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Effects of elastase from a *Bacillus* strain on the tenderization of beef meat

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Abstract

The tenderization effect of a new elastase from *Bacillus* sp. EL31410 was investigated on beef meat. Meat tenderization was done by dipping the meat cut in a solution containing proteolytic enzymes after freeze-dehydration. It was found that a marination time of 4 h was enough for enzyme adsorption. The samples were treated for 4 h in different enzyme solutions and then was stored at 4 °C for 24, 48, 72 h, and subjected to texture measurement, sensory evaluations, biochemical analysis and histological observations. A marked decrease in hardness, by texture measurements, was observed in the meats with papain and elastase and higher sensory scores for tenderness were observed in the meats treated with enzymes than in the control. The papain-treated beef meat received the highest score for tenderness, but the scores given for juiciness and taste were lower than that of the control. Rapid increases of fragmentation of myofibrils from the enzyme-treated meat were observed in the first 24 h of storage, especially for papain-treated meat. Meantime, elastin of myofibrilar structure was selectively degraded by elastase compared with the control when stored at 4 °C for 48 h as shown by electron microscopy. These findings suggest that *Bacillus* elastase (EL31410) is a promising substitute for papain as a favourable meat tenderizer.

Keywords: Meat tenderization; Papain; Elastase; Freeze-dehydration; Myofibrils; Intramuscular connective tissue

1. Introduction

Of all the attributes of eating quality of meat, consumers rate tenderness as the most important. 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. Recently, most studies have focussed on clarifying understanding of the role of connective tissues in meat and meat products. It is also found that the structure of collagen and elastin is a significant factor that affects the texture of meat (Takagi et al., 1992). There are several means for tenderizing meat, chemically or physically, which mainly reduce the amounts of detectable connective tissues without causing extensive degradation of myofibrillar proteins. Treatment by proteolytic enzymes is one of the popular methods for meat tenderization. At present, most enzymes used are derived from plants: e.g., papain and bromelain, have been widely used as meat tenderizers in America and Europe (Kang & Rice, 1970; Liu & Tang, 2001). However, these enzymes often degrade the texture of the meat, due to the broad substrate specificity, and develop unfavourable taste due to over-tenderization (Cronlund & Woychik, 1986, 1987). Consequently, 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, that would act either at the low temperature at which meat is stored or at the high temperature

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achieved during cooking (Gerelt, Ikeuchi, & Suzuki, 2000).

Although most studies have focussed on the identification and purification of elastase-producing strains, these enzymes are not successful in meat tenderization, mainly because of safety problems, such as pathogenicity, or other disadvantageous effects. We isolated a new elastase from *Bacillus* sp. EL31410 (Chen & He, 2002) this enzyme was a protease with very high elastolytic activity. Therefore, it is interesting to further study its structural and functional relationships, including the difference in substrate specificity. In the present study, we investigated elastase, applied to beef meat tenderization, in comparison with other non-specific proteases, such as papain, and evaluated the feasibility of using it for this purpose.

2. Materials and methods

2.1. Materials

The elastase was purified from *Bacillus* sp. EL31410 culture. Papain (salt precipitation, 200,000 U/ml) was obtained from the Biochemical Co., Ltd. Of Guangxi, China. The Congred elastin from bovine neck ligament was purchased from the Sigma Company. Milk casein was purchased from Shanghai Biochemical Co., Ltd. (Shanghai, China). Beef meat was excised from the shoulder part of a culled-cow carcass after slaughter and stored at −25 °C (Hangzhou meat process factory, China). Before use, it was tempered overnight in a cold room (4 °C) and cut into small pieces (50 × 50 × 30 mm). Three small pieces of beef meat were prepared for each experiment.

2.2. Analytical methods

2.2.1. Dehydration and enzyme treatment

Each piece of beef meat was freeze-dried at −50 °C overnight until it appeared porous. After the dehydration, each sample was dipped for different times (in a cold room) in 5 volumes of a solution containing proteolytic enzymes, such as papain (from plant) and elastase (*Bacillus* sp. EL31410). The concentrations of enzyme solution were 0.1% and 1% for papain and 1% for elastase. Elastase activity of the preparation was 300 U/ml by column chromatography. Papain activity of preparation was 200,000 U/ml. An untreated sample (control) was dipped in deionized water instead of an enzyme solution after freeze-dehydration.

2.2.2. Enzyme preparation

Crude elastase and purified elastase were prepared as described by Takagi et al. (1992). Briefly, a *Bacillus* sp. EL31410 was aerobically cultured in a medium containing glucose and casein and corn steep flour, at 37 °C for 30 h. Ammonium sulfate precipitation of the culture fluid was performed to obtain the partially purified enzyme, and this fraction was then further purified, using SephadexG-100 column chromatography.

