

Improved Tenderness of Restructured Beef Steaks by a Microbial Collagenase Derived from *Vibrio* B-30

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ABSTRACT

To reduce connective tissue toughness *Vibrio* B-30 collagenase was evaluated for its ability to degrade muscle collagen in a restructured beef (RSB) product. Dissected muscles pooled from clods of 4 USDA choice chucks were made into three RSB products: untrimmed control (C), hand trimmed of epimysium (T) and untrimmed and treated with 0.01% collagenase (E). RSB products were held for 24 hr at either 2°C or 11°C and were analyzed either raw or after heating (40°C for 1 hr). Aerobic microbial load remained low ($1-2 \times 10^2$ cfu/g) during the 24 hr 11°C incubation. Heated samples of enzyme treated RSB had lower Lec-Kramer shear values than controls. In both raw and heated samples enzyme treatment increased hydroxyproline solubility. An enzyme treatment of 24 hr at 11°C followed by heating produced maximum tenderization. Although optimum treatment conditions need to be defined by further research, *Vibrio* B-30 collagenase appeared to be efficacious for the tenderization of RSB products.

INTRODUCTION

ALTHOUGH connective tissue transfers muscle work to bones in the live animal, its sensory characteristics seriously limits maximum use of over one third of the beef carcass. While restructured beef (RSB) products could increase utilization of these undervalued primals, especially the chuck, they have failed to win widespread consumer acceptance because of high levels of objectional connective tissue (Secrist, 1982). Strange and Whiting (1989) determined that, while peri- and endomysium were acceptable, epimysium, tendon and gristle were most objectionable to sensory panelists. At present, processors lack a method for large scale, economical removal of these connective tissues.

Enzymes have been used for centuries to tenderize tough cuts of meat. Papain, ficin and bromelin are the most frequently employed proteases since they offer the advantage of established safety, availability, and economy. They often produce inferior quality products, however, because of nonspecific proteolysis, degrading myofibrillar and stromal proteins somewhat indiscriminately. Microbial collagenases from *Clostridium histolyticum* (EC 3.4.24.3—Gallop et al., 1957) and *Achromobacter iophagus* (EC 3.4.24.8—Woods et al., 1972) have been studied for their ability to degrade collagen in meat, generally under conditions which approached ideal temperature, pH and time (Foegeding and Larick, 1986; Bonnet and Kopp, 1984). In a comparative study on beef muscle, the most effective microbial collagenase observed was derived from the marine bacterium *Vibrio* B-30 (Cronlund and Woychik, 1987). To expand on these preliminary findings, the objective of this study was to determine if *Vibrio* B-30 collagenase degrades collagen under conditions simulating commercial production of RSB steaks.

MATERIALS & METHODS

Chemicals

All chemicals and solvents were purchased from major suppliers and were used without further purification.

Collagenase

Two custom batches of *Vibrio* B-30 collagenase (Merkel et al., 1975) were prepared according to the method described by Merkel and Dreisbach (1978). Briefly, bacteriological medium containing undenatured calf-skin collagen was used to stimulate collagenase production by *Vibrio* B-30 (ATCC, 21250). Partial purification of the enzyme was obtained using DEAE-cellulose and Sephadex G-200 column chromatography. Collagenolytic activity (Terato et al., 1976) of 136 and 102 units/mg and nonspecific protease activity (Prescott and Willms, 1960) of 1.20 and 0.885 units/mg were observed for batches one and two, respectively. Enzyme production, purification and characterization was performed at the laboratory of Dr. J. R. Merkel, Dept. of Chemistry, Lehigh Univ., Bethlehem, PA. Upon receipt at ERRC, each batch was confirmed to exhibit collagenase activity without accompanying non-specific proteolysis, using myosin as a test protein (Cronlund and Woychik, 1987).

