

# Solubilization of Collagen in Restructured Beef with Collagenases and $\alpha$ -Amylase

ANNE L. CRONLUND and JOHN H. WOYCHIK

## ABSTRACT

Three microbial collagenases were evaluated for their ability to degrade collagen structure in a restructured beef product. Collagenase preparations with and without  $\alpha$ -amylase were added to flaked meat, and their effect on collagen solubility was determined by quantitating the percent soluble hydroxyproline following cooking of product slices. Results demonstrated statistically significant increases in collagen solubility above that of a control product (no added enzymes) for purified *Clostridium histolyticum* and partially purified *Vibrio* B-30 collagenases alone and for purified *Achromobacter iophagus* and partially purified *Clostridium histolyticum* collagenases with the addition of  $\alpha$ -amylase. Thus, the use of collagenase may prove valuable in the production of quality-enhanced meat products.

## INTRODUCTION

INSOLUBLE COLLAGEN in muscle contributes significantly to meat toughness (Bailey, 1972). One approach to increasing meat tenderness is to degrade the intrinsic collagen structure to an extent which produces the desired tenderness and texture, i.e., without extensive degradation of muscle fibers.

Proteolytic enzymes such as bromelain, ficin and papain have been tested as meat tenderizers. The best tenderization effects are achieved with papain (Brocklehurst et al., 1981). Although papain does digest insoluble meat collagen (Cronlund and Woychik, 1986), its predominant action is proteolysis of the myofibrillar proteins (Kang and Rice, 1970), especially of actin and myosin (Rattie and Regenstein, 1977). This action can result in extensive degradation of the meat structure and undesirable textural quality.

The ideal enzyme for degrading insoluble collagen in a meat system must not only demonstrate specificity for collagen but must also function at the relatively low pH of meat (5.5) and at either the low temperatures at which meat is held during storage or at the higher temperatures reached during cooking. While true collagenases have the desired specificity, they are generally optimally active near neutrality and unstable above 40–45°C. Bonnet and Kopp (1984) found a small increase in collagen solubility with post mortem injection of *Achromobacter iophagus* collagenase into freshly slaughtered beef muscle. The low collagenase activity was attributed to unfavorable conditions of pH and temperature in the stored meat.

Inclusion of another enzyme which could increase the exposure of insoluble meat collagen to the added collagenase might result in improved collagenase action even under these conditions.  $\alpha$ -Amylase preparations from various sources have been used to disperse collagen fibrils from tissues such as skin, cartilage and tendon (Grant and Jackson, 1968; Quintarelli et al., 1969; Dixon et al., 1972) and in production of sausage casings (Tsuzuki and Lieberman, 1972). The collagen has then been more extractible and susceptible to other enzymes, such as pepsin and collagenase. The mode of action of  $\alpha$ -amylase on these tissues has not been determined but is speculated to be cleavage of carbohydrate/collagen linkages to ground substance which surrounds the collagen fibers, thereby making the fibers more accessible to proteolytic attack (Steven, 1965).

Amylase preparations may be contaminated with proteases, though, which could cause general muscle structure degradation if used in a meat product.

The present study utilized a model restructured beef system to test the ability of collagenases in partially purified and purified states to solubilize collagen during a brief cooking period. Collagenolytic activity and solubilization of collagen fragments were measured by determining hydroxyproline concentrations in hydrolysates of both the soluble and insoluble fractions of the cooked meat, since hydroxyproline is a rare amino acid found most abundantly in collagen in this tissue. Purified  $\alpha$ -amylase was also evaluated for a possible complementary role to the collagenolytic activity of the microbial enzyme preparations.

## MATERIALS & METHODS

### Enzymes

Purified collagenase (Type VII) from *Clostridium histolyticum* and purified papain (Type III) were purchased from Sigma Chemical Co., St. Louis, MO. Partially purified collagenase from *C. histolyticum* was donated by Amano International Enzyme Co., Inc., Troy, VA. Purified collagenase from *Achromobacter iophagus* was purchased from Boehringer/Mannheim, Indianapolis, IN. Partially purified collagenase from *Vibrio* B-30 (batch purified on DEAE-cellulose) was a gift of Dr. Joseph Merkel, Dept. of Chemistry, Lehigh Univ., Bethlehem, PA. Purified  $\alpha$ -amylase (706 U/mg) was purchased from Calbiochem/Behring Diagnostics, LaJolla, CA.

