Functional Characterization of Purified Pear Protease and Its Proteolytic Activities with Casein and Myofibrillar Proteins

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Abstract This study was performed to characterize pear protease proteolytic activity and investigate the use of pear protease as a meat tenderizer. Pear protease was purified and stabilized by 5% dextran during lyophilization (dry) or concentration (liquid). Pear protease was further characterized with respect to pH, thermodynamics, and enzyme kinetics. Pear protease was stable at a pH range of 5-8 with an optimum pH of 6.5. From Arrhenius plots, liquid protease showed higher temperature dependency (23.49 kJ/mol) than dry protease (18.62 kJ/mol) due to its higher activation energy. The k_{cat}/K_m , catalytic efficiency of enzyme, was similar with 2.9 and 2.7 $\mu\text{M}/\text{min}$ with dry and liquid proteases. Pear protease was evaluated for its proteolytic activities with casein and beef myofibrillar proteins by individually and combination with fig and kiwifruit proteases. These result indicated that pear and kiwifruit proteases could be complementary to be a desirable product for meat tenderization.

Keywords: pear protease, functional characterization, proteolytic activity, myofibrillar proteins

Introduction

Pear (*Pyrus spp.*) is one of the commonly consumed fruits and is broadly distributed throughout the world, especially in Europe and Asia. It is typically eaten fresh and often consumed in processed products such as juice, puree, or jams. For many years, pear has been used as one of the most common fruits and a meat tenderizer in food systems. Crude extract from Asian pear and its meat tenderization activity has been previously reported (1-3).

The plant origin proteases extensively were studied, and specifically, cysteine proteases are common in the food industry for meat tenderization, bread processing, flavor enhancement, and beer clarification (4,5). Plant cysteine proteases were studied including papain (papaya), ficin (fig), bromelain (pineapple), zingibain (ginger), actinidin (kiwifruit), and turmeric protease (rhizomes of *Curcuma longa*) (6-9). They are well studied for their high proteolytic functions. However, enzymes such as actinidin, bromelain, and ficin showed practical limitations because of their non-uniform or overactivity on meat proteins (10). Among plant cysteine proteases, pear protease was recently focused as the ideal meat tenderizer to prevent overactivity or mushy spots in meat in comparison with bromelain or actinidin (10). Crude pear protease has been characterized with

respect to proteolytic properties on casein and meat tenderization (1-3, 10).

The caseinolytic activity of crude pear protease previously studied showed optimum pH at 7.0 and temperatures of 60°C (11). The proteolytic activity of crude pear extract showed optimum pH at 5.3-7.0 and temperatures of 40-70°C on chicken actomyosin (11-13). Crude pear protease from Asian pear showed optimum concentration of 0.1% in pork loins and 5% in beef round for tenderization with high soluble nitrogen content and tenderness (12,13). Another study reported that partially purified pear protease showed an optimum activity at 10% concentration on chicken actomyosin with improved meat tenderizing activity by combination with pineapple or kiwifruit extracts (3,10).

Recently, our group first evaluated total protein and cysteine protease content among whole fruit, flesh, and peel of nine different Asian pear cultivars. Pear proteases were identified by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), in-gel activity staining, and MALDI-TOF MS (matrix assisted laser desorption/ionization-time of flight mass spectrometry) analysis and further characterized for their proteolytic activities with three different substrates (14). Also, optimum purification method of pear protease was studied by comparison of ethanol precipitation, two phases

partitioning, or ion exchange chromatography (DEAE Sepharose alone or followed by Mono Q). Purified proteases from each method were further evaluated for purification efficiency by SDS-PAGE analysis and enzyme activity with succinylated casein substrate (15) and, 60% ethanol precipitation resulted in a 1.78 purification fold with a 55.6% yield.

To date, most studies have investigated the proteolytic activity of crude pear extract with either casein or myofibrillar proteins. No study has been investigated to characterize purified pear protease for thermodynamics or enzyme kinetics properties. In this paper protease was purified and then stabilized by the addition of 5% dextrin to determine the enzymatic characteristics of the influence of pH on activity, thermodynamics, and enzyme kinetics. Furthermore, purified pear protease was evaluated for its proteolytic activity using casein and myofibrillar proteins in combination with purified fig and/or kiwifruit protease, thus providing valuable information for the practical use of pear protease as a meat tenderizer.

