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Proteolysis of intramuscular connective tissue during postmortem conditioning of beef muscle.

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Introduction.

The extent to which connective tissue of muscle is weakened during conditioning and whether such weakening makes a contribution to the tenderization process observed in meat conditioning remain unclear (Penny, 1980; Etherington, 1984). It is generally believed that this tenderization is related only to the breakdown of myofibrillar and perhaps cytoskeletal muscle proteins and not to changes in connective tissue (Davey, 1984). However, the enzymatic degradation of collagen may play a role in meat tenderization. Kruggel and Field (1971) and Pfeiffer et al. (1972) reported that structural changes in intramuscular collagen took place after stretching, conditioning, or stretching and subsequent conditioning of muscle. Stanley and Brown (1973) found that postmortem conditioning resulted in an increase in total amount of extractable collagen and in a replacement of large, less charged molecules by small, highly charged molecules in elution patterns of extracted collagen. In addition, collagenase and lysosomal enzymes have been shown to produce structural changes similar to those involved in postmortem conditioning of meat (Eino and Stanley 1973a; Dutson, 1974; Robbins and Cohen, 1976; Robbins et al., 1979).

Changes in thermal stability of intramuscular connective tissue (Chizzolini et al., 1975) and tendon collagen (Ledward et al., 1975) may also occur during conditioning. More recently, a postmortem reduction in denaturation temperature of collagen was reported by Judge and Aberle (1982) and by Mills et al. (1984). Bailey (1985) stated that if a reduction of tension generated during collagen thermal shrinkage is proved to occur during conditioning, a role for collagen in the postmortem tenderization of meat could be established. Some reduction in collagen isometric tension after 3 week storage at 4°C has been observed by Etherington (1984). These authors agree that for the collagen fibers to lose some thermal and mechanical stability only a few bonds would have to be broken.

The use of an exogenous collagenolytic enzyme to tenderize meat postmortem has been attempted by several workers. Non-specific proteinases (i.e., papain) are known to result in mushy, overtenderized muscles (Brooks et al., 1985). Kopp and Valin (1980) reported that isometric tension developed during heating of epimysial connective tissue was reduced upon incubation of the muscle with a crude preparation of lysosomal enzymes. Similar results were obtained by Bonnet and Kopp (1984a) upon incubation with Achromobacter iophagus collagenase. Postmortem injection of this bacterial collagenase into collagen-rich bovine muscle has been shown to produce a reduction in shear force and an increase in extractable collagen (Bonnet and Kopp, 1984b).

The objectives of the present work were to examine possible postmortem modifications in thermal and mechanical stability characteristics of intramuscular collagen occurring during conditioning and to determine the effect of the postmortem injection of bacterial collagenase preparations from Clostridium histolyticum and A. iophagus on these characteristics.

Materials and methods.

Beef sternomandibularis muscle samples were obtained from 18 month-old Charolais steers immediately after slaughter at the University of Guelph abbatoir. Epimysium was carefully removed from the muscle and the cleaned samples were diced into small cubes ($3\ cm)^3$. Some samples were injected with C. histolyticum collagenase (Type IV, Sigma Chemical Co., St. Louis MO) suspended in 0.05 M Tris hydroxymethyl-methyl-2-amino-ethane sulfonate (TES) buffer with 0.36 mM $CaCl_2$, pH 7.5, or A. iophagus collagenase (a gift from Institut Pasteur, Paris) in 0.02 M Tris hydroxymethylamino methane (Tris) buffer with 0.5 M NaCl and 3 mM $CaCl_2$, pH 7.4. The total injection volume was 5 ml in 100 g of muscle, netting a final enzyme concentration of 30 mg/100 g muscle. All samples were conditioned at 21°C for 24 hrs. and then at 4°C for nine days.

After homogenization in a Sorvall Omnimixer with 0.6 M KCl, intramuscular connective tissue was carefully dissected from the collagenous material adhered to the rotor helix. Since virtually all myofibrilar and epimysial materials had been removed from the muscle samples, the isolated material was assumed to contain primarily perimysium and some endomysium.

DSC experiments were performed as described elsewhere (Bernal and Stanley, 1986). Freshly isolated connective tissue samples (5-15 mg) plus approximately 10 ul of Tris buffer (pH 7.4) were heated at 5°C/min from 25 to 100°C in the DSC cell of a Du Pont 1090 Thermal Analyzer (Du Pont Instruments, Wilmington, DE). Tensile strength of collagen fibers was measured in an Instron Universal Testing Machine using plastic clamps to hold the samples. Collagen fibers (20 x 0.3-0.5 mm, 2.5-3.5 mg) were kept in 0.06 M KCl until tested. Maximum force and work of rupture (force times distance) were recorded at a crosshead velocity of 2 mm/min and a chart speed of 50 mm/min.

