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Effects of Crude Proteases Extracted from *Bacillus polyfermenticus* on Tenderizing Pork Meat

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Abstract

The purpose of this study was to examine the effect of a crude protease from *Bacillus polyfermenticus* on tenderizing pork meat. A *B. polyfermenticus* protease was characterized, and pork loin samples were treated in solutions containing different enzymes (papain and proteases from *Aspergillus oryzae* and *B. polyfermenticus)* and stored for 24, 72, or 168 h at 4°C. Each treated sample was subjected to a quality assessment. *B. polyfermenticus* protease activity was lower than that for other enzymes tested, although it easily hydrolyzed the meat protein. The optimum temperature and pH for the activity of this protease were 50°C and pH 7.0. The meat tenderizing activity of the protease from *A. oryzae* was higher than that of papain and the *B. polyfermenticus* protease. The fragmentation index of the enzyme-treated with the *B. polyfermenticus* protease was higher than that of the control. A sensory evaluation was not different between meat treated with proteases, but the overall tenderness of enzyme-treated meats was higher than that of the controls. Therefore, the *B. polyfermenticus* protease, papain, and the *A. oryzae* protease appear to be suitable for use as meat tenderizers.

Key words: meat tenderizer, *Bacillus polyfermenticus*, protease, tenderness

Introduction

The quality attributes of meat can be assessed in terms of leanness, appearance (color), texture (tenderness), and flavor (Bhaskar *et al*., 2006). In particular, meat tenderness is one of the most variable aspects of meat quality (Kim *et al*., 2008) because it plays a major role in meat palatability. There are several ways to chemically or physically tenderize meat. The tenderizing methods used for treating tough meat are mechanical (Pietrasik and Shand, 2005), heating (Christensen *et al*., 2004), high pressure (Macfarlane, 1985), marination (Sheard *et al*., 2005), proteolytic enzymes (Naveena and Mendiratta, 2004), and others (Tappel *et al*., 1956). The use of proteolytic enzymes is one of the more popular methods to tenderize meat throughout the world (Kim *et al*., 2008).

The perfect meat tenderizer would contain a protease with specificity for connective tissues (collagen and elastin), would be active at the low pH of meat, and at low storage temperatures or high temperatures during cooking (Gerelt *et al*., 2000). Proteolytic enzymes derived from plants such as papain, bromelain, ficin, and actinidin (Gerelt *et al*., 2000) have been widely used as meat tenderizers. Plant proteases from *Cucumis trigonus* Roxb (Kachri) and *Zingiber officinale* Roscoe (*Ginger rhizome*) have the ability to tenderize through proteolytic activity (Naveena *et al*, 2004). However, these enzymes often degrade the texture of the meat due to their broad substrate specificity, and an unfavorable taste due to over-tenderization often develops (Kim *et al*., 2008). Furthermore, several elastases and collagenases from microbes have been isolated and characterized for use as tenderizers in meat and meat products; however, the safety of these enzymes is a concern for use in meat products (Chen *et al*., 2006).

Microbial proteases from bacteria, yeasts, and molds are more important as industrial enzymes than plant proteases, such as papain and bromelain, due to the ease of isolation and purification and a higher yield. Proteases

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from *Bacillus* sp. are the most important industrial enzymes based on worldwide enzyme sales (Ward, 1985). *B. polyfermenticus* strains, which are commonly referred to as "Bispan", have been effectively used for treating long-term intestinal disorders, as live strains in the form of active endospores successfully reach the target intestine (Jun *et al*., 2000). Many studies have reported on the properties of *B. polyfermenticus,* including its industrial utility and safety (Jun *et al*., 2002; Paik *et al*., 2002), capacity to inhibit carcinogen-induced DNA damage (Park *et al*., 2004), and anticarcinogenic and antigeno-toxic effects (Park *et al*., 2004). However, no studies have been conducted regarding the use of a protease from *B. polyfermenticus* as a meat tenderizer.

Therefore, the purpose of this study was to examine the effect of a protease from *B. polyfermenticus* on tenderizing pork meat.