2.2.3. Enzyme assay

Elastolytic activity was assayed by the colorimetric method of Sachar (1955). Enzyme preparation was incubated with 20 mg of Congo-red elastin in 2 ml of 0.2 M boric acid buffer (pH 7.4) with shaking for 20 min at 37 °C. The reaction was stopped by adding 2 ml of 0.7 M sodium phosphate buffer (pH 6.0), and immediately filtered. Absorbency of the filtrate was read at 495 nm against a control (no enzyme). One unit of elastase activity was defined as the amount of enzyme required to solubilize 20 mg elastin-congo-red under the tested conditions.

2.2.4. Preparation of myofibrils and fragmentation index

Myofibrils were made from each muscle according to the procedure described by Busch, Stromer, Goll, and Suzuki (1972). The ground muscle was suspended in 5 volumes (w/v) of 50 mM Tris–HCl buffer (pH 7.6) containing 100 mM KCl and 5 mM EDTA by using a blender for 1 min. The myofibrils were sedimented in a centrifuge at 1000*g* for 10 min and suspended again in 5 volumes of the same buffer by use of a blender for 1 min. The re-suspended myofibrils were sedimented at 1000*g* for 10 min, and the resuspension-sedimentation process was repeated three more times. After the fifth wash, the myofibrils suspended in the same buffer were passed through a 20-mesh nylon net to remove connective tissue. The strained myofibrils were sedimented at 1000 g for 10 min washed three times in 1000 mM KCl and finally suspended in 100 mM KCl. After adjusting the protein concentration to 0.5 mg/ml of 100 mM KCl, turbidity at 540 nm of the solution was measured as fragmentation index by HP spectrophotometer 751GW.

2.2.5. Mechanical texture measurement

A Rheometer, NRM 2002, measured meat tenderness with a conical plunger according to the procedure described by Okabe (1979).

2.2.6. Electron microscopic studies

Specimens for scanning electronmicrograph (JSM-T300JEOL) of intramuscular connective tissue were prepared by the cell-maceration method of Ohtani, Ushiki, Taguchi, and Kikuta (1988). Briefly, small pieces from the control and enzyme-treated muscles were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 24 h, the NaOH solution being replaced everyday by a fresh one and then rinsed in distilled water for 5 days at room temperature. Then the pieces were

put in a solution of 1% tannic acid for 3 h, rinsed in distilled water for several hours, and post-fixed in 1% osmium tetraoxide-0.1 M phosphate buffer, pH 7.0, for 1 h. The specimens were dehydrated through graded ethanol, and dried by the alcohol freeze-drying method. The dried specimens were coated with gold and examined using a SEM.

2.2.7. Sensory evaluation

The samples treated with proteolytic enzymes were stored in a cold room for 3 days and subjected to sensory evaluation. The elastases from *Bacillus* sp. EL31410 and papain were used to treat the beef meat. A no-enzyme solution was adopted as the control. Sides of the samples cut into small pieces were grilled at 100 °C for 1 min. The eight panel members were students and staff of Zhejiang University, but were not trained in the sensory analysis of meat. Panel members were asked to fill in a questionnaire, containing six questions about its traits, on appearance (B1), tenderness (B2), juiciness (B3), bitterness (B4), flavour (B5), and taste (B6). Each trait except bitterness was scored on a 7-point scale from −3 to +3: very poor, fairly poor, a little poor, average, a little good, fairly good, and excellent. In the case of bitterness, a 7-point scale from −3 to +3 means an increase of bitterness (Prusa, Chambers, Bowers, Cunningham, & Dayton, 1981). In this test, the scores were evaluated in comparison to known control (non-treated).

3. Results and discussion

3.1. Specific activity of elastin and collagen

Elastin is an important connecting component of striated muscle such as beef meat, which is verified to be the main factor affecting animal muscle tissue tenderness (Thomas & Patridge, 1966). Consequently, it is necessary to investigate the actions of different tenderizers on elastin. Bacterial elastase, belonging to the family of metalloproteinases, has been studied for many years. These proteinases require Zn^{2+} atoms for activity, and in contrast to the serine proteinases, they cleavage peptide bonds on the amino-terminal side of the amino acid that determines specificity (Robert et al., 1997). In this study, the specific activities for elastin and casein were under investigation, using authentic substrates (Table 1). Under optimum buffer and reaction conditions, both elastin and casein degradative activities of the elastase were greater than those of other enzymes, based on the results of Table 1 (0.53). Elastase showed relatively more elastin degradative activity than other enzymes. On the other hand, papain showed higher specific activity on collagen when compared with elastase and bromelain. It is apparent that elastase has strong activity for selectively cleaving and degrading elastin.

