

Full Length Research Paper

Biochemical and microstructural characteristics of meat samples treated with different plant proteases

Saroat Rawdkuen^{1*} and Soottawat Benjakul²

¹Food Technology Program, School of Agro-Industry, Mae Fah Luang University, Muang, Chiang Rai 57100, Thailand.

²Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.

Accepted 29 August, 2012

This study was conducted to compare the efficiency of different plant proteases for changing biochemical and microstructural characteristics in muscle foods. The meat samples from chicken, giant catfish, pork and beef were treated with four types of proteolytic enzymes: *Calotropis procera* latex proteases, papaya latex proteases, commercial papain and bromelain at the concentrations of 2×10^3 to 6×10^3 activity units/100 g of muscle. The pH, collagen solubility, trichloroacetic acid (TCA) soluble peptides, protein patterns and muscle microstructures of the treated samples were evaluated after 24 h at 4°C. A decrease of muscle pH in chicken, giant catfish and pork was observed when the enzymes were added ($p < 0.05$). A significant increase in collagen solubility was also found in all of the muscle samples (chicken increased from 37.64 to 83.59%; giant catfish increased from 52.82 to 84.14%; pork increased from 14.34 to 86.78; and beef increased from 26.02 to 86.18%; $p < 0.05$). An increase in TCA-soluble peptides (from 0.90 to 18.53 $\mu\text{mole/g}$ sample), and myofibrillar protein degradation was observed in all of the enzyme treated samples as compared to the control ($p < 0.05$). The electrophoretic pattern of the muscle proteins also revealed extensive proteolysis and reduction of protein bands in all of the treated samples. At the microstructural level, tissue fibers were broken, and the connections between the sarcolemma and the myofibrils were loosened when each enzyme was applied. When comparing all proteolytic enzymes used, papaya latex proteases showed the highest hydrolysis activity in all muscle types, which was followed by *C. procera* latex proteases, commercial papain, and then bromelain. The results show that these proteolytic enzymes could be used as an effective meat tenderizer.

Key words: Proteases, muscle foods, collagen, tenderization, toughness.

INTRODUCTION

Meat tenderness is generally considered one of the most important attributes of meat quality. The toughness of meat depends on the amount of intramuscular connective tissue, the length of sarcomere, and also the activity of endogenous proteolytic enzymes (Kemp and Parr, 2012). Actomyosin toughness is attributable to changes in myofibrillar proteins, whereas background toughness is due to the connective tissue (Chen et al., 2006).

Myofibrillar toughness is affected by the development of rigor-mortis and tenderization caused by the enzymatic breakdown of the contractile proteins (Naveena et al., 2011). The collagen in connective tissue becomes more complex and stronger when the animal gets older. Additionally, as collagen accounts for about 80% of the connective tissue (Gelse et al., 2003), any proteolytic enzyme that could hydrolyze collagen has been sought out for meat tenderization. A number of attempts have been made to improve muscle food tenderness. All of the methods focus on disrupting the myofibrillar proteins and/or connective tissues.

Treatment of meat by exogenous proteases is one of

*Corresponding author. E-mail: saroat@mfu.ac.th. Tel: 66-5391-6752. Fax: 66-5391-6739.

the methods used for tenderization. Currently, five exogenous enzymes (papain, bromelain, ficin, *Aspergillus oryzae* protease and *Bacillus subtilis* protease) have been approved as 'generally recognized as safe' (GRAS) by the USDA. Three of these enzymes are derived from plants (Sullivan and Calkins, 2010). These enzymes can digest muscle proteins when they are mixed with meat. They also can hydrolyze collagen and elastin, which helps to tenderize meat. Wada et al. (2002) reported that plant thiol proteases, such as papain, bromelain, and ficin, affect the structure of myosin and actin filaments. Ketnawa et al. (2010) also confirmed that bromelain from pineapple peels can extensively degrade the collagen from beef and giant catfish skin. Protease from *Calotropis procera* latex can degrade the muscles of beef, squid and farmed giant catfish effectively (Rawdkuen et al., 2011). Plant proteases are superior to bacterial derived enzymes mainly because of safety problems such as pathogenicity or other disadvantageous effects (Chen et al., 2006). However, the proper quantity of enzymes needs to be considered because an excessive amount would result in meat decomposition.

For studies on meat tenderness, physical and chemical methods have been developed, which include measuring forces (shearing, penetrating, biting, mincing and compressing) and determining the solubility and enzymatic digestion of connective tissue. Most research on the digestion mechanism of meat proteins has been carried out by using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Besides, many sophisticated techniques have been widely used to assess the tenderness of meat, such as enzyme activity estimation, myofibrillar fragmentation index, hydroxyproline measurement, and scanning electron microscopic studies (Maiti et al., 2008). The present study investigates the application of plant proteases from different sources to determine their potential to improve tenderness through biochemical and microstructural changes.

