Effects of an Alkaline Elastase from an Alkalophilic *Bacillus* Strain on the Tenderization of Beef Meat

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The tenderizing effect of a new alkaline elastase produced by alkalophilic *Bacillus* sp. strain Ya-B was evaluated on beef meat. We first investigated differences in the hydrolysis of elastin, collagen, casein, and myofibrillar proteins by various enzymes. The elastolytic activity of the new elastase was 60–200 times greater than that of papain and bromelain, which are used as meat tenderizers. This enzyme had greater hydrolyzing activity toward both collagen and elastin but markedly lower hydrolyzing activity toward casein and myofibrillar proteins at a lower pH. We then analyzed the mode of myofibril degradation using SDS–polyacrylamide gel electrophoresis after incubation of intact samples with or without the enzymes. Elastase treatment inhibited the proteolysis of isolated myofibrils, in contrast to papain, which degraded most proteins, especially myosin heavy chain and actin, when the higher concentration ratio of substrate to enzyme was applied at refrigeration temperature. To examine the effect of enzymes on meat tenderness, mechanical hardness was measured with a rheometer. When enzyme solutions were injected into bovine thigh muscle, we found that the force required to cut the muscle fiber was reduced in enzyme-injected samples. These findings suggest that this alkaline elastase is promising as a favorable meat tenderizer.

INTRODUCTION

Tenderness is the most important sensory characteristic of meat (Bernholdt, 1975). Meat toughness can be subdivided into "actomyosin toughness", which is attributable to changes in myofibrillar proteins, and "background toughness", which is attributable to connective tissues (Marsh and Leet, 1966). Recently, attention has been focused on clarifying our understanding of the role of connective tissues in meat and meat products (Stanton and Light, 1990). The molecular structure of collagen and elastin in connective tissues is a significant factor that affects the characteristic texture of meat (Cross et al., 1973). One approach to increasing meat tenderness is to significantly reduce the amounts of detectable connective tissues without causing extensive degradation of myofibrillar proteins.

This purpose may be achieved by mechanical destruction; alternatively, proteolytic enzymes derived from plants, such as papain, bromelain, and ficin, have been widely used as meat tenderizers in America and Europe (Prusa et al., 1981). However, these enzymes often degrade the texture of the meat, due to their broad substrate specificity, and this leads to unfavorable taste due to overtenderization (Kang and Rice, 1970; Cronlund and Woychik, 1986). Therefore, the ideal meat tenderizer would be a proteolytic enzyme with specificity for collagen and elastin in connective tissues at the relatively low pH of meat which would act either at the low temperature at which meat is stored or at the higher temperature achieved during cooking (Cronlund and Woychik, 1987).

Although several microbial elastases and collagenases, in addition to pancreatic elastase, have so far been isolated and characterized (Morihara and Tsuzuki, 1967; Woods et al., 1972; Shiio et al., 1974; Terato et al., 1976), these enzymes were not successful in meat products due to safety problems, such as pathogenicity, or other disadvantageous effects (Foegeding and Larick, 1986; Bernal and Stanley, 1987; Cronlund and Woychik, 1987; Miller et al., 1989). Tsai et al. (1983) isolated a new alkaline elastase from alkalophilic *Bacillus* sp. Ya-B; this was an alkaline serine protease with very high elastolytic activity. This enzyme has the characteristics of high elastin hydrolyzing activity, high elastin binding ability, and substrate preference for aliphatic amino acid residues (Tsai et al., 1986). The amino acid sequence deduced from the DNA nucleotide sequence of this enzyme has about 50% homology to microbial alkaline serine protease, namely subtilisin from *Bacillus* strain, and the catalytic site of both enzymes is particularly well conserved (Kaneko et al., 1989). Therefore, it is interesting to study further their structural and functional relationships, including the differences in substrate specificity (Takagi et al., 1992).

In the present study regarding the application of this enzyme, we showed the characteristics of this new bacterial elastase in the tenderization of beef, in comparison with those of other nonspecific proteases, and evaluated the feasibility of using it for this purpose.