Restructured beef manufacture

Four USDA choice/#2 beef clods (#114 NAMP) were purchased from a local meat purveyor (78.9 kg total weight) and were dissected of Triceps brachii, Infraspinatis, and Supraspinatis. Seam and intermuscular fat and major tendons were removed. Muscles were cut into large chunks, divided randomly into three batches and labeled untrimmed control (C), trimmed of epimysium (T) or untrimmed and enzyme treated (E). Epimysium was trimmed by hand from the muscle chunks in the T treatment. To permit convenient slicing and cubing all meat was stored overnight at -35°C and then tempered the following morning at 11°C for 4 hr. Muscle chunks from each treatment were sliced and then cut further by hand into 0.95 cm square cubes. Weights were 9.9, 6.9, and 9.6 kg for the C, T and E treatments, respectively. Cubes from each treatment were stored overnight at -35°C, then tempered at 11°C.

Each of the three treatments (C,T,E) was subdivided into three replicates. The nine treatment/replicate combinations (1300–1400g each) were formulated into RSB with 0.75% NaCl and 0.125% sodium tripolyphosphate and then mixed for 2 min using a Hobart food mixer. Treatment E received 0.01% *Vibrio* B-30 collagenase which was suspended in 5 mL of buffer (0.02M Tris, 0.005M CaCl₂, pH 7.4) prior to mixing. The calcium containing buffer was added to stimulate action of the collagenase (Norwig, 1971). RSB product was then weighed into aluminum weighing dishes (ca 40g/dish), vacuum packaged, labeled and then immediately moved to a 2°C cooler. Upon completion of all formulations appropriate treatment/replicate combinations were stored for 24 hr at 11°C to determine collagenolytic effects at this temperature. The remaining samples were retained at 2°C. A total of 11 RSB samples was prepared for each treatment/repetition/incubation time combination to permit sufficient material for all analyses. Upon completion of the 24 hr treatment time, samples were stored at -35°C until they were analyzed.

pH determination

pH was determined on RSB samples after formulation by direct insertion into the product of a combination electrode attached to a pH meter equipped with a temperature compensator.

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Microbiology

Beef cubes, prior to formulation, and RSB samples from 2°C and 11°C (24 hr) samples were blended individually in a stomacher with 1% sterile peptone water. Samples were diluted further and aerobic microbial loads were determined by surface plating dilutions onto petri dishes containing nutrient agar medium. Triplicate plates from the three replicates were prepared. Plates were inverted and incubated at 25°C for 48 hr before enumeration.

Instron measurements

An Instron Universal Testing Machine equipped with a Lee-Kramer multiple blade shear cell was used to determine peak shear force on RSB samples. Both raw and heated samples were evaluated. For each sample, three RSB samples were thawed, removed from their aluminum dish and cut in half. Six shears were performed for each treatment/rep/incubation temperature combination. For the raw samples, thawed RSB samples in their package were kept chilled in an ice bath. Heated samples were cooked in their package in a 40°C water bath for 1 hr before cutting in half. This temperature was chosen because it potentiated enzymatic activity but minimized confounding toughening effects from actomyosin denaturation (Strange and Whiting, 1988). Shears were performed at a cross-head velocity of 50 mm/min and 500 kg full scale. Maximum peak heights and individual sample weights were measured and results were reported as Newtons per gram.

Hydroxyproline determinations

Total hydroxyproline (HYP) determinations on raw and heated RSB product were performed on samples from each treatment/rep/incubation temperature combination. Samples were thawed at 4°C and ground through a 1/16" (0.16mm) plate using a laboratory grinder. Three subsamples for the total HYP determination were cooled and maintained cold in ice water and then individually blended in a Polytron homogenizer (Brinkmann, Westbury, NY) until a uniform paste was obtained (usually within 1 min). Two 100 mg portions were weighed for a total of six determinations for each combination. For determination of soluble HYP in raw and heated samples, triplicate 5.0g samples from intact RSB samples were added to 10 mL of water in a centrifuge tube. Raw samples were kept cool and immediately homogenized for 1 min using a Polytron. Heated samples were incubated at 40°C for 1 hr in a water bath. Afterward, heated samples were cooled in ice and homogenized to a fine paste using a Polytron. For soluble HYP determinations in both cases, raw and heated, four 3 mL portions of water were used to wash the generator tip; washings were collected in the sample tube. Samples were then centrifuged at 10,000 x g for 1 hr. The supernatant fluid volume was measured and divided equally into three hydrolysis tubes and lyophilized. Soluble HYP determinations were performed on the dry contents of each tube. HYP in all samples was assayed according to Woessner (1961). A standard curve was determined each day. Soluble HYP values were estimated by first determining the sum of values for the three lyophilized tubes and then calculating percent soluble HYP from these and total HYP values.

