
<td>37.3 ± 6.9*</td>
<td>56.5 ± 9.2*</td>
</tr>
<tr>
<td>Sedimentable</td>
<td>183.6 ± 34.4</td>
<td>264.8 ± 77.9</td>
<td>246.1 ± 67.4</td>
<td>246.8 ± 65.5</td>
</tr>
<tr>
<td>Total</td>
<td>208.7 ± 34.7</td>
<td>294.3 ± 81.2</td>
<td>283.8 ± 73.5</td>
<td>303.3 ± 71.6</td>
</tr>
<tr>
<td>CDP-I</td>
<td>81.7 ± 7.4*</td>
<td>77.3 ± 8.7*</td>
<td>84.3 ± 3.1*</td>
<td>6.0 ± 0.0*</td>
</tr>
<tr>
<td>CDP-II</td>
<td>136.9 ± 5.7*</td>
<td>124.8 ± 5.5*</td>
<td>126.5 ± 1.2*</td>
<td>57.6 ± 5.7*</td>
</tr>
<tr>
<td>CDP-I + CDP-II</td>
<td>218.6 ± 8.9*</td>
<td>202.2 ± 9.0*</td>
<td>210.8 ± 2.7*</td>
<td>57.6 ± 5.7*</td>
</tr>
<tr>
<td>Inhibitor†</td>
<td>78.3 ± 7.0*</td>
<td>68.3 ± 4.5*</td>
<td>69.2 ± 7.9*</td>
<td>0.0 ± 0.0*</td>
</tr>
</tbody>
</table>

* Means within the same row with different superscripts differ (P<0.05).
* d on wet basis.
* kg/1.27 cm sone
* t units/mg protein/min.
* Low Ca²⁺-requiring Ca²⁺-dependent protease, total activity/200 g muscle (caseinolytic activity)
* High Ca²⁺-requiring Ca²⁺-dependent protease, total activity/200 g muscle (caseinolytic activity)
* Inhibitor of CDP-I and CDP-II, Aₜ₅₀/200 g muscle (inhibition of casein hydrolysis by CDP-II).

Fig. 1—SDS-PAGE of myofibrils isolated from longissimus muscle of treatments at different days postmortem. Gels 1, 2, and 3 represent Control; 4, 5, and 6 represent electrically stimulated; gels 7, 8 and 9 represent electrically stimulated then infused with water, and gels 10, 11 and 12 represent electrically stimulated then infused with CaCl₂ at 1, 3 and 6 days postmortem. Arrow indicates the position of 30,000 dalton fragment.

dation and tenderization took place prior to total loss of enzymatic activity.

Reasons for the loss of inhibitor activity with CaCl₂ infusion of carcasses are not readily apparent. It has been demonstrated that both CDP-I (Goll, personal communications) and CDP-II (Mellgren et al., 1986) are capable of hydrolyzing their specific inhibitor. However, Mellgren et al. (1986) reported that even after extensive cleavage of the inhibitor by calcium-dependent protease it remained a potent inhibitor of the protease. Mellgren et al. (1986) suggested that calcium-dependent proteolysis of inhibitor may represent an initial step in the turnover of the inhibitor and further degradation of the inhibitor may occur by other proteases.

In summary, we report a procedure that would eliminate the requirement for postmortem storage beyond 24 hr to ensure meat tenderness. The procedure involves low frequency elec-
trical stimulation of carcasses after death followed by infusion of the carcasses with a CaCl₂-containing solution prior to evisceration. However, further studies will have to be conducted to ascertain the effect of this procedure on other important meat characteristics (e.g., flavor and protein functionality) before any recommendation of its commercial application can be made.

REFERENCES


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