Results and discussion.

All three sample groups showed a significant ($P<0.05$) decrease in collagen denaturation temperature (T_D) with conditioning (Fig. 1). This T_D reduction is similar to that reported by Judge and Aberle (1982) and likely indicates that some changes in the structural characteristics of collagen have occurred during conditioning. No significant differences ($P<0.05$) in T_D were observed between the sample groups at any given conditioning time. Denaturation enthalpies (ΔH) of conditioned samples after 10 days were nonsignificantly smaller than that of the initial control (Table 1). Differences in the final ΔH values suggest that some variations existed among the conditioning treatments. The decrease in ΔH was significantly larger for the sample treated with A. iophagus collagenase, indicating that the changes in

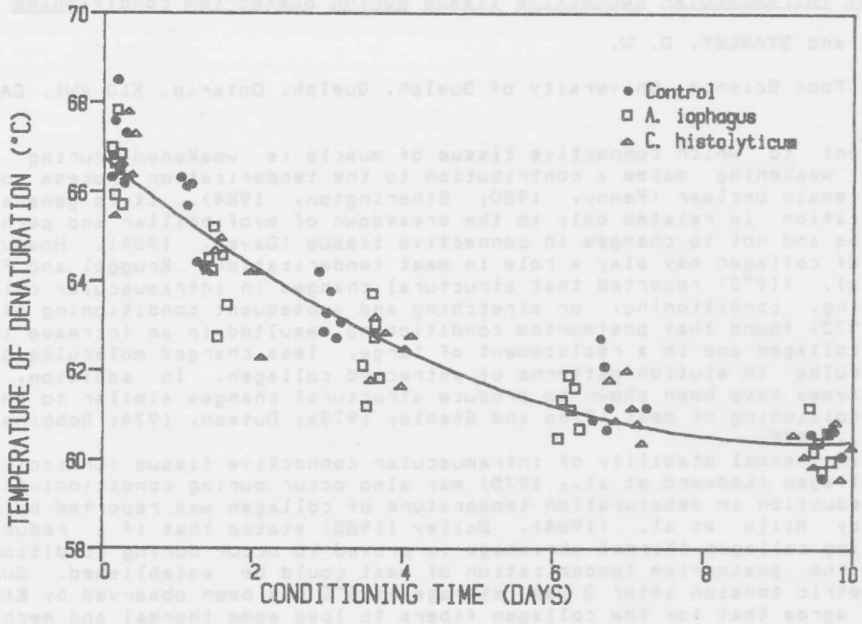

Figure 1. Temperatures of denaturation for connective tissue fractions during conditioning.

Table 1. Enthalpies of denaturation for connective tissue fractions. Means with the same letter are not significantly different at the probability level of $P<0.05$.

Sample	ΔH (J/g dry sample) [a]	
Control (initial)	52.7 ± 10.6	A
Control (10 days)	49.5 ± 10.8	A
C. histolyticum collagenase treated muscle (10 days)	45.3 ± 12.8	A,B
A. iophagus collagenase treated muscle (10 days)	32.16 ± 8.8	B

[a] ± Standard deviation of six replicates.

intramuscular connective tissue produced by this enzyme were the most severe.

Upon conditioning, the gradual appearance of a shoulder at the onset of the collagen melting peak of the samples treated with *A. iophagus* collagenase was noticed (Fig. 2). This shoulder may reflect a looser organization of the collagen fibers caused by an increase in the amount of water being able to penetrate the collagen network and a subsequent reduction in the energy of crystallization of collagen as a result of collagenolysis. The shape of the thermal curves of the other two sample groups did not change. The decrease in thermal stability of collagen may be significant enough to contribute to tenderization of meat during conditioning.

Results from the tensile strength measurements are presented in Table 2. These results provide further evidence for the changes in connective tissue apparent from the calorimetric experiments. Collagen fibers isolated from intact muscle required a higher maximum force than those from conditioned and collagenase-treated samples. Differences in work of rupture values also indicate differences in mechanical stress among the samples. Again these results suggest that the *A. iophagus* collagenase treatment may be more effective in disrupting the collagen structure than that of the *C. histolyticum* preparation. The reductions in tensile strength of muscle treated with *C. histolyticum* collagenase observed by Eino and Stanley (1973b) may be the