Statistical analysis

Data entry and initial calculations were performed using Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA) and statistical analysis was performed on SAS (SAS Institute, Inc., Raleigh, NC) using analysis of variance or general linear model procedures. Bonferroni analysis (Miller, 1981) was used to separate treatment effects where appropriate and when the analysis of variance demonstrated significance at the $p < 0.05$ level.

RESULTS

AEROBIC PLATE COUNTS for the ground beef were initially 211 CFU/g and decreased to 122 CFU/g during the 24 hr enzyme treatment period at 11°C. This decrease, however, was not statistically significant. pH of the RSB was 5.99.

Lee-Kramer shear value data from raw and heated samples from each combination of treatment, incubation temperature and final heating were subjected to an analysis of variance. Results indicated that there were treatment effects, but not

incubation temperature, heating or repetition effects. Little change in shear values would be expected from mild heating (40°C) since tenderization by cooking occurs primarily from the collagen to gelatin phase transition at 68–70°C. Data were further analyzed by pooling 2°C and 11°C results and are presented in Table 1. Raw samples from the trimmed RSB steaks (T) had significantly lower ($p < 0.05$) shear values (70.0 Newton/g) than either the control (C) or enzyme treated (E) products, which were 114.7 and 115.1 N/g, respectively; this represented an approximately 40% reduction in shear force as a result of hand trimming. Raw C and E samples were not significantly different at the 95% level. Within the heated samples all treatments were significantly different ($p < 0.05$) from each other. This indicated that the enzyme treatment was effective in reducing shear force below untrimmed control level. No interactions were detected.

Soluble HYP values (means and standard error of means) from the restructured beef steaks are presented in Table 2. A three-way ANOVA and Bonferroni mean separation test were used to evaluate the data. Enzyme treatment significantly increased ($p < 0.01$) soluble hydroxyproline levels above both trimmed and control samples. This phenomenon was evident in both raw and heated samples. Enzyme treatment increased HYP solubility above controls by 126% and 101% for raw and heated samples, respectively. Comparing raw and heated enzyme treated sample values demonstrated that most of the HYP solubilization occurred during the 24 hr incubation period. This contrasted with the shear data, which indicated enzyme effects only in heated samples. These data suggested that HYP solubility evaluation might be the more sensitive indicator of collagen degradation. Trimmed samples were not different from controls. Significant ($p < 0.01$) two and three-way interactions were also observed for treatment-by-incubation temperature, 40°C heating-by-incubation temperature and treatment-by-40°C heating-by-incubation temperature. The effect of interactions is most evident by comparing columns in Table 2. Means within and across all columns were similar except for those containing enzyme-treated data. The raw enzyme treated samples showed only the primary treatment effect discussed above. The heated enzyme treated samples showed increased HYP solubility with increased incubation and heating temperatures. Such interactions would be expected from enzymological principles.

DISCUSSION

RESULTS from the present study demonstrated that treatment with 0.01% partially purified microbial collagenase from *Vibrio* B-30 was efficacious for the tenderization of beef chuck muscles which were used to produce a RSB product. Enzyme-treated samples showed enhanced collagen solubilization and reduced shear values, with no apparent detriment to microbial load. Sensory evaluation could not be performed since this enzyme lacks approval for food use. This study expands on a previous report (Cronlund and Woychik, 1987), which demonstrated superiority of *Vibrio* B-30 over other microbial collagenases for degrading collagen in RSB.

Although the pH and temperature optima for the *Vibrio* B-

Table 1—Effect of *Vibrio* B-30 collagenase on Lee-Kramer shear force values in restructured beef

Treatment	Peak shear force (Newtons/g)	
	Raw	Heated ^d
Control (C)	114.7 (7.5) ^{*b}	126.0 (8.3) ^c
Trimmed (T)	70.0 (2.6) ^a	75.2 (2.4) ^a
Enzyme (E)	115.1 (8.1) ^b	108.4 (4.9) ^b

^{a-c} Values within the same column with no superscript in common are significantly different ($p < 0.05$).