MATERIALS AND METHODS

Materials. The enzymes, purified porcine pancreatic elastase, papain, bromelain, and collagenase from *Clostridium histolyticum*, were purchased from Sigma Chemical Co. (St. Louis, MO). The substrates, elastin–orcein, elastin from bovine neck ligament, and collagen type V from bovine achilles tendon, were also purchased from Sigma. Milk casein was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan).

Enzyme Preparation. Crude and purified elastase were prepared as described by Tsai et al. (1988). Briefly, an alkalophilic *Bacillus* was aerobically cultured in a medium containing 2% soymeal, which stimulates elastase production, at 37 °C for 24

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h. Ammonium sulfate precipitation of the culture fluid was performed to obtain the partially purified enzyme, and this fraction was then further purified, using DEAE-Sephadex and CM-Sephadex column chromatography.

Enzyme Assay. First, both elastolytic and caseinolytic activities were estimated by conventional methods. Elastolytic activity was assayed according to the colorimetric method of Sachar et al. (1955). Each enzyme was incubated with 20 mg of elastin–orcein in 1 mL of buffer, with shaking, for 1 h at 37 °C. The reaction was stopped by the addition of 2 mL of 0.7 M phosphate buffer (pH 6). The substrate was removed by centrifugation, and absorption of the supernatant was determined at 590 nm. The amount of enzyme that gave half of the absorbance at 590 nm when 20 mg of elastin–orcein was completely hydrolyzed was defined as 10 units.

When casein was used as a substrate, the caseinolytic activity was determined according to the method of Hagihara et al. (1956). One unit was defined as the quantity required to increase the absorbance at 660 nm by an equivalent of 1 μg of tyrosine/min at 37 °C.

For the determination of enzyme activity, we selected buffer solutions close to the optimum conditions for each enzyme: 50 mM $NaHCO_3$–Na_2CO_3 buffer (pH 10.5) for the elastase that was the subject of this study; 50 mM Tris-HCl buffer (pH 8.5) and 1 mM $CaCl_2$ for porcine pancreatic elastase; and 300 mM NaCl, 2 mM EDTA, 5 mM Cys, and 10 mM β-mercaptoethanol for papain and bromelain.

Hydrolysis of Elastin, Collagen, Casein, and Myofibrillar Proteins. The hydrolysis of elastin, collagen, casein, and myofibrillar proteins was determined as described previously (Tsai et al., 1983). Each enzyme was incubated with 20 mg of substrate in 1 mL of each optimum buffer or in 50 mM Tris-HCl buffer (pH 6.0), with shaking, for 1 h at 37 °C. The reaction was stopped by the addition of 2 mL of TCA solution (0.11 M trichloroacetic acid, 0.22 M CH_3COONa, 0.33 M CH_3COOH). After a 20-min incubation, the substrate was removed by centrifugation and the absorbance of the supernatant was measured at 275 nm. The buffer solution used for collagenase was 50 mM Tris-HCl (pH 7.4) and 1 mM $CaCl_2$.

SDS–Polyacrylamide Gel Electrophoresis. The effects of elastase on myofibrillar proteins were compared to those of papain, using SDS–polyacrylamide gel electrophoresis. Ten micrograms of isolated myofibrillar protein, prepared as described by Kimura and Maruyama (1983), was incubated with 1 ng of each enzyme in 30 μL of distilled water at 37 or 4 °C. At the end of the incubation time, an electrophoresis sample buffer was added, and the samples were boiled for 3 min to stop the enzymatic reaction.

Isolated collagen type V from bovine achilles tendon was also characterized by electrophoresis according to the procedures of Wu et al. (1982). Twenty-five milligrams of each powdered collagen sample was incubated with 2.5 μg of each enzyme in 1 mL of distilled water at 37 or 4 °C. At the end of the incubation time, 1 mL of dispersion solution, containing 4 M urea, 1% SDS, and 10 mM phosphate (pH 7.2), was added. After a 2-h incubation at 50 °C and centrifugation, an electrophoresis sample buffer was added to each supernatant.

Electrophoresis was carried out with 4–20% gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan), as described by Laemmli (1970).