MATERIALS AND METHODS

Chemicals and raw materials

L-Cysteine, SDS and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (β ME), Coomassie Brilliant Blue G-250, Chloramine T hydrate, 4-dimethylamino-benzaldehyde, hydroxyproline, and casein were purchased from Sigma-Aldrich Company, LLC (St. Louis, MO, USA). *N,N,N,N*-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Trichloroacetic acid (TCA), hydrochloric acid, tris-(hydroxymethyl)-aminomethane, Folin phenol reagent, and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Papain from papaya latex [1.5 to 10 units/mg solid and bromelain from pineapple stem (3 to 7 units/mg protein)] was obtained from Sigma-Aldrich Company, LLC (St. Louis, MO, USA). Latex of *C. procera* and papaya were collected from Nayong, Trang Province, Thailand. Beef, pork, and chicken muscles were purchased from

Ban Du Market, Chiang Rai Province, Thailand. Farmed giant catfish was obtained from Charan Farm, Chiang Rai Province, Thailand.

Enzyme preparation

Latex was collected in a clean tube by breaking the *C. procera* stems or from fresh papaya. The collected latex was diluted with distilled water (1:1, v/v). It was mixed well and then centrifuged at 15000 \times g at 4°C for 10 min. The obtained supernatant was filtered through a Whatman paper No. 1 and then freeze dried. This sample was referred to as the "*C. procera* latex proteases or papaya latex proteases" and was used for further study.

Enzyme activity determination

An enzyme sample of 0.10 ml was mixed with 1.10 ml of 1% (w/v) casein in 0.10 M Tris-HCl (pH 8.0) containing 12 mM cysteine. The reaction was started by incubating the mixture at 37°C for 20 min. The reaction was stopped by adding 1.8 ml of 5% (w/v) TCA. After centrifugation at 3000 \times g for 15 min, the absorption of the soluble peptides in the supernatant was measured at 280 nm. One unit of caseinolytic activity was defined as the amount of enzymes needed to produce an increment of 0.01 absorbance units per minute at the assayed condition (Rawdkuen et al., 2011).

Enzyme treatment of meat samples

Meat samples were packed in low-density polyethylene bags, and stored in a refrigerator at $4 \pm 1^\circ\text{C}$ for 24 h. After, the samples were cut into small pieces, and mixed with different types and concentrations of enzymes. Stock solutions of each enzyme were prepared to get the same starting units of enzyme activity. The meat samples were then thoroughly mixed with the enzyme solution to get the final concentrations of 2×10^3 , 4×10^3 and 6×10^3 units/100 g sample. After mixing, the resulting chunks were placed in bowls and covered with polyethylene bags and stored at 4°C for 24 h. After the treatment, the samples were evaluated for both their biochemical and microstructural properties. Two replications were done for each treatment with three measurements performed for each replication.

Biochemical properties determinations

pH

To determine pH, 10 g of the treated samples were homogenized with 50 ml of chilled distilled water. The pH values were measured with a digital pH meter (Model pH 510, Eutech Instrument, Ayer Rajah Crescent, Singapore).

Collagen solubility

Soluble collagen for the treated samples was extracted according to the method of Wattanachant et al. (2004). Muscle samples (2g) were homogenized with 8 ml of 25% Ringer's solution (32.8 mM NaCl, 1.5 mM KCl, and 0.5 mM CaCl_2). The homogenate was heated at 77°C for 70 min and then centrifuged for 30 min at 4°C. The extraction was repeated twice with supernatants combined. The sediment and supernatants were then hydrolyzed with 6 M HCl at 110°C for 24 h in an oil bath. The hydrolyzates were allowed to

Table 1. pH of meat samples treated with different sources of proteolytic enzyme.

Sample	Chicken	Giant catfish	Pork	Beef
Control	6.19±0.010 ^d	6.31±0.010 ^c	5.99±0.006 ^c	5.58±0.010 ^a
Calotropis	5.88±0.015 ^b	6.18±0.010 ^a	5.97±0.006 ^b	5.52±0.010 ^a
Papain	5.92±0.015 ^c	6.25±0.010 ^b	5.99±0.006 ^c	5.67±0.162 ^b
Papaya	5.83±0.015 ^a	6.18±0.015 ^a	5.98±0.006 ^{bc}	5.57±0.010 ^{ab}
Bromelain	5.87±0.010 ^b	6.29±0.010 ^c	5.91±0.010 ^a	5.58±0.006 ^{ab}

^{a,b,c}Different letters in the same column indicated significant differences ($p < 0.05$). The activity unit of enzyme is 6 KU/100 g of sample.

equilibrate to room temperature, and they were neutralized with an equal volume of 6 M NaOH. They were then filtered through filter paper Whatman No. 1 and diluted 10 times with distilled water. The hydroxyproline concentrations of the diluted samples were determined by measuring the absorbance at 570 nm against a standard curve of hydroxyproline. The collagen content (hydroxyproline \times 7.25) of the sediments and supernatants were determined separately (Bergman and Loxley, 1963), with total collagen content as the sum of the collagen content in the sediment in addition to that in the supernatant. The amount of heat-soluble collagen (collagen solubility) was expressed as a percentage of the total collagen.