^d 40°C for 1 hr.

^{*} Mean (SEM) of 36 pooled shares from 2° and 11°C samples.

Table 2—Effect of temperature of incubation for 24 hr and subsequent heating on solubilization of hydroxyproline (HYP) by 0.01% *Vibrio* B-30 collagenase

24 Hr incubation temp (°C)	Raw			Heated ^a		
	Trimmed	Control	Enzyme	Trimmed	Control	Enzyme
2	3.18 (0.23) ^b	2.48 (0.25)	6.06 (0.80)	3.22 (0.73)	3.31 (0.32)	4.18 (0.46)
11	3.38 (0.53)	2.47 (0.30)	5.09 (0.81)	3.46 (0.37)	2.69 (0.36)	7.88 (0.44)

^a 40°C for 1 hr.

^b Values are % soluble HYP. Each number is the mean (SEM) of 9 estimations. MSE = 2.05 (94 df). Means which differ by values exceeding 0.87 are significantly ($p < 0.05$) different.

30 collagenase are 7.6 and 37°C, respectively (Merkel and Dreisback, 1978), enzymatic activity persisted at suboptimal conditions (pH 5.99 and temperature shifts between 2-40°C) as previously described (Cronlund and Woychik, 1987). Incubation temperature at 2° or 11°C for 24 hr did not affect enzymatic release of HYP in raw samples, but interactions between temperature and time of incubation were revealed in samples which were subsequently heated to 40°C for 1 hr. HYP solubilization was, nevertheless, facilitated primarily by enzymatic activity during the initial cold incubation. The increased HYP solubilization observed in heated enzyme treated samples may result from thermally induced collagen helix instability. The observation by Engel (1987) that calf skin collagen undergoes a thermal transition midpoint (T_m) at 36°C contributes to the explanation of such interactions. It was likely that, additionally, the increased temperature (40°C) created a more favorable environment for enzymatic hydrolysis to occur. Studies need to be conducted to refine time and temperature effects on muscle collagen degradation by this microbial collagenase. Moreover, optimum levels of enzyme use should be determined. Finally, a low heating temperature of 40°C was chosen to minimize actomyosin effects on texture measurements. Future studies should include final cooking temperature of 70°C.

Correlations between beef shear values and released HYP were previously shown to be significant (Fogle et al., 1982). Data presented here, however, indicated that HYP solubility was the more sensitive index of connective tissue breakdown. The quantitative aspects of this relationship might have broad implications and should be explored further. Coupled with sensory data, this information would permit a better understanding of the role of connective tissues in muscle tenderness perception.

The observation that HYP solubilization by *Vibrio* B-30 collagenase occurs primarily during cold temperature incubation is in contrast to the action of papain, which degrades proteins primarily between 60-80°C (Tappel et al., 1956). This phenomenon may support *Vibrio* collagenase use because it offers the advantage of using refrigerated holding periods during processing and distribution to increase collagen degradation. Furthermore, unlike nonspecific proteases, such as papain, and unlike collagenases from toxigenic microorganisms, such as *Clostridium histolyticum*, *Vibrio* B-30 collagenase is a true collagenase and is derived from an organism reported to be nontoxicogenic (Merkel and Dreisback, 1978).

CONCLUSION

THE PRESENT STUDY shows that treatment of a restructured beef product with 0.01% *Vibrio* B-30 collagenase for 24 hr at 11°C produced maximum tenderization as measured by soluble

HYP and Lee-Kramer shear values. This was most clearly demonstrated after RSB products were heated. In addition to RSB products, application of this enzyme could be extended to a variety of meat products. Although usage parameters need to be optimized to maximize effectiveness, the present work demonstrated the enzyme's utility. Thus, this microbial collagenase might offer meat processors additional armamentarium in their pursuit to offer consumers high quality and economical products.

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