Materials and Methods

Preparation of materials and enzyme solutions

Fresh loins of five large white crossed Landrace pigs (castrated male pig) were purchased from a local market at 48 h post mortem. Loin samples were carefully trimmed off all subcutaneous fat, epimysium and peripheral muscles so that only the completely trimmed *longissimus dorsi* (LD) muscle was left. To equally allocate pork loin according to treatments, the LD muscle was cut into small pieces (30×50×50 mm) from the front to the back of five pork loins and arranged at a time. Enzyme activity of each protease used in this experiment followed as; protease from *B. polyfermenticus* SCD (97.2 units/mg), protease from *A. oryzae* (947.7 units/mg), and papain (457.9 units/mg). For the activity of enzymes added to the enzyme solutions, *B. polyfermenticus* SCD protease and papain (P4762, EC 3.4.22.2, Sigma Chemical Co., USA) were added in 9.7, 2.1 volumes (w/w) of protease from *A. oryzae* (Sigma Chemical Co.), respectively. That is, the concentration of enzyme solution was 16.7% for protease from *B. polyfermenticus* SCD, 1.7% for protease from *A. oryzae*, and 3.5% for papain. The treated LD muscle was immersed in 0.5 volumes (w/w) enzyme solutions (meat: solution = 1 0.5, the meat chunks were completely dipped with solution). Treated samples were aerobically packed during 24, 72, and 168 h at 4°C and then analyzed (Kim *et al*., 2008).

Preparation in crude protease of *B. polyfermenticus*

Tenderizers were prepared with the crude protease from *B. polyfermenticus,* which is used mainly to tender the pork meat. The crude protease from *B. polyfermenticus* was purified according to previous study (Kim *et al*., 2006). Briefly, this strain was cultivated in tryptic soy broth (Difco Laboratories, MI, USA) at 37°C for 12 h. The culture broth was then centrifuged at 15,000 *g* for 30 min at 4°C and the supernatant prepared the crude protease. Solid ammonium sulfate was slowly added to the culture supernatant (1,000 mL) up to 75% saturation at 4°C. Precipitated proteins were pelleted by centrifugation at 15,000 *g* for 30 min at 4°C, re-suspended in 10 mM phosphate buffer (pH 7.0) and dialyzed in 3 L of 10 mM phosphate buffer (pH 7.0) for 12-18 h in Spectra-Por No. 3 dialysis tubing (Molecular weight cutoff, 3,500 Da; Spectrum Medical Industries, USA). The dialyzed samples were concentrated using a rotary vacuum evaporator (Eyela, Japan). The protease of *B. polyfermenticus* exhibited multiple proteases of molecular weight 28 and 44 kDa.

Myofibrillar fragmentation index (MFI)

Myofibrils were obtained according to the method of Olson *et al*. (1976) and suspended in MFI buffer (20 mM K-phosphate, pH 7.0, 100 mM KCl, 1 mM EDTA, 1mM NaN$_3$). An aliquot of myofibril suspension was diluted with MFI buffer to a protein concentration of 0.5 mg/mL, and the absorbance of this suspension measured at 540 nm. MFI values were recorded as absorbance units per 0.5 mg/mL of myofibril protein sample multiplied by 200.

Fragmentation Index
= Optical density at 540 nm × 200

Cooking loss

The 3.0 cm thick pork loin samples treated with proteolytic enzymes for 24, 72, and 168 h were put into a polyethylene bag. The packages were heated in a water bath at 75°C for 30 min and cooled at room temperature for 30 min. Cooking loss percentage was determined by weighing the samples before and after cooking.

Shear force

Five representative 1.27 cm diameter cores per sample were removed from each sample parallel to the muscle fiber after cooking. Shear force values were determined with a Warner-Bratzler shear attachment on a Texture Analyzer (TA-XT2*i*, Stable Micro System Ltd., UK). Test speeds were set at 2 mm/s. Data were collected and ana-

lyzed from the shear force values to obtain the maximum force required to shear through each sample and converted into kg.

Sensory evaluation

Samples were treated with proteolytic enzymes for 24, 72, and 168 h and subjected to sensory evaluation. After heating in a water bath at 75°C for 30 min, cooked samples were served to 10 panel members with previous experience. Panelists were presented with four randomly coded samples. The appearance color, flavor, tenderness, and overall acceptability of the cooked samples were evaluated using a 10-point descriptive scale (1 = extremely undesirable, 10 = extremely desirable). This analysis was conducted using the Hedonic test described by Choi *et al.* (2010). Panelists were required to cleanse their palate between samples with water.

Statistical analysis

An analysis of variance was performed on all the variables measured using the general linear model (GLM) procedure of the SAS statistical package (2008). Duncan's multiple range test ($p < 0.05$) was used to determine differences between treatment means.