### Collagenase activity

Collagenase activity of each of the collagenase preparations was determined by incubation of 10  $\mu$ g of the preparation ( $\pm$  5  $\mu$ g  $\alpha$ -amylase) with 1.0 mg insoluble connective tissue (collagen) from meat (prepared as in Cronlund and Woychik, 1986) in a total volume of 1.0 mL buffer (0.02M Tris, 0.005M CaCl<sub>2</sub>, pH 7.4) for 2 hr at 37°C. The effects on this activity of the higher temperature and lower pH expected in the meat experiment were examined by similar incubations for 15 min at 65°C at both pH 7.4 and pH 6.2. After incubation the tubes were centrifuged in a Beckman Microfuge 12 for 3 min. Aliquots of the supernatant (800  $\mu$ L) were mixed with an equal volume of 12N HCl and were hydrolyzed at 110°C for 16 hr. Hydroxyproline concentration of the hydrolysates was measured by the method of Woessner (1961), and the amount of hydroxyproline released into the supernatant by each enzyme preparation was determined.

### Proteolytic activity

The proteolytic effect of each of the enzyme preparations on myofibrillar proteins was compared to that of papain by incubation for 2 hr at 37°C of 10  $\mu$ g enzyme with 10 mg isolated myofibrillar proteins (prepared as in Cronlund and Woychik, 1986) in 250  $\mu$ l of buffer, either 0.02M Tris, 0.005M CaCl<sub>2</sub> at pH 7.4 or 0.02M sodium acetate at pH 5.5. At the end of the incubation time an equal volume of electrophoresis sample buffer (0.13M Tris-HCl at pH 6.8, 5% sodium dodecylsulfate (SDS), 1.4M  $\beta$ -mercaptoethanol and 25% by volume glycerol) was added, and the samples were heated at 100°C for 2 min to inactivate the enzymes and to prepare the samples for electrophoresis. The extent of proteolytic activity was assessed by comparing electrophoretic patterns of samples incubated with enzymes with patterns from control samples (no enzymes). Proteolytic activity was demonstrated as loss of major structural protein bands and appearance of lower molecular weight degradation product bands.

**Electrophoresis**

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the discontinuous system of Laemmli (1970) as modified by Basch et al. (1985), using a 12.5% acrylamide separating gel with a 4% acrylamide stacking gel both at 2.6% crosslinking.

**Restructured beef preparations**

USDA Choice chuck was purchased from a local supermarket and, after removal of the epimysium, was cut into 1-in (2.5 cm) square columns which were chilled in a -20°C freezer to stiffen slightly before being flaked either by hand cutting or with a Cuisinart food processor equipped with a slicing blade. Chilled, irregularly-shaped meat flakes (200g) were then mixed in a Hobart mixer with 1 g NaCl, 500 mg sodium tripolyphosphate (Na<sub>5</sub>P<sub>3</sub>O<sub>10</sub>) and 20 mg collagenase (± 10 mg α-amylase) until the meat pieces began to adhere to each other (approximately 1-1.5 min). The mixture was placed quickly into a sausage casing [1 1/2-in (3.8 cm) diameter] and frozen at -20°C for at least 16 hr before use.

Slices (1/2 in (1.3 cm)) were cut from various locations along each frozen restructured beef log over a 2-month period. After the casing was removed, each slice was weighed, sealed in a plastic bag and held at 4°C until all the slices were prepared. The bags were immersed in a water bath at 65°C for 30 min to simulate cooking. Bag contents were then transferred into polypropylene centrifuge tubes, including two 15 mL bag washes with 1 mM EDTA, and were homogenized with a Polytron homogenizer (Brinkmann Instrument Co., Westbury, NY). Homogenates were centrifuged at 10,000 rpm for 1 hr at 5°C in a DuPont Sorvall RC-5B refrigerated centrifuge. After the resulting top solid layer of lipid was carefully removed with a spatula, the supernatant was decanted and supernatants and pellets were lyophilized. Portions of the lyophilized supernatants (soluble fraction) and pellets (insoluble fraction) were hydrolyzed in 6N HCl at 110°C for 16 hr. Hydroxyproline content of the hydrolysates was determined as above and % soluble hydroxyproline was calculated.