TCA-soluble peptides

The TCA-soluble peptides content of the samples was measured by the method used in Ketnawa and Rawdkuen (2011). The samples (2g) were weighed and then homogenized with 18 ml of 5% (w/v) TCA for 1 min and stored at 4°C for 1 h before they were centrifuged at 8000 \times g for 5 min. The soluble peptides in the supernatant were measured by using the Lowry assay (Lowry et al., 1951). The TCA soluble peptides content was calculated as the μ mole of tyrosine/g of the samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method in Laemmli et al. (1970). Samples (2g) subjected to different treatment conditions were mixed with 18 ml of 5% (w/v) SDS solution (85°C). The mixture was then homogenized using a homogenizer (IKA Ultra Turrax, T25D, Germany). The homogenate was incubated at 85°C in a water bath for 1 h to dissolve the protein. It was then centrifuged at 8000 \times g for 5 min at room temperature to remove the un-dissolved debris. The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol, and 10% β ME) and then boiled for 3 min. The samples (20 μ g protein) were placed onto a poly-acrylamide gel (10% running and 4% stacking gels). Electrophoresis was performed at a constant current of 15 mA per gel by using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gels were stained over night with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. The protein patterns were then visualized after de-staining the gel until a clear background was achieved.

Microstructure determination

The microstructure of the samples was evaluated by using a

scanning electron microscope (LE01450VP, Cam-bridge, UK). The muscle specimens were cut with a thickness of 2 to 3 mm and then fixed with 2.5% (v/v) glutaraldehyde in a 0.2 M phosphate buffer (pH 7.2) for 2 h. The samples were then rinsed for 1 h with distilled water before being dehydrated in ethanol with a serial concentration of 50, 70, 80, 90, and 100% (v/v). They were then critical-point-dried (Balzers mod CPD 030, Blazers Process Systems, Vaduz, Liechtenstein) by using CO₂ as a transition fluid. The dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater, SC7620, Polaron, UK). The specimens were observed with a scanning electron microscopy (SEM) with a magnification of 250 \times at an acceleration voltage of 10 kV.

Statistical analyses

The obtained data was statistically analyzed with the SPSS program for Windows (SPSS version 11.5, SPSS Inc., Chicago, IL, USA). Duncan's multiple-range test was used to compare the difference between means. The accepted level of significance for all comparisons was $p < 0.05$. Experiments were conducted in two replications.

RESULTS AND DISCUSSION

Effect of plant proteases on muscle pH

The pH value of the meat samples treated with different sources of proteolytic enzymes at a level of 6 KU/100 g sample are presented in Table 1. Without enzyme addition, beef was shown to be the lowest in pH (5.58), followed by pork (5.99), chicken (6.19) and giant catfish (6.31). A decrease in pH was observed in the treated chicken and giant catfish when compared to the control ($p < 0.05$). However, no significant decrease in pH was found in pork and beef for all types of enzymes applied ($p > 0.05$). Pawar et al. (2007) reported that a lower pH value of treated chevon when compared with the control could be due to the effect of the additives (acidity of ascorbic acid) on the ionic strength of the system. Moreover, the enzymatic hydrolysis of the muscle may result in releasing acidic amino acids that can increase the pH of the system.

Effect of plant proteases on collagen solubility

The collagen solubility of meat samples treated with

Table 2. Collagen solubility of meat samples treated with different sources of proteolytic enzyme.

Sample	Enzyme account (unit) KU	Collagen solubility (%)			
		Chicken	Giant catfish	Pork	Beef
Control	-	37.64 ± 5.47 ^a	52.82 ± 2.34 ^a	14.34 ± 4.59 ^a	26.02 ± 2.22 ^a
Calotropis	2	69.85 ± 1.90 ^d	80.42 ± 2.09 ^{de}	59.84 ± 2.63 ^d	63.87 ± 1.50 ^e
	4	70.36 ± 2.09 ^{de}	82.17 ± 1.92 ^{ef}	65.34 ± 1.17 ^{ef}	70.79 ± 1.53 ^f
	6	81.30 ± 1.17 ^f	82.09 ± 0.97 ^{ef}	82.53 ± 3.55 ^{gh}	81.80 ± 1.42 ^g
Papain	2	56.67 ± 1.61 ^c	77.93 ± 1.57 ^d	42.05 ± 4.09 ^c	59.34 ± 2.05 ^d
	4	60.19 ± 3.50 ^c	79.96 ± 1.01 ^{de}	56.02 ± 4.99 ^d	60.67 ± 0.65 ^{de}
	6	69.28 ± 2.26 ^d	80.04 ± 0.55 ^{de}	60.84 ± 2.93 ^{de}	63.33 ± 2.50 ^e
Papaya	2	71.42 ± 3.29 ^{de}	81.81 ± 0.48 ^{ef}	69.49 ± 2.16 ^f	83.91 ± 2.07 ^{gh}
	4	75.36 ± 1.32 ^e	82.17 ± 1.47 ^{ef}	81.26 ± 0.10 ^g	85.92 ± 1.79 ^h
	6	83.59 ± 3.48 ^f	84.14 ± 1.12 ^f	86.78 ± 1.59 ^h	86.18 ± 1.97 ^h
Bromelain	2	44.09 ± 3.29 ^b	60.74 ± 3.87 ^b	28.01 ± 1.52 ^b	26.17 ± 4.53 ^a
	4	59.40 ± 3.61 ^c	72.65 ± 2.20 ^c	38.03 ± 1.91 ^c	37.20 ± 2.29 ^b
	6	61.09 ± 1.01 ^c	78.43 ± 0.19 ^d	42.29 ± 3.55 ^c	47.32 ± 1.01 ^c