Mechanical Texture Measurement. A silverside cut of beef imported from Australia was purchased from a local supermarket and was cut into several cubes (4 × 4 × 3 cm). Enzyme treatment was carried out by injecting an amount of the enzyme solution (0.01% enzyme in distilled water) equivalent to 5% of the meat on a weight basis. After incubation under the various conditions, each meat sample was heated at 70 °C for 20 min in a water bath and was subjected to mechanical texture measurement with a rheometer (NRM-2005J, Fudo Kogyo, Japan). Cores (12.5-mm diameter), parallel to the grain of the muscle fibers, were removed from each meat sample. The force required to cut the muscle fiber was measured when a plunger was pressed with 5-kg force.

Measurement of Myofibril Fragmentation. Myofibril fragmentation was measured indirectly by carrying out spectrophotometric measurements of myofibril suspensions, as described by Moller et al. (1973). Each myofibrillar protein specimen prepared from the enzyme-treated meat was adjusted to a concentration of 0.25 mg/mL, and the absorbance was measured at 540 nm.

Collagen Solubility. Collagen solubility was determined according to the method of Hill (1966). Each enzyme-treated meat sample was frozen and powdered. Five volumes (v/w) of 0.03 M NaCl was added to the mixture, which was heated at 77 °C for 60 min, following which time it was centrifuged, after which the supernatant was collected. The supernatant and residue fractions were individually hydrolyzed with 100 volumes (v/w) of 6 N HCl at 110 °C for 24 h. After neutralization of the hydrolysates with 6 N NaOH, the hydroxyproline content of each fraction was determined according to the procedure of Woessner (1961). The hydroxyproline content of the residue and the supernatant was multiplied by 7.25 and 7.52 (Cross et al., 1973), respectively, to yield the collagen content. The total collagen content of the sample was taken as the sum of the collagen content of both residue and supernatant. The percentage of soluble collagen was calculated by dividing the collagen content of the supernatant by the total collagen content.

Table I. Specific Activity of Various Enzymes with Elastin and Casein as Substrates

enzyme	elastolytic,[a] units/mg[b]	caseinolytic,[a] units/mg[c]
alkaline elastase	2400	1900
pancreatic elastase	500	670
bromelain	37	360
papain	12	270

[a] Variations in values were below 5%. [b] The amount of enzyme which gave half of the absorbance at 590 nm when 20 mg of elastin–orcein was completely hydrolyzed was defined as 10 units. [c] Units of caseinolytic activity are expressed as micrograms of tyrosine released per minute.

RESULTS AND DISCUSSION

Specific Activity for Elastin and Casein. The elastolytic and caseinolytic activities of the various enzymes were determined according to a conventional method, using the authentic substrates (Table I). In comparison with porcine pancreatic elastase, papain, and bromelain at their optimum pH, the purified alkaline elastase had very high specific activity, 2400 units/mg of protein for elastin and 1900 units/mg of protein for casein, at pH 10.5. In particular, the elastolytic activity was about 60–200 times higher than that of the commercially used meat tenderizers, papain and bromelain. Recent findings (Tsai et al., 1983) indicate that this enzyme, at the pH of meat, ranging from 5 to 6, still showed about 5–20 times higher elastolytic activity than the other enzymes at the optimal pH.

The proteolytic activity of each enzyme on myofibrillar proteins was not determined in this experiment, since no appropriate method for measuring specific activity with regard to myofibrillar proteins has yet been developed. Thus, to investigate the effects of enzyme on myofibrillar proteins, we carried out two assays, as described below, hydrolysis and electrophoresis (Table II and Figure 1, respectively).

Hydrolysis of Elastin, Collagen, Casein, and Myofibrillar Proteins. The degradation of elastin, collgen, casein, and myofibrillar proteins by each enzyme was determined by another method, using the optimum pH and pH 6 buffer solutions. Table II shows the results. In the optimum buffer (Table IIA), both the elastin and the casein degradative activities of the alkaline elastase were more potent than those of the other enzymes and showed the same tendency as that indicated in Table I. This enzyme showed relatively more potent elastin degradative activity than porcine pancreatic elastase and was at least 10 times more potent than papain or bromelain. Although

Table II. Hydrolysis of Elastin, Collagen, Casein, and Myofibrillar Proteins[a]

