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ULTRASTRUCTURAL AND TEXTURAL PROPERTIES OF RESTRUCTURED BEEF TREATED WITH A BACTERIAL CULTURE AND SPLENIC PULP

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Abstract

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and Instron measurements were used to evaluate the effects of an Achromobacter iophagus culture (BC) and splenic pulp (SP) treatments on the structural and textural properties of flaked and restructured beef steaks. Both treatments improved the textural characteristics of the product when conditioned at 35°C. Electron microscopy studies revealed that the bacterial culture treatment caused a greater effect than SP on the connective tissue elements, with a degradation of the endomysial sheath and sarcolemma. Treatment with splenic pulp produced an overall excessive disruption at the Z-lines with little definition of the A-bands.

Introduction

Tenderness is one of the most important characteristics that render restructured products acceptable to consumers. Collagen has been implicated in providing the so-called "background" toughness of meat (Bailey, 1972). Connective tissue fibers can be partially broken down by flake-cutting. This particle reduction procedure and accompanying restructuring technology enables the use of lower priced cuts of meat. However, the incorporation of different cuts of meat with varying amounts of connective tissue into the same product may reduce textural uniformity.

Collagenases selectively degrade connective tissue elements (Eino and Stanley, 1973). Postmortem injection of bacterial collagenases into muscle (Bernal and Stanley, 1986) or blending with a restructured beef product (Cronlund and Woychik, 1987) caused an increase in the collagen solubility and reduction in thermal stability. In a recent study*, Elkhalifa and Marriott (Submitted Manuscript, 1988) found similar results by injecting a bacterial culture into restructured beef steaks. Cathheptic enzymes of bovine spleen have also been shown to degrade myofibrillar Z-bands and sarcolemma (Robbins and Cohen, 1976; Cohen et al., 1982) as well as collagen (Etherington, 1976; Elkhalifa and Marriott, submitted manuscript, 1988*) and they improved the textural uniformity of precooked, freeze-dried meat (Cohen et al., 1978). The objectives of this study were to observe the effect of bacterial culture and splenic pulp treatments on the collagen of restructured beef as determined by the shear force and structural changes occurring within collagen and muscle fibers.

Materials and Methods

Sample preparation and treatment
Muscle samples were obtained from animals slaughtered at the Virginia Polytechnic Institute and State University Meat Science Laboratory. Postmortem muscle samples were removed from the longissimus dorsi (LD) to represent a low collagen (LC) control treatment and the extensor carpi radialis, flexor carpi radialis, flexor carpi ulnaris, superficial digital flexor and deep digital flexor muscles to represent high collagen (HC) muscles of U.S. Choice steer carcasses that were stored at 2°C for 48 hr postmortem. Epimysium was not removed and samples were cut into 3.5 x 3.5 cm pieces which were frozen at -20°C and later tempered to -4°C before flaking. The

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tempered samples were flaked with an Urschel Comi- 
control (Model 3600) using a head opening size of 6.1 x 
17 mm. The flaked HC particles were formulated to 
contain 1.8% NaCl and 0.25% sodium tripolyphosphate 
(STP) and blended in a Hobart (Model No. 512) Mix- 
er (in unit - CDB 0615) for 10 min. Three portions were assigned 
to one of the following treatments: (a) HC-control; 
(b) HC-bacterial culture treated; (c) HC-3% (W/W) 
splenic pulp (SP). The SP samples were divided into 
two groups. One group was conditioned at 35°C for 
3 h and stored at 4°C for 7 days. The other group 
was stored at 4°C for 7 days. Both treatments were 
wrapped and stored in waxed freezer paper. Splenic 
pulp was prepared by separating the connective tis- 
uue from bovine spleen. Samples from the LD pro- 
ducts were flaked and formulated with 1.0% NaCl and 
0.25% STP in a similar manner as the LC control. 
The mixed meat samples from each treatment 
group were stuffed into 110 mm diameter casings. 
The stuffed 4.2 kg logs were frozen to -20°C and 
then tempered to -4°C over a 16 h period. The 
tempered meat logs were pressed in a Ross press 
(Superform 720) at a setting of 37 kg/cm² with 2 sec 
dwell time into the shape of a ribeye, and sliced 
with a Hobart (Model 512) slicer to produce 
70 g, 12.5 mm thick steaks which were then wrapped 
in waxed freezer paper and stored at -20°C until 
testing.