**RESULTS**

**COLLAGENASE ACTIVITY** of the enzyme preparations, with and without α-amylase, was measured to determine the specific activity using insoluble meat collagen as the substrate. Under generally optimal conditions for collagenase activity (37°C, pH 7.4), the purified collagenases demonstrated similar specific activities (Table 1). The partially purified collagenases demonstrated higher specific activities than those of the purified preparations. At 65°C and pH 7.4 all the *C. histolyticum* collagenase mixtures exhibited decreased activities, except for the purified enzyme alone whose activity was unchanged. *A. iophagus* and *Vibrio* B-30 collagenase mixtures all exhibited increased activities at 65°C. When the pH of incubation was lowered in addition to the increased temperature, only the *Vibrio* B-30 samples demonstrated high levels of collagenase activity.

Since the specific activity measurements of the collagenase preparations indicated reasonable levels of activity at both 37°C and 65°C and the actual conditions in the meat product could not be well defined, the amount of collagenase preparations added to meat flakes was set at 0.01% the weight of the meat.

Neither amylase nor any of the collagenase preparations demonstrated proteolytic activity against isolated myofibrillar proteins at either pH 5.5 or 7.4, in contrast to papain which degraded all of the proteins when incubated at equal and 1/10th the enzyme concentrations (Fig. 1). These data suggest that even the partially purified collagenase preparations maintain the desired specificity. Further evidence of this specificity was that all of the restructured beef samples retained shape and bind throughout the cooking step.

Each collagenase preparation solubilized meat collagen at a level greater (p < 0.05) than that of the no enzyme control, although *A. iophagus* and partially purified *C. histolyticum* preparations required the addition of α-amylase to do so (Table 2). No difference was found between *C. histolyticum* collagenase meat samples containing α-amylase and the corresponding meat samples without α-amylase using Student's two-tailed t test. Significant differences were found between the other amylase-containing samples and corresponding collagenase samples. However, amylase appeared to have a stimulatory effect with purified *A. iophagus* collagenase and an inhibitory effect with partially purified collagenase from *Vibrio* B-30. No pattern emerged, then, relating significant results to the purification state of the collagenase or to the addition of α-amylase.

**DISCUSSION**

THE PRESENT STUDY demonstrated that collagenases from various microorganisms and in differing states of purity could function in a meat product to increase the fraction of total collagen in the soluble phase after cooking. Additional increases might be expected with modifications in processing, such as extension of holding time of the meat/enzyme mixture before freezing, microbiologically safe elevation in temperature at some point in the processing, or use of different enzymes in combination with collagenase.

Addition of enzymes prior to mixing of flaked beef provided maximum accessibility of added collagenase to the connective tissue in the meat. Indeed, the range of % soluble hydroxyproline measured in the cooked meat products was comparable to that which could be derived from data of the insoluble collagen preparations incubated under ideal conditions of temperature and pH (values not shown).

Processing of a restructured meat product has required the mixing in of salts to promote the binding of meat flakes. Trout and Schmidt (1984) demonstrated that addition of 0.5% sodium tripolyphosphate to ground meat increased the meat pH to at least 6.0, which would certainly improve the conditions for collagenase activity. The actual pH at the site of enzyme activity could not be determined but one might expect that if the pH was 6.2 or lower, little connective tissue would be affected by these collagenases with the exception of the *Vibrio* B-30 preparation. In fact, data of the *Vibrio* preparation and *Vibrio* preparation + amylase combination in meat correlated well with the activity data on isolated connective tissue at 65°C and pH 6.2, but *C. histolyticum* preparation results were slightly

Table 1—Collagenase activity of enzyme preparations on insoluble collagen of meat<sup>a</sup>