(A) In Optimum Buffer

enzyme	A_{275}/mg[b] elastin	collagen	casein	relative activity elastin/casein	collagen/casein
alkaline elastase	220	50	420	0.53	0.12
pancreatic elastase	39	39	180	0.22	0.22
bromelain	10	62	220	0.045	0.28
papain	4.9	45	140	0.035	0.32
collagenase	1.5	39	17	0.088	2.3

(B) In Tris-HCl (pH 6.0) Buffer

enzyme	A_{275}/mg[b] elastin	collagen	myofibril	relative activity elastin/myofibril	collagen/myofibril
alkaline elastase	34	20	4.2	8.1	4.8
pancreatic elastase	17	30	11	1.5	2.7
bromelain	2.8	5.7	6.5	0.43	0.88
papain	1.8	3.3	4.8	0.38	0.69
collagenase	0.7	3.6	1.0	0.70	3.6

[a] Hydrolysis activity was assayed as described under Materials and Methods. The following buffer systems were used as the optimum buffer: 50 mM $NaHCO_3$–Na_2CO_3 (pH 10.5) for alkaline elastase; 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ for pancreatic elastase; 300 mM NaCl, 2 mM EDTA, 5 mM Cys, and 10 mM mercaptoethanol (pH 6.5) for bromelain and papain; and 50 mM Tris-HCl (pH 7.4) and 1 mM $CaCl_2$ for collagenase from *Clostridium hystolyticum*. [b] Variation in values was below 5%.

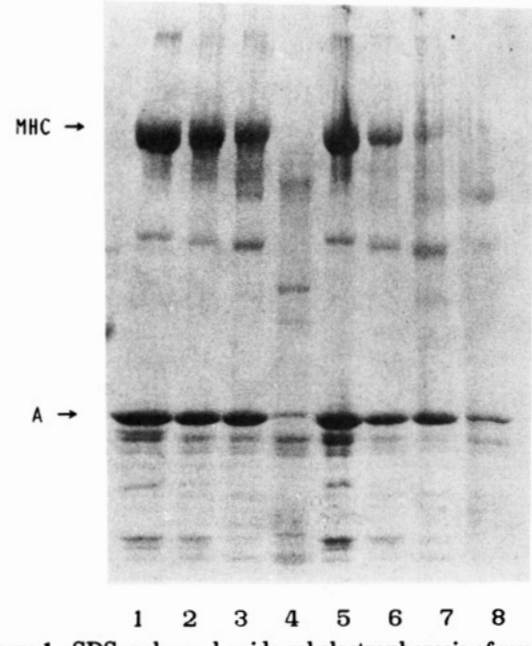

Figure 1. SDS–polyacrylamide gel electrophoresis of myofibrillar proteins incubated with enzymes in distilled water: substrate:enzyme = 10 000:1; (lanes 1–4) incubated at 4 °C for 15 h; (lanes 5–8) incubated at 37 °C for 1 h; (lanes 1 and 5) myofibrillar proteins incubated in the absence of enzyme; (lanes 2 and 6) incubated with purified elastase; (lanes 3 and 7) incubated with partially purified elastase; (lanes 4 and 8) incubated with papain. MHC, myosin heavy chain; A, actin. The gel was stained with Coomassie Blue.

these results may appear to conflict slightly with the data shown in Table I, this would seem to be attributable to differences in the assay procedures or the substrates used in those experiments. These findings indicate that the enzyme, compared to other proteases, has strong activity for selectively cleaving and degrading elastin.

When the enzyme reaction was allowed to proceed at pH 6, i.e., a pH close to that of meats, as it would be in commercial use, the relative degradation of elastin and collagen by the alkaline elastase increased markedly as casein and myofibril degradative activities were markedly reduced, suggesting that this enzyme shows marked preference for elastin and collagen over other proteins. These enzyme properties are thus advantageous for specifically cleaving elastin and collagen, which are the main components of connective tissues in meat. This enzyme therefore seems to be more effective than papain or bromelain, which have previously been used to tenderize meat.