### Results

The instrumental shear measurements are pre- 
sented in Table 1. The LC-control samples required 
less (P less than 0.05) shear force than the HC sam- 
plies regardless of the treatment. Shear force values
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of the HC-control samples at 4°C were not different (P greater than 0.05) from the bacteria and splenic pulp treated samples. The shear force value for the HC-control samples at 35°C was higher (P less than 0.05) than for the bacterial culture and spleen treated samples; however, there was no significant difference between the BC and SP treated samples. Both the BC and SP samples had lower standard errors than the control which suggests more uniformity among the treated samples.

Table 1. Mean shear force values for cooked control and texture-modified restructured beef steaks

<table>
<thead>
<tr>
<th>Samples</th>
<th>Shear Stress (Newtons/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C2</td>
</tr>
<tr>
<td></td>
<td>X</td>
</tr>
<tr>
<td>LC* -control</td>
<td>52.8 ± 6.66Ca</td>
</tr>
<tr>
<td>HC** -control</td>
<td>70.5 ± 9.84ABB</td>
</tr>
<tr>
<td>HC + A. Ilophagus culture³</td>
<td>74.7 ± 10.06AA</td>
</tr>
<tr>
<td>HC + splenic pulp⁴</td>
<td>64.3 ± 13.54Ba</td>
</tr>
</tbody>
</table>

1 N=18 steaks per treatment.
2 Products were stored at 4°C for 7 days, or conditioned at 35°C for 3 h and then stored at 4°C for 7 days.
3 6% (V/W) bacterial culture injected into steaks.
4 3% splenic pulp added before forming steaks.

A,B,C Mean values in the same column with identical upper case superscripts are not different (P greater than 0.05).

a,b Mean values in the same row with identical lower case superscripts are not different (P greater than 0.05).

* LC=low collagen.
** HC=high collagen.

Scanning electron Microscopy (SEM)

Results and discussion are slightly abbreviated by discussion of only the SP samples that were stored at 4°C, since the data in Table 1 were not different (P greater than 0.05) between the two temperatures. Fig. 1 illustrates the SEM results of the muscle surface of untreated and treated restructured muscle samples. The LC-control (Fig. 1A) which possessed 4.7 mg/g tissue of collagen, contains few collagen fibers which allows the muscle fiber surface to be observed directly although the muscle fiber observed is a small part of the micrographic field shown. The HC-control (Fig. 1B) which had 20.2 mg/g tissue of collagen, is more heavily covered by collagen fibers than the other treatments. The structure of the HC-muscle tissue appeared to be looser and less compact than the LC-control. Representative HC-samples injected with the bacterial culture and conditioned at 35°C (Fig. 1C) showed major effects of this treatment by exhibiting aggregated and tight entanglements of randomly selected collagen fibers within the muscle tissue. In addition, Fig. 1C shows an area of muscle tissue where collagen fibers appear beaded on the surface and the sarcolemma has been degraded exposing the underlying myofibrils. These ultrastructural changes were not observed in the HC-samples treated with bacteria at 4°C (Fig. 1D), which showed structural characteristics similar to the HC-control. Samples treated with splenic pulp revealed some degradation of the sarcolemma exposing the underlying myofibrils (Fig. 1E). The muscle fibers appeared more degraded and disorganized than those of the HC-control. In addition, collagen fibers appeared somewhat fragmented and more loosely aggregated.