^{a,b,c}Different letters in the same column indicate significant differences ($p < 0.05$).

different sources of plant proteases is shown in Table 2. The collagen solubility was significantly affected by both the type and concentration of enzyme and meat. Significantly higher collagen solubility values were observed in all enzyme treated samples when compared to the control ($p < 0.05$). The control pork showed the lowest collagen solubility (14.34%), while the highest value was found in the control giant catfish sample (52.82%). Markedly, increased collagen solubility was observed in all muscle types when papaya latex proteases were added. Bromelain showed a low efficiency for increasing collagen solubility for all muscle types. However, when comparing papain and papaya latex proteases, the former showed less effect on collagen solubility than the latter. This result may have been caused by the content and numbers of proteolytic enzymes that exist in the latex of papaya. Sullivan and Calkins (2010) also reported that papain-treated samples exhibited greater collagen solubility than the other treatments (bromelain, ficin, and *B. subtilis* proteases). Takagi et al. (1992) also reported significantly higher collagen solubility in beef treated with papain compared to a water-treated control and an alkaline elastase-treated sample. A more than 50% increase in collagen solubility was found in all muscle types when compared with the control where about 4 KU of papain, protease from *C. procera* and papaya latex, was applied.

Increased collagen solubility of enzyme treated samples might be due to an increase in permeability of the connective tissue, which will disintegrate easily. In addition, proteases may also promote structural alterations through action on intermolecular cross-links.

The solubilization of collagen to gelatin is an indicator of tender meat. The role of collagen is of particular interest, as it has been proposed that collagen is actually the determining factor in the textural differences among various muscles. The solubility of connective tissue rather than total amount of connective tissue is more highly associated with sensory characteristics (Naveena et al., 2011). Naveena et al. (2004) observed that in buffalo meat, when adding ginger rhizome extract, it resulted in extensive muscle fiber and connective tissue degradation and a decrease in shear force values.

Effect of plant proteases on TCA-soluble peptides content

The TCA-soluble peptides content of muscle samples treated with different sources of plant proteases is shown in Table 3. The lowest content of TCA-soluble peptides was found in the control sample of giant catfish (0.90), followed by beef (2.09), pork (2.90), and chicken (4.13), respectively. When the enzyme activity unit was increased, the TCA-soluble peptides content in the entire treated sample also increased ($p < 0.05$). The increase in TCA-soluble peptides content in treated samples might be due to an increase in permeability of myofibrillar structures, resulting in disintegration and then the release of peptides. Ketnawa and Rawdkuen (2011) showed that the high TCA-soluble peptides content in bromelain treated samples was due to greater muscle protein hydrolysis. The bromelain extract applied to the meats resulted in collagen hydrolysis into small peptides

Table 3. TCA-soluble peptides content of meat samples treated with different concentrations of plant proteases.