Table 1
Hydrolysis of elastin, casein and collagen[a]

Enzyme	OD275/mg			Relative activity	
	Elastin	Collagen	Casein	Elastin/casein	Collagen/casein
Elastase	220	50	420	0.53	0.11
Papain	4.9	45	140	0.035	0.32
Bromelain	10	62	220	0.045	0.28

[a] Elastase reaction conditions: 200 mM boric acid buffer (pH 7.4), temperature 37 °C; papain and bromelain reaction conditions: 200 mM NaCl, 2 mM EDTA, 5 mM Cys and 10 mM mercaptoethanol (pH 6.5) for bromelain and papain, temperature 37 °C.

3.2. Dehydration and absorption of enzyme solutions

The absorption ratio of enzyme solution is shown in Table 2. As shown in the table, the absorption ratio was around 80% of the water removed in dehydration. Differences between absorption ratios were observed among enzyme solutions, the absorption ratio of elastase was higher than those of 1% papain and control. The absorption ratio almost linearly increased with the increase of dipping time up to the four hours, then gradually increased and reached about 87%, but the fading of meat colour due to the release of myoglobin from meat into enzyme solution increased markedly over 4 h of dipping time (as shown in Fig. 1). Therefore, considering the time cost and meat quality, 4 h of

Table 2
Adsorption ratio of enzyme solution for beef meat at different dipping times

Treats	Absorption ratio (%)		
	3 h	4 h	8 h
Control	75.0 ± 1.21	76.5 ± 1.78	77.1 ± 2.01
Papain (1%)	80.2 ± 1.45	82.5 ± 1.96	83.1 ± 2.11
Papain (0.1%)	76.7 ± 1.32	78.2 ± 1.43	78.9 ± 1.65
Elastase	82.6 ± 1.58	85.9 ± 1.69	86.2 ± 1.87

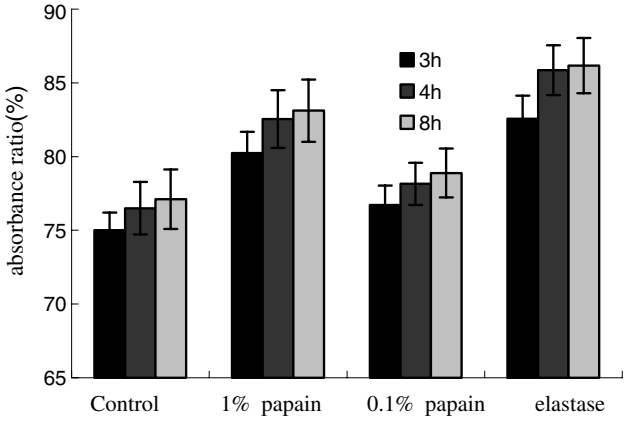

Fig. 1. Effects of different kinds of enzymes on adsorption of enzyme solution for beef meat at different dipping times.

dipping time was chosen as the treatment time in the following study.

3.3. Texture measurements

The changes in the relative hardness of enzyme-treated meat, as expressed as a percentage of that of the control (untreated meat) stored for 24 h, are shown in Fig. 2. Significant decrease in the relative hardness was observed during storage, irrespective of enzyme treatment or not. However, it occurred more rapidly and completely in the enzyme-treated meats, especially for 1% papain-treated meat compared with elastase and the control. The relative hardness of elastase-treated meat was near to that of papain-treated meat, and its relative hardness change is slower than that of papain. However, tenderness is only one of many meat sensory characterizations and all should be equally important in the meat tenderization process. In view of the results for meat texture and sensory quality in the next part, elastase-treated meat received a high score for sensory evaluation compared with papain-treated meats.

3.4. Sensory evaluations

The results of the sensory evaluation of grilled meats treated with enzymes are demonstrated in Tables 3 and 4. The papain-treated meat received the highest score in tenderness; however, the scores given for juiciness and

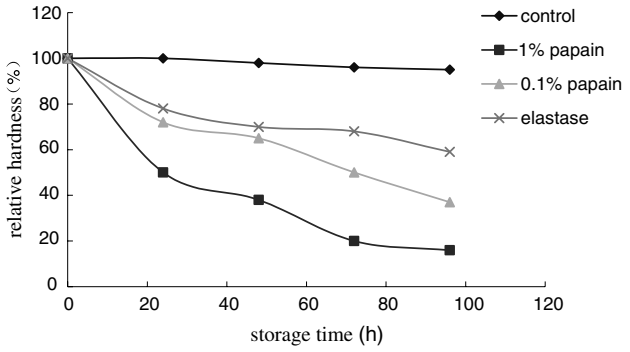

Fig. 2. Changes in hardness of enzyme-treated beef meat.