Effects of Enzyme Treatment on Myofibrillar Proteins and Collagen. To examine the mode of myofibril degradation by enzymes, we examined their electrophoretic patterns. After the large sinews were removed from commercially available Japanese round beef, myofibrillar protein was prepared, and the elastase (partially purified and purified enzyme preparations) or papain was added. The reaction was allowed to proceed at 37 °C for 1 h or at 4 °C overnight. The weight ratio of substrate protein to each enzyme was 10 000:1. As shown in Figure 1, in the intact enzyme group, several proteins, including myosin and actin, were observed. When papain was added, all of the proteins were highly degraded, the degradation of myosin, which amounts to 60% of the myofibrillar protein, being especially marked. This action can result in extensive degradation of the meat structure and can thus produce an undesirable texture (Cronlund et al., 1986). Since both bromelain and ficin belong to the family of thiol proteases and have properties similar to those of papain, they may also, like papain, excessively degrade myofibrillar protein (Bernholdt, 1975; Rolan et al., 1988).

On the other hand, little difference in gel banding patterns was seen between the control and each of the elastase preparations after incubation with myofibrils, obviously indicating that excessive degradation of protein does not occur at refrigeration temperature in particular. The same results were obtained irrespective of the degree of purification of the enzyme and the reaction conditions. However, the myosin heavy chain was degraded more rapidly than the other proteins when it was incubated with the partially purified preparation at a lower substrate concentration ratio to enzyme (1000:1, data not shown). It seems likely that contamination with other proteases in the crude preparation causes the loss in the myosin heavy chain. It should also be noted that the concentration ratios for substrate:enzyme at which the enzyme was completely ineffective were quite different for the purified and partially purified enzymes.

Generally, those enzymes capable of hydrolyzing peptide bonds in the triple-helical region of undenatured collagen molecules are known as collagenases; general proteases, such as trypsin and papain, are not considered to be collagenases, since they attack the nonhelical regions or degrade denatured collagen molecules (Bernal and Stanley, 1986; Tunick, 1988). Therefore, we investigated, electrophoretically, under the same conditions as those for myofibrillar proteins, the pattern of degradation of insoluble collagen resulting from treatment with each enzyme (Figure 2). The gel banding pattern obtained from the elastase treatment showed limited hydrolysis of the collagen (indicated by arrows in Figure 2), whereas papain may bring about excessive degradation of the denatured collagen molecule during heat treatment (50 °C, 2 h). At present, since it is not at all clear how this elastase cleaves native collagen molecules and what the differences between elastase and papain are in their action on collagen, much more research (for example, calorimetric experiments)

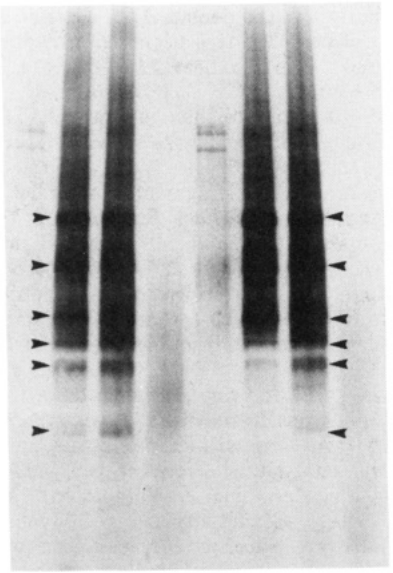

Figure 2. SDS–polyacrylamide gel electrophoresis of insoluble collagen incubated with enzymes in distilled water: substrate:enzyme = 10 000:1; (lanes 1–4) incubated at 4 °C for 15 h; (lanes 5–8) incubated at 37 °C for 1 h; (lanes 1 and 5) insoluble collagen incubated in the absence of enzyme; (lanes 2 and 6) incubated with purified elastase; (lanes 3 and 7) incubated with partially purified elastase; (lanes 4 and 8) incubated with papain. Solid arrows show the position of the limited-hydrolytic collagen. The gel was stained with Coomassie Blue.