Results and Discussion

Fragmentation of myofibrils

Measurement of myofibril fragmentation is one of the most widely used methods to determine meat proteolysis (Soltanizadeh *et al.*, 2008). Myofibril fragmentation involves myofibril breakdown near the Z-disk during aging (Olson *et al.*, 1976), and a close correlation exists between meat tenderness and myofibril fragmentation (Moller *et al.*, 1973). The myofibrillar fragmentation index of different enzyme-treated meats aged for 168 h at 4°C are shown in Fig. 1. During storage, the fragmentation index of the enzyme-treated meats was higher than that in the control ($p < 0.05$). Additionally, the meat samples treated with *A. oryzae* and papain proteases showed a higher myofibrillar fragmentation index than that of the control and samples treated with *B. polyfermenticus* protease ($p < 0.05$). The fragmentation index of meat treated with protease was the lowest of the enzyme treated samples ($p < 0.05$). Compared with myofibrils from the control, rapid increases in myofibril fragmentation in the samples treated with *A. oryzae,* papain and the *B. polyfermenticus* protease were observed at 24 h of storage. Gradual increases in myofibril fragmentation occurred in the myofibrils of samples
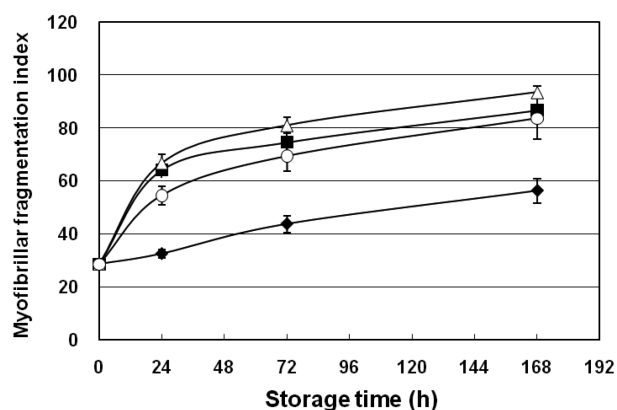

Fig. 1. Changes in the myofibrillar fragmentation index of different enzyme-treated meats aged for 168 h at 4°C. Control (◆), not treated; T-P (■), treated with papain; T-A (△), treated with protease from *Aspergillus oryzae*; T-B (○), treated with protease from *Bacillus polyfermenticus* KD21

treated with protease and the control without protease at up to 168 h of storage. These results agreed with those of Chen *et al.* (2006) who found that the myofibrillar fragmentation index of enzyme-treated meats was higher than that of a control. Gerelt *et al.* (2000) reported that the fragmentation index was in good agreement with that of texture measurements due to the acceleration of myofibril fragmentation in meats treated with proteolytic enzymes. Soltanizadeh *et al.* (2008) indicated that the myofibrillar fragmentation index is a very useful indicator of meat tenderness and is particularly strongly related to shear force and sensory tenderness.

Cooking loss and shear force

The cooking losses of different enzyme-treated meat samples aged for 168 h at 4°C are shown in Table 1. As storage time increased, significant cooking loss was observed in the control and all enzyme-treated samples ($p < 0.05$), but no significant differences were observed among the treated samples. This was because the enzyme curing solution absorbed by the meat was released during cooking. This result was in agreement with that of Han and Chin (2004) who reported that the cooking loss of enzyme-treated meats is not significantly higher than that of a control. In contrast, an increase in cooking yield in enzyme-treated meat compared to control meat was reported by Naveena *et al.* (2004) and Bhaskar *et al.* (2006).

As storage time increased, a decrease in shear force was observed in all treated samples and the control (Table 1). However the shear force of enzyme-treated meats decreased faster than that of the control. In particular, the *A. oryzae* protease treated meat had the lowest shear

Table 1. Changes in cooking loss and shear force of different enzyme-treated meats aged for 168 h at 4°C

Item	Storage time (h)	Control	T-P	T-A	T-B
Cooking loss (%)	0	26.79±2.55[d]	26.79±2.55[d]	26.79±2.55[d]	26.79±2.55[d]
	24	34.38±1.78[c]	37.02±1.74[c]	35.82±1.90[c]	35.37±1.74[c]
	72	37.89±2.26[b]	40.13±2.97[b]	39.54±3.28[b]	39.00±1.92[b]
	168	41.28±3.06[a]	43.09±3.10[a]	42.32±2.34[a]	41.79±2.29[a]
Shear force (Kg)	0	5.39±0.55[a]	5.39±0.55[a]	5.39±0.55[a]	5.39±0.55[a]
	24	5.03±0.45[Ab]	4.61±0.55[Bb]	4.31±0.49[Cb]	4.76±0.57[Bb]
	72	3.77±0.58[Abc]	3.26±0.51[BCc]	3.09±0.52[Cb]	3.40±0.53[Bc]
	168	3.02±0.41[Ac]	2.98±0.38[Bc]	2.74±0.39[Cc]	2.02±0.31[Bc]