SEM was also used to study the morphology of isolated collagen fibers from the control and treated samples (Fig. 2). The LC-control samples (Fig. 2A) contained thin collagen fibers which appeared loosely aggregated and structurally disorganized. Collagen of the HC-control samples appeared as dense sheets of long unfragmented fibers (Fig. 2B). Because of their tight association, the collagen fibers appeared coarser than those of the LC-control. This is in striking contrast to the HC-treated with bacterial culture at 35°C which (Fig. 2C) appears to contain rather loose separated fibers and fiber bundles no longer in compact dense sheets. A similar arrangement of loose collagen fibers was also observed in the bacteria-treated HC at 4°C (Fig. 2D) and splenic pulp treated (Fig. 2E) samples. However, Fig. 2D does show some areas of loosely packed sheets of collagen and, like Fig. 2E, there is disorganization and loss of integrity of the fiber bundles. These observations support the differences in the Instron values of the LC- and HC-control samples.

Transmission electron microscopy (TEM)

Transmission electron micrographs representative of the control and treated restructured muscle samples are shown in Fig. 3. As seen in Fig. 3A and 3B, restructuring produced irregularities in the orientation of the myofibers. For the LC-control sample (Fig. 3A), the sarcolemma and the endomysium have some degradation. The endomysial sheath of the HC-control sample (Fig. 3B) appears to remain intact. However, the 35°C BC treated samples (Fig. 3C) reflect excessive degradation of the endomysium and sarcolemma. A slight degradation of the myofibrils occurred in certain areas. As illustrated in restructured samples treated with bacteria (Fig. 3D), some degradation occurred in the endomysium as well as the myofibrils. The SP samples (Fig. 3E) reflect considerable overall fiber disruption. The Z-lines and A and I bands are not well defined. Less degradation was evident in the endomysium and sarcolemma components than in the HC-controls (Fig. 3B). TEM micrographs of isolated collagen fibers from control and treated samples are shown in Fig. 4. Collagen fibrils of LC-control samples have intact fibrils with separation of some fibrils into protofilaments (Fig. 4A). In Fig. 4B, collagen fibrils of HC-control samples are intact and tightly packed. The
Fig. 1. SEM Micrographs of: (A) A low collagen (LC-control) restructured control sample showing muscle tissue and associated collagen (C). Note the lack of individual collagen fibers over the surface of the muscle fiber (M). (B) A high collagen (HC-control) restructured sample. The surface of muscle fibers is thickly covered by collagen sheets. (C) A high collagen sample (HC-bacteria) treated with bacteria at 35°C. An area is shown in which the collagen fibers beaded on the surface (arrows) and underlying muscle fibers (M) are exposed. (D) A high collagen sample treated with bacteria at 4°C. Note the presence of collagen fibers (CF) on the surface of the tissue and the structural similarity to the control shown in Figure 2B. (E) A high collagen sample treated with splenic pulp and stored at 4°C. An area is shown in which the sarcolemma appears to have lost some of its integrity resulting in the exposure of what may be underlying myofibrils (arrow).

Bars = 50 μm (for A, D and E), 10 μm (for B) and 5 μm (for C).
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Fig. 2. SEM micrographs of collagen fibers isolated from: (A) LC restructured control sample. The sample contains a high proportion of thin collagen fibers (arrows) which appear disorganized and loosely arranged. (B) HC restructured control sample. Collagen appears as dense sheets (C) composed of highly compacted fibers. (C) HC bacteria sample treated with bacteria at 35°C. Note the loose arrangement and separation of both thick and thin collagen fibers resulting from the degradative action. (D) HC sample treated at 4°C. Partial loosening of collagen fibers implies less bacterial degradation at 4°C than at 35°C (Figure 2C). (E) HC sample treated with splenic pulp and stored at 4°C. The loose arrangement of the collagen fibers appears similar to that seen in the samples treated with bacteria at 4°C (Figure 2D).

Bars = 10 μm (for A, B and E); = 5 μm (for C and D).