Materials and Methods

Chemicals and plant materials Polyethylene glycol (PEG 4000), sodium dodecyl sulfate (SDS), L-cysteine, and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). β -Mercapto-ethanol and Coomassie Brilliant Blue G-250 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Magnesium sulfate (MgSO_4), potassium phosphate (K_2HPO_4), and other chemicals of analytical grade were obtained from Merck (Kenilworth, NJ, USA). For protease extraction, ripe pear (*Pyrus pyrifolia*), fig (*Ficus carica*), and kiwifruit (*Actinidia deliciosa*) were harvested from an orchard at the Agricultural Research Institute (Chonnam, Korea).

Proteases extraction Pear, fig, or kiwifruit was washed, and then rapidly cut into thin slices. Fruit slices were lyophilized by freeze-drying (FD8512; Ilshin Lab Co. Ltd, Yangju, Korea) until 90% (w/w) of moisture content was removed. Dried samples were then further ground by a pulverizer (FM-681C; Hanil Electric Co., Ltd., Seoul, Korea) and stored at -20°C in polyethylene bags. Proteolytic enzyme from pear, fig, or kiwifruit was extracted as described previously with slight modifications (14, 16). Each powder (10 g) was mixed with extraction buffer (1 mM cysteine, 1 mM EDTA, 50 mM sodium phosphate, pH 6.5) at a ratio of 1:9 (w/v) and then incubated with gentle shaking at 4°C for 1 h. The extract was centrifuged at 4°C for 30 min at 15,000 $\times g$ to remove insoluble materials (Avan-Tr J-E; Beckman Coulter, Brea, CA, USA). The supernatant (crude extract) was filtered through Whatman No. 1 filter paper at 4°C . Beakers containing crude extracts (10 mL) and deionized water (30 mL) were cooled to 0°C using a refrigerated water bath (VWR PolyScience model 1162; PolyScience Inc., Warrington, PA, USA). The addition of absolute ethanol (60 mL) was performed by consecutive application for a final ethanol concentration of 60%. The temperature variations

with time were monitored every 20 s by a precision digital thermometer (Omega 5831A; Omega Engineering Inc., Stamford, CT, USA), and the total elapsed time for each experiment was 20 min. After ethanol addition, the sample was centrifuged at 8,000 $\times g$ for 20 min at 0°C , and the precipitate was solubilized in 10 mL of extraction buffer and then used as protease extract.

Protease quantification and enzyme activity Total protein in protease extracts was determined using a protein determination kit (BCA protein assay reagent kit #23227; Thermo Fisher Scientific, Waltham, MA, USA) based on a modified Bradford technique (17). Bovine serum albumin, in the range of 50–200 $\mu\text{g/mL}$, was used as the standard, and the absorbance of samples at 595 nm was recorded using a spectrophotometer (Beckman/Coulter Du 7500; Beckman Coulter, Brea, CA, USA).

Protein profiles of each protease extract were determined by SDS-PAGE analysis. Proteases from pear, fig, and kiwifruit extracts were quantified by densitometry scanning of protein bands with expecting molecular masses of 36 and 38 (pear proteases) (9), 25 (ficin, 12), or 24, 28–30 kDa (actinidin) (18) in each lane using a NIH program (Scion Image; Scion Corp., Frederick, MD, USA). The molecular mass of each extracted protease was estimated by comparison with the prestained protein standard (Dual color; Bio-Rad, Hercules, CA, USA).

Protease extracts were assayed for proteolytic activity using the substrate succinylated casein as described previously (16). Briefly, caseinolytic activities of the extracted proteases from pear, fig, or kiwifruit (100 μg) were determined using a Quanti-protease Assay kit (Protease assay kit Quanticleave; Thermo Fisher). The assay was based on the ability of proteases to hydrolyze succinylated casein, and the measurements were carried out using a microplate reader (Powerwave XS; Biotek, Winooski, VT, USA). The 96 well plate was temperature equilibrated in an oven at 37°C for 3 min. Succinylated casein solution (100 μL of 1 mg/mL) and protease extract (50 μL) were added. Then 50 mM sodium borate buffer, pH 8.5 (100 μL) and TNBSA (2,4,6-trinitrobenzene sulfonic acid) solution (50 μL) were added to each well for purple color development over 20 min followed by the addition of 0.1 N NaOH (50 μL). The absorbance at 450 nm was read immediately after the plate was gently shaken for 3 s. Total or relative enzyme activity ($\Delta A/\text{min}$) was obtained by an increase in the absorbance at 450 nm, compared to a control without substrate under the above conditions.