Table 3
Sensory evaluations of different enzyme-treated meats[a]

Traits	Treatments		
	A1 (elastase)	A2 (papain)	A3 (the control)
B1 (apperance)	0.250	0.250	−0.125
B2 (tenderness)	−0.125	0.250	−0.500
B3 (juciness)	0	−0.125	−0.500
B4 (bitterness)	0.375	0.125	0
B5 (flavor)	0.125	0.250	−0.375
B6 (taste)	0.500	0	−0.250

[a] A means different treatments; B shows different meat traits; The data from each meat sensory trait were means from eight panellists.

Table 4
ANOVA results of different treatments and meat traits

Source	Sum of square	DF	MS	*F* value	*P* value
Block	12.4375	7	1.7768	1.0509	0.3997
Factor A	36.6806	2	18.3403	10.8481	0.00005
Factor B	5.3681	5	1.0736	0.6350	0.6734
A × B	127.1528	10	12.7153	7.5209	0.00000
Error	201.1875	119	1.6907		
Total	382.8260	143			

taste are significantly lower than those of the control and elastase. The panel also gave a high score for bitterness in papain-treated meats. The meats treated with elastase from *Bacillus* sp. EL31410 received higher scores in appearance, taste and tenderness. From the ANOVA results of different factors, it is obvious that different treatments had significant effects on meat sensory scores ($p < 0.01$); however, different traits showed no significant differences ($p > 0.1$). The interaction of different treatments and meat traits is very effective for meat sensory characteristics ($p < 0.01$).

Based on the Duncan's multiple range test (Tang & Feng, 1997) results of Table 5, meat treated with elastase showed the same effect on meat sensory scores as that treated with papain, but both had highly significant effects on sensory properties of meat when compared with the control. Different kinds of traits showed no significant differences in this experiment, which implies that the meat sensory scores is determined by different treatments.

The sensory evaluation score for tenderness is in good agreement with the results of texture measurement. The increase of bitterness and the decrease of juiciness and taste are problems when using papain as a meat tenderizer. On the other hand, the elastase from *Bacillus* sp. EL31410 seems better as a meat tenderizer to this point. In view of these data, it was apparent that elastase prefers elastin and/or collagen to myofibrillar proteins as a substrate, whereas papain tends to degrade, not only collagen, but also myofibrillar proteins, which could result in the over-tenderization of meat.

Table 5
Results of Duncan's multiple range test between means of the fixed effects for the two factors (A and B) under elastase treatment

Sources	Mean value	Significant level (5%)	Highly significant level (1%)
A (treatments)			
A2	0.50	a	A
A1	0.2708	a	A
A3	−0.6667	b	B
B (traits)			
B2	0.3337	a	A
B5	0.1667	a	A
B6	0.1250	a	A
B4	−0.0833	a	A
B1	−0.0837	a	A
B3	−0.25	a	A

3.5. Fragmentation of myofibrils

The degradation of myofibrils was examined by measuring the relative fragmentation index. Myofibril fragmentation involves the shortening of the 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 (Moller, Vestergaard, & Wismer-Pedersen, 1973). The changes in the relative fragmentation of myofibrils prepared from enzyme-treated meat, expressed as a percentage of that of the control, are shown in Table 6. As compared with the control, the rapid increases of fragmentation from the enzymes-treated meats were observed in the first 24 h of storage. The relative fragmentation index of the myofibrils, treated with elastase and papain reached about 230% and 200%, respectively. After that gradual increases of the fragmentation were observed in the myofibrils treated with elastase from 24 to 72 h. In contrast, a slight increase of the fragmentation was found in the myofibrils treated with papain. Based on the case of the control, gradual increase of the fragmentation was observed during all storage, but the ratio of the fragmentation was always lower than that of the enzyme-treated myofibril, especially in the first 24 h storage period, the fragmentation index of the control was about half that of the enzyme-treated myofibrils. According to the differences of RFI between the papain-treated and elastase-treated samples, it is clear that elastase had the same fragmentation ability against beef meat as did papain. However, the elastase prefers elastin and/or collagen to myofibrillar proteins as a substrate, whereas papain tends to degrade, not only collagen, but also myofibrillar protein, which could result in the over-tenderization of meat.