Fig. 3. TEM micrographs of: (A) Low collagen restructured control sample showing small remnants of the sarcolemma (S) and endomysium (E). There is some disruption of the structural organization of Z-lines and myofibrils due to the restructuring of tissue. (B) High collagen restructured control sample. In this oblique section, the endomysial sheath (E) appears to be intact. Structural disorganization of the myofibrils and associated components has resulted from restructuring or other forms of mechanical disruption of the muscle tissue. (C) High collagen restructured sample treated with bacteria at 35°C showing complete degradation of the endomysium, sarcolemma and Z-line (Z) configuration of the myofibrils. The space between the myofibers contains collagen remnants (small arrows). (D) High collagen restructured sample treated with bacteria at 4°C. Notice slightly less degradation of endomysium (E), myofibrils and Z-lines as compared to Fig. 3C. Cross-sections of collagen fibers occupy the space external to the endomysia. (E) High collagen restructured sample treated with splenic pulp and stored at 4°C. Z-lines and A and I bands are no longer apparent due to excessive disruption of the myofibrillar structure caused by the action of hydrolytic enzymes. Remnants of the endomysium (E) and sarcolemma (S) are present.

Bars = 1 μm (for A, B, C, and D) and 0.5 μm (for E).
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Fig. 4. TEM micrographs of collagen fibrils isolated from:

(A) A low collagen restructured control sample showing intact fibrils separated with some fibrils into protofilaments (arrows). (B) A high collagen restructured control sample. The collagen fibrils appear to be intact and tightly packed. (C) A high collagen restructured sample treated with a bacterial culture at 35°C. Amorphous regions can be seen within a single fibril (arrows). (D) A high collagen restructured sample treated with a bacterial culture at 4°C. Orientation of collagen fibrils has been disrupted as evidenced by numerous cross-sections relative to the control. This may have been caused by normal factors i.e., placement in block (4B). There is some separation of fibrils into protofilaments (arrow) similar to the low collagen control (4A). The diameter of the collagen fibrils is smaller than controls. Note bacteria (B). (E) A high collagen restructured sample treated with splenic pulp and stored at 4°C. This micrograph reveals that collagen fibril diameters are less than the controls. Cross-sections of collagen can be observed as being dispersed among longitudinal segments of banded collagen.

Bars = 1 μm (for A and D), 0.5 μm (for B and E), and 0.1 μm (for C)
BC treatment (35°C) resulted in amorphous regions along a single fibril (Fig. 4C). Figures 4D and 4E suggest that treatment with the bacterial culture at 4°C or splenic pulp (Figs. 4D and 4E) did not result in amorphous regions. These observations reveal that treated samples (Figs. 4C, 4D and 4E) have large proportions of relatively small diameter collagen fibers and predominantly short segments of normally banded collagen, suggesting effects of the BC and SP treatments.

Discussion

The reduction in shear force of the bacteria treated samples (35°C) is a reflection of the selective degradation of collagen (Figs. 3C and 4D) by a collagenase enzyme in the bacterial culture. Treatment with splenic pulp at either temperature resulted in excessive disruption of the myofibrillar structure as evidenced by the degradation of Z-lines and A and I bands (Fig. 3E) which was attributable to the lysosomal enzymes in spleen. In work on the effect of catheptic enzymes from spleen, Cohen et al. (1979) found that enzyme-treated samples were more tender and more uniform in texture.

As seen in the SEM micrographs, treatment with either the bacterial culture or splenic pulp gave rise to surface structural changes of the restructured samples. Using SEM, Eino and Stanley (1973) observed similar hydrolytic changes in the connective tissue and sarcolemma when rabbit psoas muscle was incubated with a bacterial collagenase. In a study on the muscle fiber surface of restructured beef, Cohen et al. (1982) demonstrated that catheptic enzymes from splenic pulp caused degradation of the sarcolemma and exposure of the myofibrils in restructured beef. Results from this investigation support what earlier researchers have reported and add to the knowledge base of the effects of these two treatments.