Sample	Enzyme account (unit) KU	TCA-soluble peptide content ($\mu\text{mole Tyr/g sample}$)			
		Chicken	Giant catfish	Pork	Beef
Control	-	4.13 \pm 0.07 ^a	0.90 \pm 0.07 ^a	2.90 \pm 0.03 ^a	2.09 \pm 0.04 ^a
	2	11.64 \pm 0.22 ^g	7.83 \pm 0.15 ^h	9.35 \pm 0.09 ^h	12.50 \pm 0.15 ^h
	4	13.64 \pm 0.07 ^h	9.62 \pm 0.14 ⁱ	11.90 \pm 0.13 ⁱ	15.31 \pm 0.22 ⁱ
	6	14.25 \pm 0.25 ⁱ	12.04 \pm 0.03 ^k	13.66 \pm 0.12 ^j	17.44 \pm 0.16 ^k
Papain	2	6.51 \pm 0.04 ^e	2.33 \pm 0.07 ^c	5.32 \pm 0.11 ^e	5.53 \pm 0.09 ^e
	4	8.01 \pm 0.05 ^f	4.26 \pm 0.09 ^f	6.66 \pm 0.02 ^f	6.78 \pm 0.08 ^f
	6	8.08 \pm 0.06 ^f	5.64 \pm 0.14 ^g	7.87 \pm 0.59 ^g	8.43 \pm 0.11 ^g
Papaya	2	13.49 \pm 0.10 ^h	9.89 \pm 0.08 ^j	12.18 \pm 0.15 ⁱ	16.75 \pm 0.12 ^j
	4	15.62 \pm 0.08 ^j	13.03 \pm 0.23 ^l	14.02 \pm 0.18 ^k	18.53 \pm 0.24 ^l
	6	16.96 \pm 0.21 ^k	15.25 \pm 0.22 ^m	15.15 \pm 0.15 ^l	18.32 \pm 0.09 ^l
Bromelain	2	4.94 \pm 0.06 ^b	1.71 \pm 0.06 ^b	3.41 \pm 0.04 ^b	3.65 \pm 0.03 ^b
	4	5.80 \pm 0.06 ^c	2.56 \pm 0.07 ^d	4.05 \pm 0.03 ^c	4.64 \pm 0.06 ^c
	6	6.30 \pm 0.02 ^d	3.48 \pm 0.12 ^e	4.76 \pm 0.14 ^d	5.00 \pm 0.04 ^d

^{a,b,c}Different letters in the same column indicate significant differences ($p < 0.05$).

(Ketnawa et al., 2010). According to the results, beef showed the highest content of TCA-soluble peptides when added with papaya latex proteases, while the lowest content was found in bromelain addition for all of the muscle samples. Papaya latex proteases showed to be the most effective for producing peptides from all of the meat samples, which was followed by *C. procera* latex proteases, papain, and then bromelain. More than three-times of TCA-soluble peptides content were found in the sample treated with 2 KU/100 g sample of plant proteases, especially with papaya and *C. procera* latex proteases. This result suggested that the proteolytic enzymes that exist in the latex had hydrolytic activity towards the degradation of protein. The tyrosine level indicated that the oligopeptides and/or free amino acids, as well as the degradation products, all accumulated after being mixed with proteolytic enzymes. From these results, high TCA-soluble peptides content indicated a greater muscle protein hydrolysis, which was generated by proteolytic enzymes.

Effect of plant proteases on protein patterns

Representative protein patterns by SDS-PAGE for the meat samples mixed with different sources and concentrations of plant proteases are presented in Figure 1. The myosin heavy chain (MHC) and actin (AC) are the major proteins in all muscle types. There was increased proteolysis of the muscle proteins in all of the treated samples as evidenced by the reduction in the number and intensity of the protein bands when each enzyme

was added. At higher enzyme activity units, these proteins were totally degraded or even disappeared, especially at 4 to 6 KU/100 g sample. The breakdown of proteins in high amounts was more visible in the chicken sample than in the others. When comparing the treatments (enzyme treated) to the control, the MHC band was markedly degraded into lower molecular weight products as shown at the bottom part of the gel. Jorgova et al. (1989) reported that bacterial proteases treatment of muscle protein showed a reduction in the level of higher molecular weight fractions due to the degradation of myosin. The degradation of muscle protein plays a major role in determining the tenderness and WHC of meat during postmortem storage (Melody et al., 2004). Moreover, degradation of the AC (band disappearance) from the treated sample was also observed on the SDS-PAGE, especially in chicken, pork, and giant catfish muscles added with enzymes activity > 4 KU/100 g sample. Papaya latex proteases showed the highest hydrolysis activity against all muscle types, followed by proteases from *C. procera* latex, papain, and bromelain. Ha et al. (2012) reported that papain, bromelain and zingibian preparations appeared to have a similar hydrolysis profiles to both beef connective tissue and topside myofibril extracts. According to Wada et al. (2002), plant thiol proteases affect the structure of MHC and the AC filaments of myofibrillar proteins. Furthermore, these enzymes have very broad specificities; and therefore, they indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), resulting in over-tenderization to a mushy-textured product.