Table 6
Changes in the relative fragmentation index of myofibrils prepared from enzyme-treated beef meats during storage period[a]

Storage time (h)	Control (RFI, %)	Papain (RFI, %)	Elastase (RFI, %)
0	100	100	100
24	111	200	230
48	140	230	245
72	138	240	240

[a] The experimental results were averaged from three replicates.

These results are good in agreement with results of texture measurement of Fig. 2. The appearance of fragmentation of myofibrils from the meats treated with proteolytic enzymes is one of the reasons for the meat tenderization, as confirmed in the texture measurements.

3.6. Ultrastructure of intramuscularly connective tissue

Scanning electronmicrographs of the intramuscular connective tissue in the meats with elastase and the control (no enzyme) are shown in Fig. 3. Marked deformation and disruption of honey-like structure were observed at 48 h of storage as compared with that of the untreated meat, based on the results of the 5000 times magnification.

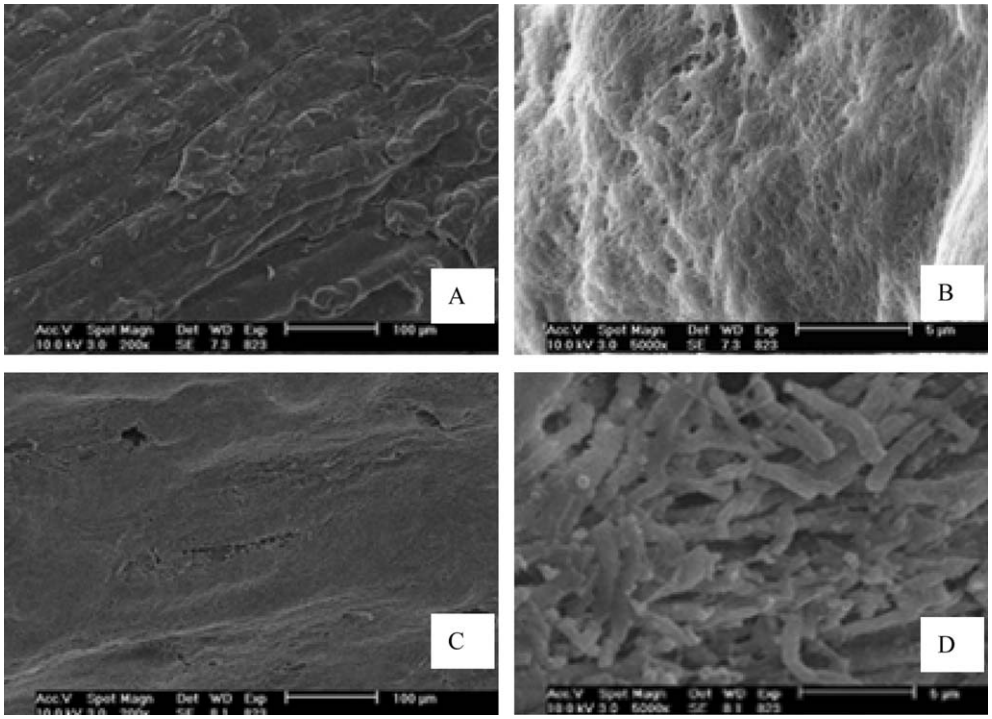

Fig. 3. Scanning electronmicrographs of intramuscular connective tissue prepared from the elastase-treated beef meats and the control (a, control 200×; b, control 5000×; c, elastase 200×; d, elastase 5000×; the treatment time is 48 h, other tenderization conditions shown in methods).

In this study the structure from papain-treated meat was not investigated, consequently it is difficult to compare with the effect of the papain-treated and the elastase-treated. However, disruption of the structure of intramuscular connective tissue is another reason for meat tenderization by the proteolytic enzymes.

4. Conclusion

In conclusion, some findings in the present experiment showed that the new elastolytic enzyme produced by a *Bacillus* sp. is a promising meat tenderizer. It had a marked preference for elastin and collagen, which can contribute to meat hardness, over other myofibrillar proteins at the pH of meat, usually ranging from 5.5 to 6.0. This elastase had the same tenderization effect on beef meat as papain, based on the results of texture, sensory and structure analyses. However, much still remains to be done before this elastase can be put to practical use; for example, studies of the changes in various proteins in muscle and connective tissues and determination of the optimum conditions for the enzyme are necessary. Furthermore, there were many problems under investigation if this enzyme, is to be applied to large-scale meat industry, such as elastase safety and elastase stabilization in the meat tendering process.

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