Enzyme preparation	Specific activity <sup>b</sup>					
	Purified			Partially purified		
	pH 7.4		pH 6.2	pH 7.4		pH 6.2
37°C	65°C	65°C	37°C	65°C	65°C	
<i>C. histolyticum</i> collagenase	380	390	100	780	310	100
<i>C. histolyticum</i> collagenase + α-amylase	280	160	100	1330	320	100
<i>A. iophagus</i> collagenase	280	530	0	—	—	—
<i>A. iophagus</i> collagenase + α-amylase	240	330	50	—	—	—
<i>Vibrio</i> B-30 collagenase	—	—	—	470	770	650
<i>Vibrio</i> B-30 collagenase + α-amylase	—	—	—	420	1010	310

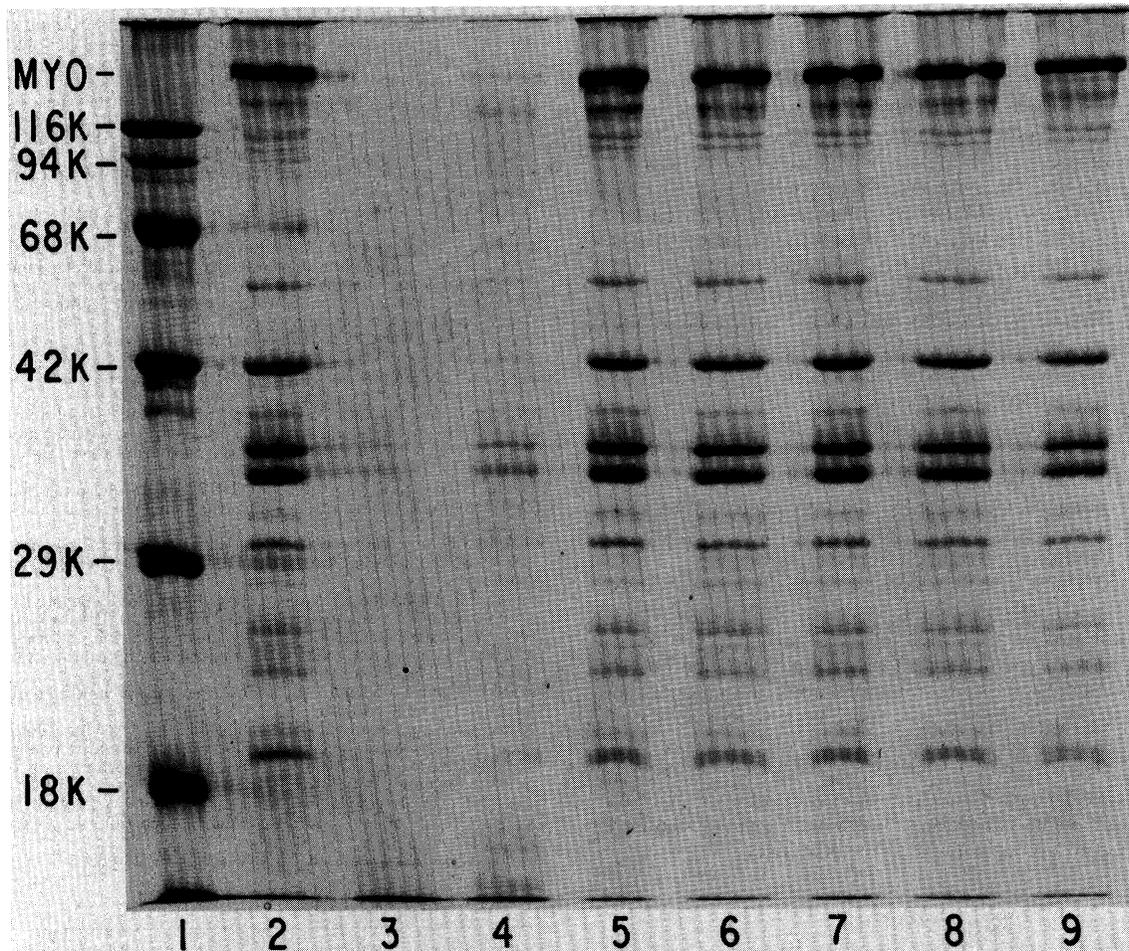


Fig. 1—SDS-PAGE of myofibrillar proteins incubated with enzymes at pH 5.5. (1) Molecular weight standards from top to bottom:  $\beta$ -galactosidase, phosphorylase b, bovine serum albumin, actin, carbonic anhydrase and  $\alpha$ -lactoglobulin; (2) myofibrillar proteins at 0 time and (3-9) incubated with enzyme preparations; (3) papain; (4) papain at 1/10 concentration; (5)  $\alpha$ -amylase; (6) purified *C. histolyticum* collagenase; (7) partially purified *C. histolyticum* collagenase; (8) purified *A. iophagus* collagenase; and (9) partially purified *Vibrio* B-30 collagenase. MYO indicates the position of myosin. Gel of pH 7.4 incubation mixtures was identical and, therefore, is not shown. Incubations carried out as in Materials & Methods.