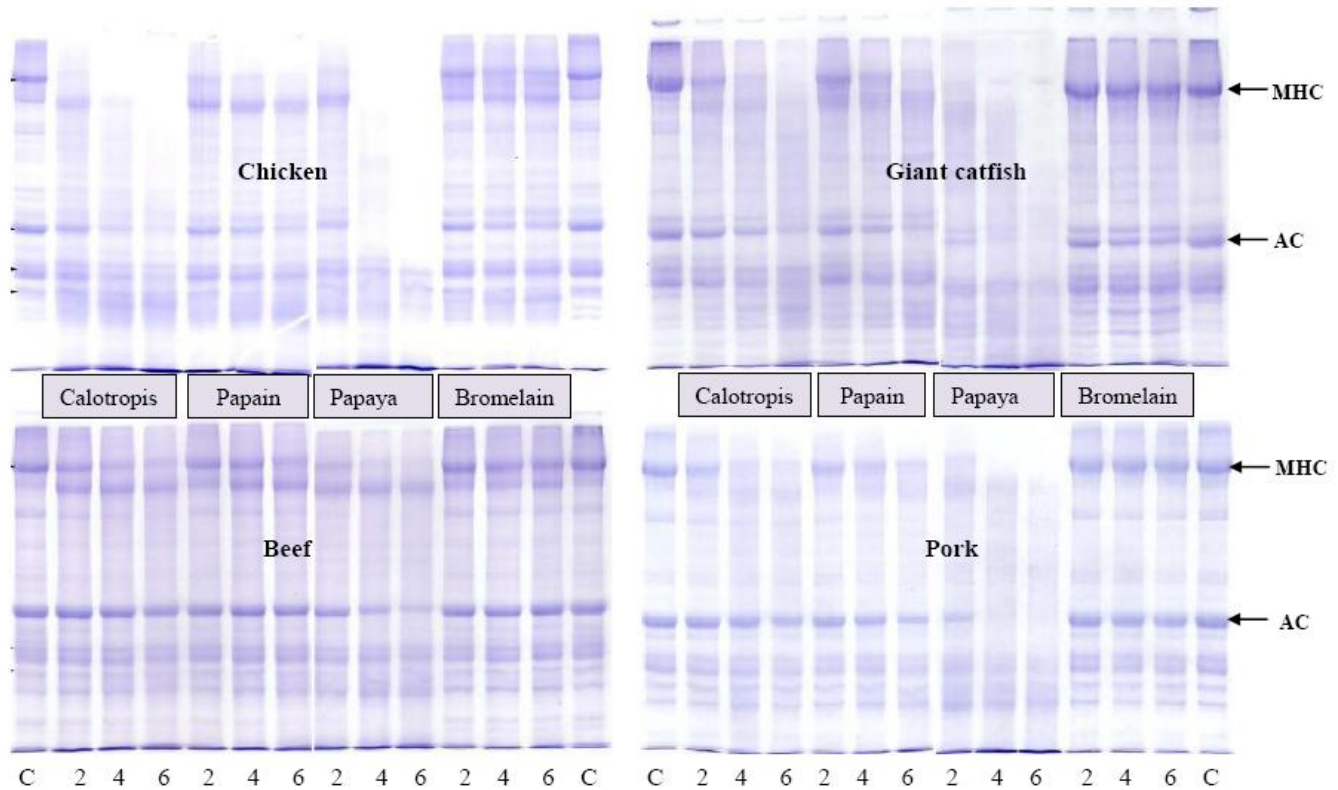


Figure 1. Protein patterns of meat samples treated with different sources of proteolytic enzymes. Numbers represent the enzyme activity unit (KU), C, control; MHC, myosin heavy chain; AC, actin.

Zhao et al. (2012) reported that almost all of the myofibrillar proteins (including MHC and AC) were degraded into fragments with molecular weights lower than 20 kDa when beef was treated with bromelain or papain at 37°C for 1 h. Moreover, they also said that papain and bromelain are all thiol proteases, and their strong activity towards all the myofibrillar proteins may result in extensive degradation of myofibrillar proteins and meat structure. Fragmentation of both myofibrillar proteins and collagen tissue when treated with ammonium hydroxide resulted in the tenderization of buffalo meat (Naveena et al., 2011). When the breakdown of myofibrillar protein occurred, small peptides with low molecular weights were generated and therefore reduced the firmness of the meat samples. However, the ultimate tenderness of meat is dependent on the degree of alteration and weakening of myofibrillar structures (Kemp et al., 2010).

Effect of plant proteases on muscle microstructure

Scanning electron micrographs of the meat samples treated with different plant proteases are shown in Figure 2. Based on the above results, the level of enzyme activity at 4 KU/100 g sample and the control (without enzyme) was considered for microstructural study. The

control samples of all the muscle fibers had well organized structures and were closely bound to each other. The most compact structures were found in giant catfish. Transverse sections of SEM photographs clearly indicated a loss of muscle fiber interaction and a reduction in diameter of muscle fibers in enzyme treated meats when compared to the control. Extensive breakdown of endomysial connective tissue layers surrounding muscle fibers without gaps between fibers is clearly evident in papaya and *C. procera* latex proteases treated samples. This indicated proteolytic enzyme activity on connective tissue and/or myofibrillar proteins. Strong muscle fibers were broken and the cell membranes were severely degraded. In addition, for transverse sections of treated beef, SEM photographs showed a wide gap between fibers indicating complete breakdown and solubilization of collagen layers (endothelium) surrounding individual muscle fibers. Similar findings were observed in a previous study by Naveena and Mendiratta (2004) about buffalo meat tenderization using ginger extract. Disruption of the intramuscular connective tissue structure is another cause for meat tenderization. The microstructure of the enzyme treated samples showed a strong correlation with the previous results in terms of collagen solubility, TCA-soluble peptides content, and SDS-PAGE patterns. Naveena (2004) studied the effect of ginger extract on the microstructure of buffalo muscle