All data are means ± standard deviation.
[A-C] Means sharing different letters in the same row are significantly different ($p<0.05$).
[a-d] Means sharing different letters in the same column are significantly different ($p<0.05$).
Control, not treated; T-P, treated with papain; T-A, treated with protease from *Aspergillus oryzae*; T-B, treated with protease from *Bacillus polyfermenticus* KD21

force, whereas no difference in shear force was observed between papain and *B. polyfermenticus* protease-treated meat. A reduction in shear force values with enzyme-treated meats has also been reported by Bhaskar *et al.* (2006). Gerelt *et al.* (2000) found that a decrease in shear force was observed during storage, but that it occurred more rapidly and completely in samples treated with proteases. Among the enzymes tested in this study, the meat tenderizing activity of *B. polyfermenticus* had the best result. Overall, these results show that *B. polyfermenticus* protease may be useful as a tenderizer.

Sensory evaluation

The sensory evaluation of the different enzyme-treated meats aged for 168 h at 4°C are shown in Table 2. When aged for 168 h, the appearance color and flavor of meat treated with papain received the lowest evaluation ($p<0.05$), but it did not differ significantly from the other treated samples or the control. However, the tenderness of enzyme-treated meats was higher than that of the control, and the tenderness of meat aged more than 72 h was rated higher than meat aged for 24 h ($p<0.05$). The sensory evaluation score for tenderness was in good agreement with the texture measurements. The overall acceptability of the control was lower than meat treated with *A. oryzae* or *B. polyfermenticus* proteases. Gerelt *et al.* (2000) reported that meat treated with papain had the highest score for tenderness and juiciness, but overall acceptability was lower than that in the control. These results are similar to the results of the present study. Mestre Prastes

Table 2. Changes on sensory properties of enzyme-treated pork meats during storage at 4°C

Items	Storage time (h)	Control	T-P	T-A	T-B
Appearance color	24	7.63±2.71[a]	8.13±0.64	8.38±0.52	7.88±0.83
	72	8.00±0.65[a]	8.00±0.82	8.14±0.90	7.86±0.90
	168	7.00±1.92[Bb]	8.00±0.76[A]	8.00±0.76[A]	8.13±0.83[A]
Flavor	24	7.75±0.89	7.75±0.71	7.88±0.83	8.00±0.53
	72	7.86±0.90	7.71±0.76	7.57±0.79	7.29±0.76
	168	7.50±1.53	7.50±0.53	7.25±0.46	7.50±0.76
Tenderness	24	7.38±0.52[B]	8.38±0.52[A]	8.00±0.53[AB]	7.88±0.83[AB]
	72	7.71±0.49	8.43±0.53	8.14±0.90	8.00±0.82
	168	7.13±0.64[B]	8.63±0.52[A]	8.38±0.74[A]	8.00±0.53[A]
Overall acceptability	24	7.38±0.52[Ba]	8.13±0.35[A]	8.25±0.46[A]	7.88±0.64[AB]
	72	7.57±0.53[a]	8.00±0.54	8.14±0.90	8.00±0.58
	168	7.13±0.35[Bb]	7.88±0.64[A]	7.81±0.60[A]	7.94±0.18[A]

All data are means±SD.
[A-C] Means sharing different letters in the same row are significantly different ($p<0.05$).
[a,b] Means sharing different letters in the same column are significantly different ($p<0.05$).
Control, not treated; T-P, treated with papain; T-A, treated with protease from *Aspergillus oryzae*; T-B, treated with protease from *Bacillus polyfermenticus* KD21

et al. (2002) reported that as tenderness is the most important sensory characteristics meat attribute for consumers, an understanding of structural collapse of tenderness is required to optimize meat proteases and processing in the industry. Kim *et al.* (2008) indicated that the tenderness of a meat product treated with a crude protease was greater than that in a control without protease, as was samples treated with 0.01% crude protease such as *B. polyfermenticus* SCD and *Streptomyces griseus*. According to Prusa *et al.* (1981), the more meat protease added, the stronger the off-flavor and brittle texture occurred with excessive meat tenderness (Prusa *et al.*, 1981). Thus, *A. oryzae* and *B. polyfermenticus* proteases appear to be good meat tenderizers.

The results showed that treatment with a crude protease from *B. polyfermenticus* SCD improved pork meat tenderness. Furthermore, a 168 h marination period with the crude protease did not have a significant effect on meat quality. The tenderness of enzyme-treated meats was higher than that of the control. Therefore, papain, and the *A. oryzae* and *B. polyfermenticus* proteases seem to be legitimate choices as meat tenderizers.

Acknowledgements

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