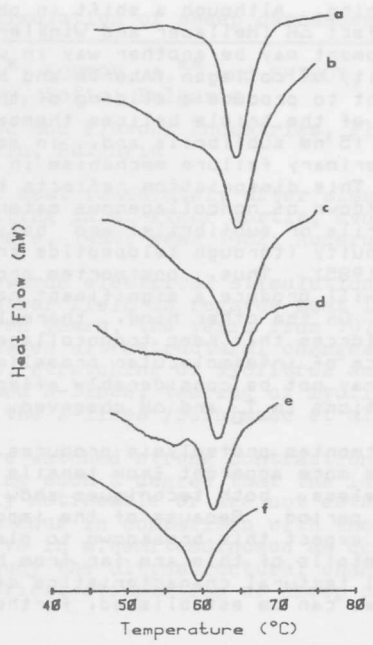

Figure 2. Thermal curves for the muscle samples treated with A. iophagus collagenase. Conditioning time: (a) 2 hours; (b) 1 day; (c) 2 days; (d) 4 days; (e) 7 days; (f) 10 days.

Table 2. Tensile strength measurements from the connective tissue samples. Means with the same letter are not significantly different at the probability level of $P<0.05$.

Sample	Maximum force (g)[a]	Work of rupture (g.mm)[a]
Control (initial)	252.5 ± 37.4 A	16837 ± 2513 A
Control (10 days)	179.8 ± 35.2 B	14947 ± 2608 A,B
C. histolyticum collagenase treated muscle (10 days)	146.5 ± 36.8 B	11970 ± 2148 B,C
A. iophagus collagenase treated muscle (10 days)	78.7 ± 29.2 C	4790 ± 1138 C

[a] ± Standard deviation of 10 replicates.

result of a decrease in connective tissue tensile strength. In the present study, the reduced mechanical stability found in the control samples after 10 days suggests a role for connective tissue breakdown in the tenderness development taking place during meat conditioning. The injection of bacterial collagenase enhanced the reductions in thermal and mechanical stability observed.

Connective tissue changes are believed to be the result of lysosomal enzyme activity and are known to be affected by the temperature at which conditioning takes place. While low (1–4°C) temperatures may not facilitate these changes, conditioning at 37°C is known to promote release of lysosomal enzymes, enhancing postmortem connective tissue changes and collagen solubility (Wu et al., 1981). In this study, lysosomal enzymes released during the first 24 hr, may remain active throughout the whole conditioning period, and be responsible for the connective tissue changes observed in the control. These enzymes may also contribute to the changes observed in the other two sample groups. The degradation of mucopolysaccharides or proteoglycans associated with collagen fibrils in connective tissue may be required before enzymes with collagenolytic activity (lysosomal cathepsins B, L and N) are able to degrade these fibers (Wu et al., 1981; Etherington, 1984). Lysosomal glycosidases may have been active in the muscle samples, facilitating collagenolytic activity of both native lysosomal proteases and microbial preparations injected into the muscle. Microbial proteolytic activity may take

place at the latter stages of conditioning. Although a shift in pH from 7.0 to 6.0 does not produce comparable changes in T_D nor affect ΔH (Hellauer and Winkler, 1975), the redistribution of ions that occurs during rigor development may be another way in which postmortem conditioning affects the thermal stability of collagen (Aberle and Mills, 1983).

Applied tensile stress is thought to produce a sliding of the collagen triple helices relative to each other and a stretching of the triple helices themselves (Mosler et al., 1985). Progressive fibrillar dissociation into 15 nm subfibrils and, in some instances, further into 3.5 nm microfibrils, seems to be the primary failure mechanism in native tendon subjected to mechanical stress (Torp et al., 1975). This dissociation reflects the collapse of the collagen constituents of the fiber and not breakdown of noncollagenous materials (Torp et al., 1975). Although no single units (either fibrils or subfibrils) are believed to run continuously through a collagen fiber, some continuity (through telopeptide crosslinking) is believed to exist along the fiber (Mosler et al., 1985). Thus, postmortem proteolysis, by breaking the continuity throughout collagen fibers, will produce a significant reduction in the tension that these fibers can stand before rupture. On the other hand, thermal stability of collagen will reflect the strength of noncovalent forces that keep tropocollagen and individual helices aligned together rather than maintenance of intermolecular crosslinks through which collagen polymerizes. These noncovalent forces may not be considerably affected during conditioning, as indicated by the relatively small reductions in T_D and ΔH observed, particularly in the control.

From our work it seems that postmortem proteolysis produces changes in intramuscular collagen stability. These changes are more apparent from tensile strength measurements than from calorimetric experiments. Nevertheless, both techniques show significant alterations in connective tissue over the conditioning period. Because of the importance of connective tissue in meat texture it would be logical to expect this breakdown to play a role in postmortem meat tenderization; however, the precise details of this are far from being understood. Before the importance of these changes to the final textural characteristics of conditioned meat and their possible impact on its sensory attributes can be established, further research is necessary.

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