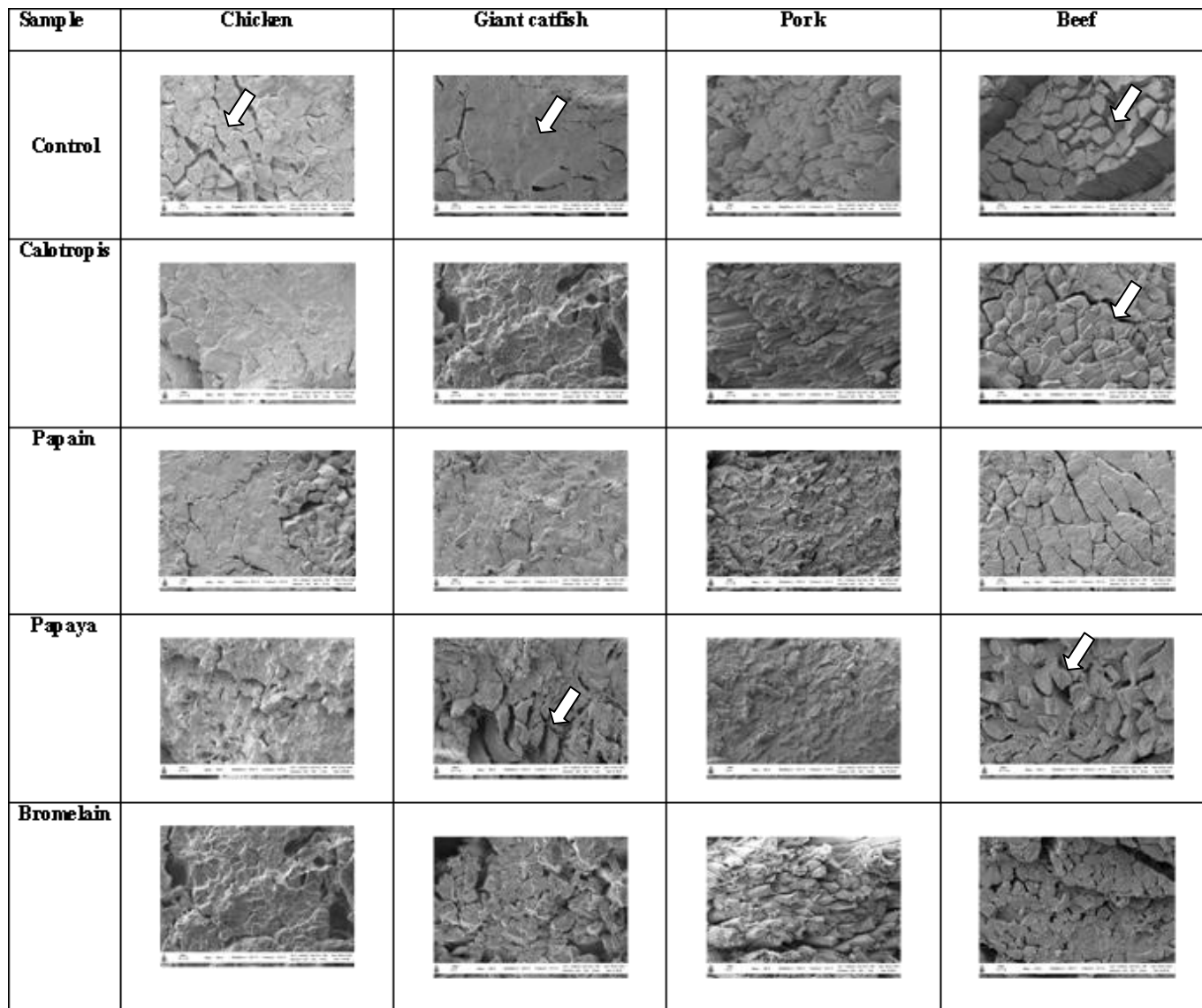


Figure 2. Microstructure of meat treated with different sources of proteolytic enzymes at the level of 4 KU/100 g sample.

by using SEM, and found that the ginger extract treatment broke muscle fibers into different bundles and also increased the space between the bundles. In addition, there was marked deformation and disruption of honey-like structure observed in the beef treated with elastase from the *Bacillus* strain (Chen et al., 2006).

Conclusion

The results obtained in this experiment shows tenderness improvements of proteolytic enzyme treated meats by monitoring biochemical and microstructural changes. Papaya latex proteases provided the greatest ability to improve tenderness in all tested samples. Bromelain used in this experiment showed the lowest capacity when

compared with others. Increasing the unit of enzyme activity resulted in marked changes in all characteristics of the treated samples. Caution must be taken when high levels of proteolytic enzymes are applied, because negative results will be obtained.