Ultrastructure of muscle and connective tissue of a restructured product have not been studied specifically by TEM. The structural differences between control and treated samples seen in the TEM micrographs (Figs. 3 and 4) are the degradation of the endomysial sheath, myofibrils and the sarcolemma. The degradation of sarcolemma and endomysium due to the bacterial culture treatment has important implications. Offer (1984) suggested a mechanism for water loss from muscle during cooking that involves shrinkage of actomyosin within muscle cells to leave free fluid which can then purge out. Since the structure of the endomysium is in intimate contact with such shrunken actomyosin components in samples cooked to 77°C, it may accelerate this passive process. Light et al. (1985) suggested that the endomysium may present a barrier to transverse fracture of muscle pieces which can develop a compressive force as shrinking constricts muscle cells, thus squeezing free water out of the cut ends of the muscle in an active process and thereby contributing to increased toughness. This hypothesis may be correct since the degradation of the endomysial sheath in BC samples at 35°C may contribute to the reduction in shear force. The improvement in tenderness of HC samples from the SP is evidence of the extensive degradation of the myofibrillar structure.

In view of the results presented here, there is no doubt that there was a greater effect on the connective tissue elements and sarcolemma by the 35°C bacteria treatment than by the SP treatment which produced microstructural changes within the myofibrils. It is reasonable to expect the technology of both treatments to be quite useful as exogeneous meat tenderizers to permit the use of less costly cuts of meat at significant monetary savings.

Acknowledgments

The authors thank Dr. K. C. Diehl for assistance with the instrumental texture analysis. Assistance from Mr. Steve Phelps is also acknowledged. The authors express their appreciation to the Virginia Cattle Industry Board and the Virginia Agricultural Council for financial support. Appreciation is extended to Ross Industries, Inc. for use of the Ross Superform 720 Press and to Urschel Laboratories, Inc. for loan of the Comitol 3600.

References

Reynolds ES (1963). The use of lead citrate at high pH as an electron-opaque stain in electron
Authors: The mean collagen content of the ftbrous samples was 4.7 mg/g tissue. The LC samples had a mean collagen content of 20.2 mg/g tissue.

Reviewer I: Is one of your conclusions that more fibrous collagen occurs in the HC control?

Authors: This answer is based on our assumption that the reviewer meant more fibrous collagen occurs in the HC control than the LC control and HC samples treated with bacterial culture or splenic pulp. Yes, we concluded that more fibrous collagen occurs in the HC control. The LC samples had less collagen as evidenced by another study that has been submitted for publication separately.* The BC and SP samples had less fibrous collagen because of collagen degradation that occurred.

Reviewer I: Are the control microscopy samples randomly selected from your cuts? In samples such as these there must be considerable variation in the "terrain" to be viewed on a sample. How extensive was your microscopy? How many samples?

Authors: The samples were selected randomly. Fifteen samples per treatment were observed.

Reviewer I: How can you tell the difference between endomysium and sarcolemma in some of your TEM micrographs?

Authors: The endomysium is thicker since it surrounds the muscle fiber. The sarcolemma is thinner and is the innermost membrane. By the morphology location and measurement of the structures, we were able to distinguish between the endomysium and sarcolemma.

Reviewer IV: What are casamino acids? Are they available from a commercial supplier or must they be prepared in the laboratory?

Authors: Casamino acids is acid hydrolyzed casein recommended for use in microbial culture media which require a completely hydrolyzed protein as a nitrogen source. Casamino acids is well suited for the preparation of "synthetic" or chemically defined media. We incorporated this compound for the maintenance and growth of the A. Iophagus culture used in this study. It is available commercially through Difco in Detroit, Michigan.

D.N. Holcomb: Please provide more details of the testing fixture used in measuring the shear forces with the Instron testing machine.

Authors: A cast aluminum alloy housing was incorporated with sample space size as follows: 67 mm between smooth walls, 66 mm between slotted walls and a depth of 62 mm. Ten blunt-ended, teflon-coated blades with a width of 3 mm and depth of 70 mm are guided into the slots, spaced at 3 mm apart, by a removable cover plate which rests on the top of the cell. The blades are suspended from an aluminum alloy housing which attaches to the load cell on the Instron testing machine.

*This companion paper has been submitted for publication. Preprints are available from N. G. Marriott, 103 Food Science Building, VPI & SU, Blacksburg, VA 24061.