Table III. Mechanical Texture Measurement Score

treatment	force,[c] kg	tenderness,[c] cm/100 g
control	3.41 (0.49)[a]	0.056 (0.014)[a]
alkaline elastase		
4 °C, 17 h	2.49 (0.42)[b]	0.074 (0.013)[b]
37 °C, 1 h	2.45 (0.43)[b]	0.082 (0.014)[b]
papain		
4 °C, 17 h	1.79 (0.66)[b]	0.094 (0.016)[b]
37 °C, 1 h	2.01 (0.20)[b]	0.073 (0.005)[b]

[a,b] Values within the same column with no superscript in common are significantly different ($p < 0.05$). [c] Mean (SD) of four samples.

should be performed to study the effects of the changes produced by enzymes on the thermal behavior of collgen.

These findings suggest that when the lower concentration ratio of substrate to enzyme is employed at lower temperatures, even a partially purified elastase preparation has the desired specificity for meat tenderization, i.e., low specificity toward myofibrillar proteins and high specificity toward elastin and collagen.

Mechanical Texture Measurement. The tenderization of meat achieved with the enzymes was examined by measuring physical properties through breakage tests (Table III). The force required for cutting the meat was reduced by alkaline elastase treatment, and the meat became tender, as when commercially available papain was used. Elastase is favorable as a meat tenderizer, since its efficiency in tenderizing was almost the same at the relatively low pH of meat and at the low temperature at which meat is stored. Moreover, the same effect was noted not only with the purified enzyme after treatment with CM-Sephadex (the final stage of purification) but also with the crude enzyme after salting out with ammonium sulfate. In this experiment, 0.5 mg of elastase was injected into 100 g of meat. Assuming that approximately 20% of the meat on a weight basis is composed of myofibrillar proteins, the concentration ratio of substrate (myofibrillar proteins) to enzyme was shown to be 40 000:1, a concentration for which we have no hydrolysis data. Therefore, degradation of the myofibrillar proteins at 40 000:1 is as little that observed at 10 000:1 and 4 °C overnight (Figure 1).

Table IV. Fragmentation Index of Myofibrils and Collagen Solubility

treatment	% fragmentation index[d]	% soluble collagen[d]
control	100[a]	5.6[a]
water	96[a]	5.0[a]
alkaline elastase	107[a]	14.6[b]
papain	184[b]	59.0[c]

[a–c] Values within the same column with no superscript in common are significantly different ($p < 0.05$). [d] Mean of four samples.

To confirm this proposition, we also determined the fragmentation index of myofibrils prepared from the enzyme-treated meat; this was done indirectly, by spectrophotometry (Table IV). Myofibril fragmentation involves the shortening of myofibril length and reduction of the sarcomere number, due to the destruction of the Z lines. These structural changes occur post-mortem and are correlated with meat tenderness (Sayre, 1970). The results of this experiment clearly indicate that there was no difference between elastase-treated samples and controls (no enzyme and water treatment), while papain treatment significantly increased the fragmentation index by 180% above that of the controls.

Collagen solubilization by enzyme treatment is also shown in Table IV. Although both enzymes solubilized meat collagen at a level greater than that shown in the no enzyme and water-treated control, papain appeared to have a marked capacity to solubilize hydroxyproline through a 1-h heating at 77 °C, in agreement with the results of the gel electrophoresis study (Figure 2). Such differences would be expected from the differences in enzymological characteristics between elastase and papain. These data suggest that, even though considerable tenderization was obtained by both enzyme treatments, the elastase prefers elastin and/or collagen to myofibrillar proteins as a substrate, whereas papain tends to degrade not only collagen, denatured due to heat treatment, but also myofibrillar proteins, which could result in the overtenderization of meat.

In conclusion, several findings in the present study show that the new elastolytic enzyme produced by an alkalophilic *Bacillus* sp. is promising as a favorable meat tenderizer; it has a marked preference for elastin and collagen, which contribute to meat toughness, over other myofibrillar proteins at the pH of meat, usually ranging from 5.5 to 6.0. In this respect it is unlike other nonspecific proteases such as papain. However, much still remains to be done before this new elastase can be put to practical use; for example, studies of the changes in various proteins in muscle and connective tissues, sensory evaluation of enzyme-injected meat, and determination of the optimum conditions for the enzyme are necessary.

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