higher and *A. iophagus* collagenase + amylase results were much higher than expected from the same activity data. These results suggested that the pH at the site of enzyme activity was higher than 6.2.

Partially purified collagenase preparations were originally tested with thoughts of the economic advantage afforded the meat processor over use of more costly purified collagenases and of additional tenderization of the product due to activity

of protease contaminants on actin and myosin. There was no evidence of proteolytic activity, however, when these partially purified preparations were incubated with myofibrillar proteins at the concentration used in the meat product. Although the amount of collagenase per weight of the preparation was less in these partially purified preparations than in the purified ones, the partially purified preparations had much higher specific activities than the purified collagenases on an insoluble collagen substrate. Perhaps, contaminating proteins merely improved the stability of the collagenase in solution since the same results are not seen when the collagenases are used in a meat product (Table 2). In this case, the partially purified collagenase from *C. histolyticum* did not appear to be more active than the purified enzyme. The *Vibrio* collagenase preparation had 1.7 times the effect of the *C. histolyticum* preparations, again with less collagenase present. Collagenase from *A. iophagus* combined with  $\alpha$ -amylase demonstrated 2.3 times the effect of the *Vibrio* preparation, suggesting that less of this enzyme might be used if the effect of the *Vibrio* preparation was adequate for tenderization. However, the decreased quantity of *A. iophagus* collagenase needed may still not be low enough to make its use cost effective.

The results of the purified  $\alpha$ -amylase combination with the various collagenases were anomalous and did not resolve the question of its usefulness in this process. The data suggested an apparent synergy between the amylase and collagenase of *A. iophagus* and an inhibitory effect with the partially purified

Table 2—Collagen solubility of enzyme-treated restructured meat samples

Enzyme preparation	% Soluble collagen <sup>a</sup>
No enzyme control	6.2
$\alpha$ -Amylase	4.0
<i>C. histolyticum</i> collagenase	9.6*
<i>C. histolyticum</i> collagenase + $\alpha$ -amylase	8.0
PP <sup>b</sup> <i>C. histolyticum</i> collagenase	7.8
PP <i>C. histolyticum</i> collagenase + $\alpha$ -amylase	9.3*
<i>A. iophagus</i> collagenase	6.0
<i>A. iophagus</i> collagenase + $\alpha$ -amylase	37.4*
PP <i>Vibrio</i> B-30	16.0*
PP <i>Vibrio</i> B-30 + $\alpha$ -amylase	7.4

<sup>a</sup> Means of four samples. Calculated as

$$\frac{(\mu\text{g Hyp in supernatant})}{(\mu\text{g Hyp in supernatant}) + (\mu\text{g Hyp in pellet})} \times 100$$

<sup>b</sup> PP = partially purified

\* Significantly greater ( $p \leq 0.05$ ) from control mean as determined by Dunnett's Procedure (Steel and Torrie, 1980). RMS error = 1.47.

## COLLAGENASE EFFECT IN RESTRUCTURED BEEF . . .

collagenase of *Vibrio* B-30. Differences in specific cleavage sites on collagen for the two collagenases and the identity of other proteins in the partially purified enzyme preparation might aid in explaining these results, but further study of these enzymes would be required. Other enzymes like hyaluronidase and chondroitinases which are known to cleave ground substance materials may be better enzymes to test.