ACKNOWLEDGEMENTS

This research was supported by Mae Fah Luang University and the Thailand Research Fund under the TRF Senior Research Scholar program. We also thank Matthew Robert Ferguson, Mahidol University International College for kindly providing suggestions and corrections for the manuscript. The help of Mr. Samart Sai-UT was also appreciated for his work as a research

assistant in this project.

REFERENCES

- Bergman L, Loxley R (1963). Two improved and simplified methods for the spectrophotometric determination of hydroxyroline. *Anal. Chem.* 35:1961-1965.
- Chen QH, He GQ, Jiao YC, Ni H (2006). Effects of elastase from a *Bacillus* strain on the tenderization of beef meat. *Food Chem.* 98:624-629.
- Gelse K, Pöschl E, Aigner T (2003). Collagens-structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* 28:1531-1546.
- Ha M, Bekhit AEA, Carne A, Hopkins DL (2012). Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins. *Food Chem.* 134:95-105.
- Jorgova VK, Danchev S, Kostov A (1989). Effect of bacterial enzyme preparation on the solubility and electrophoretic properties of muscle proteins. *Proc. Int. Cong. Meat Sci. Technol.* 35:913-917.
- Kemp CM, Parr T (2012). Advances in apoptotic mediated proteolysis in meat tenderisation. *Meat Sci.* 92:252-259.
- Kemp CM, Sensky PL, Bardsley RG, Buttery PJ, Parr T (2010). Tenderness-an enzymatic view. *Meat Sci.* 84:248-256.
- Ketnawa S, Rawdkuen S (2011). Application of bromelain extract for muscle foods tenderization. *Food Nutr. Sci.* 2:393-401.
- Ketnawa S, Rawdkuen S, Chaiwut P (2010). Two phase partitioning and collagen hydrolysis of bromelain from pineapple peel *Nang Lae* cultivar. *Biochem. Eng. J.* 52:205-211.
- Laemmli UK, Beguin F, Gujer-Kellenberger G (1970). A factor preventing the major head protein of bacteriophage T4 from random aggregation. *J. Mol. Biol.* 47:69-74.
- Lowry QH, Rosebrough NJ, Farr LA, Randall R J (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:256-275.
- Maiti AK, Ahlawat SS, Sharma DP, Khanna N (2008). Application of natural tenderizers in meat- a review. *Agric. Rev.* 29:226-230.
- Melody JL, Lonergan SM, Rowe LJ, Huiatt TW, Mayes MS, Huff-Lonergan E (2004). Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. *J. Anim. Sci.* 82:1195-1205.
- Naveena BM (2004). The tenderization of buffalo meat using ginger extract. *J. Muscle Food.* 15:235-244.
- Naveena BM, Kiran M, Reddy KS, Ramakrishna C, Vaithiyanathan S, Devatkal SK (2011). Effect of ammonium hydroxide on ultrastructure and tenderness of buffalo meat. *Meat Sci.* 88:727-732.
- Naveena BM, Mendiratta SK (2004). Tenderization of buffalo meat using ginger extract. *J. Muscle Food.* 15:235-244.
- Naveena BM, Mendiratta SK, Anjaneyulu ASR (2004). Tenderization of buffalo meat using plant proteases from *Cucumis Trigonus Roxb* (Kachri) and *Zingiber Offi-cinale Roscoe* (Ginger Rhizome). *Meat Sci.* 68:363-369.
- Pawar VD, Mule BD, Machewad GM (2007). Effect of marination with ginger rhizome extract on properties of raw and cooked chevon. *J. Muscle Food.* 18:349-369.
- Rawdkuen S, Pintathong P, Chaiwut P, Benjakul S (2011). The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its hydrolytic pattern on muscle proteins. *Food Bioprod. Process.* 89:73-80.
- Sullivan GA, Calkins CR (2010). Application of exogenous enzymes to beef muscle of high and low-connective tissue. *Meat Sci.* 85:730-734.
- Takagi H, Arafuka S, Inouye M, Yamasaki M (1992). The effect of amino acid deletion in subtilisin E, based on structural comparison with a microbial alkaline elastase, on its substrate specificity and catalysis. *J. Biochem.* 111:584-588.
- Wada M, Suzuki T, Yaguti Y, Hasegawa T (2002). The effect of pressure treatments with kiwi fruit protease on adult cattle semitendinosus muscle. *Food Chem.* 78: 167-171.
- Wattanachant S, Benjakul S, Ledward DA (2004). Composition, color, and texture of Thai indigenous and broiler chicken muscles. *Poult. Sci.* 83:123-128.
- Zhao GY, Zhou MY, Zhao HL, Chen XL, Xie BB, Zhang XY, He HL, Zhou BC, Zhang YZ (2012). Tenderization effect of cold-adapted collagenolytic protease MCP-01 on beef meat at low temperature and its mechanism. *Food Chem.* 134:1738-1744.