How the increased solubility of collagen relates to tenderness of the meat is not known. Certainly one expects that a change in the state of collagen from insoluble to soluble increases the perceived tenderness of meat. A test of this hypothesis is currently impossible, however, since none of the collagenases studied has been evaluated for safety as food additives. *C. histolyticum* is a pathogenic organism; *A. iophagus* is purported to be nonpathogenic; and *Vibrio* B-30 has not been tested for pathogenicity. Taste panel evaluations of products containing enzymes from these microorganisms can, therefore, not be undertaken. Alternative physical tests using the Instron Universal Testing Machine for evaluating the tenderness of restructured beef products have provided highly variable results (U.S. Agricultural Research Service, 1984).

The present study demonstrated that collagenase additions could increase the present soluble collagen and, presumably, improve tenderness of a restructured meat product. The demonstrated collagenolytic activity of the partially purified *Vibrio* enzyme was encouraging for commercial application, since the partially purified collagenase was more economical and provided greater solubilization of collagen than use of the purified *C. histolyticum* collagenase. Further study of these collagenases and effects of processing parameters on their activity in restructured meats may provide industry with a new technique for production of quality enhanced meat products.

### REFERENCES

Bailey, A.J. 1972. The basis of meat texture. *J. Sci. Food Agric.* 23: 995.  
Basch, J.J., Douglas, F.W., Procino, L.G., Holsinger, V.H., and Farrell,

- H.M., Jr. 1985. Quantitation of casein and whey proteins of processed milks and whey protein concentrates, application of gel electrophoresis and comparison with Harland-Ashworth procedure. *J. Dairy Sci.* 68: 23.  
Bonnet, M. and Kopp, J. 1984. Essai d'attendrissage de la viande: influence de l'injection d'une collagenase bacterienne non pathogene sur la tendrete de muscles riches en collagene. *Sci. Aliments* 4: 213.  
Broklehurst, K., Baines, B.S., and Kierstan, M.P.J. 1981. Papain and other constituents of *Carica Papaya* L. In "Topics in Enzyme and Fermentation Biotechnology." A. Wiseman (Ed.), p. 262. Ellis Horwood Limited, Chichester.  
Cronlund, A.L. and Woychik, J.H. 1986. Effect of microbial rennets on meat protein fractions. *J. Agric. Food Chem.* 34: 502.  
Dixon, J.S., Hunter, J.A.A., and Steven, F.S. 1972. An electron microscope study of the effect of crude bacterial  $\alpha$ -amylase and ethylenediaminetetraacetic acid on human tendon. *J. Ultrastructure Res.* 38: 466.  
Grant, M.E. and Jackson, D.S. 1968. Carbohydrate content of bovine collagen preparations. *Biochem. J.* 108: 587.  
Kang, C.K. and Rice, E.E. 1970. Degradation of various meat fractions by tenderizing enzymes. *J. Food Sci.* 35: 563.  
Laemmli, U.K. 1970. Cleavage of structured proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* 227: 680.  
Quintarelli, G., Dellovo, M.C., Balduini, C., and Castellani, A.A. 1969. The effects of alpha amylase on collagen-proteoglycans and collagen-glycoprotein complexes in connective tissue matrices. *Histochemie* 18: 373.  
Rattrie, N.W. and Regenstein, J.M. 1977. Action of crude papain on actin and myosin heavy chains isolated from chicken breast muscle. *J. Food Sci.* 42: 1159.  
Steel, R.G.D. and Torrie, J.H. 1980. "Principles and Procedures of Statistics — A Biometrical Approach," 2nd ed. McGraw-Hill Book Co., New York.  
Steven, F.S. 1965. The cleavage of tyrosyl peptides by pepsin from collagen solubilized by the Nishihara technique. *Biochim. Biophys. Acta* 97: 465.  
Trout, G.R. and Schmidt, G.R. 1984. Effect of phosphate type and concentration, salt level and method of preparation on binding in restructured beef rolls. *J. Food Sci.* 49: 687.  
Tsunami, T. and Lieberman, E.R. 1972. Process for making edible collagen casings. U.S. patent 3,681,093.  
U.S. Agricultural Research Service. 1984. "End Product Quality Assessment Systems for Flaked and Formed Steaks," Final Report prepared for U.S. Army Natick Research & Development Laboratories by USDA Meat Science Research Laboratory, Beltsville, MD.  
Woessner, J.F., Jr. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys.* 93: 440.  
Ms received 11/1/86; revised 1/30/87; accepted 1/30/87.

---

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

---