Cryoprotectors for pear protease extract Pear protease extract (PPE) was adjusted up to 1 mg/mL by diluting in 10 mM sodium phosphate buffer (pH 6.5) containing the different cryoprotectors (10%, w/v) glucose, sucrose, dextrin, glycerol, or PEG 4000 in order to evaluate the product stability during freeze-drying. Among cryoprotectants, dextrin and glucose in phosphate buffer (pH 6.5) were added at 0, 2, 5, and 7% (w/v) to PPE followed by freeze-drying (dry form) or evaporation with rotary evaporator at $65\text{--}70^\circ\text{C}$ (liquid form) to determine optimum concentration of cryoprotectors for the

prevention of enzyme activity loss. After this process, PPE was resolubilized in 10 mM phosphate buffer (pH 6.5) and the enzymatic activity was determined before and after drying and concentration. Dextrin (5%, w/v) was also added to fig and kiwifruit protease extract followed by freeze-drying (dry form) for further studies.

Functional characterization of pear protease extract Dry and liquid PPE with 5% dextrin (PPED) (w/v) were prepared at 1 mg/mL in 10 mM phosphate buffer (pH 6.5) for enzyme assays (9). The optimum pH for PPED was determined by measuring reaction rates over a pH range of 3-10. The buffers used were: sodium acetate buffer (pH 3-4), sodium phosphate buffer (pH 5-8), and Tris-HCl buffer (pH 10).

PPED (100 mg) was incubated at 4, 16, 25, 37, 45, 60, 70, and 80°C in a water bath for 120 min. Aliquots of enzyme solution at each temperature were removed, and the residual enzyme activity was measured using succinylated casein at 1, 3, 5, 10, 15, 30, 45, 60, and 120 min during the incubation period. The activation energy (E_a) for enzyme inactivation was obtained from two graphs as described by Nam *et al.* (9). First, the log of the percent residual activity of heat treated pear protease was graphed against the incubation time for each temperature analyzed. The slope of these lines resulted in a rate constant, k , for enzyme inactivation. Next, the natural log of the k values at each temperature was plotted against the reciprocal of the temperature in degrees Kelvin (K). The slope of the best-fit line gave an estimate of the value $-E_a/R$, where, E_a is the activation energy and R is the universal gas constant (8.314 J/mol K).

Various concentrations of succinylated casein substrate (0.02-20.00 μ M) were used to determine kinetic constants. Trypsin (20 μ g) (as a positive control) and PPED (100 μ g protein) were used for kinetic analysis. The kinetic parameters V_{max} , K_m , and k_{cat} for succinylated casein were determined from Michaelis-Menton and Lineweaver-Burk plots. The reciprocal of V was graphed versus the reciprocal of $[S]$ and the best-fit line was obtained. Values of V_{max} and K_m were determined using the equation: $1/V = K_m/V_{max}[S] + 1/V_{max}$. The turnover number, k_{cat} was obtained by dividing V_{max} by the total enzyme concentration.

Proteolytic activity of purified pear, fig, and kiwifruit proteases

Dry and liquid PPED were at 0, 0.1, 0.2, 0.4, 1.0, 2.0, and 4.0 mg/mL of protein in 10 mM phosphate buffer (pH 6.5) for activity detection. In addition to PPED, fig and kiwifruit proteases (dry form) were prepared at 0-4.0 mg/mL protein in 10 mM phosphate buffer of pH 6.5. As a control, the commercial meat tenderizer, composed of bromelain extract (pineapple protease), potassium phosphate, and pyrophosphate, was applied for proteolytic activity detection at same concentration. For optimum combination of proteases from pear, fig, and kiwifruit, PPED was mixed with fig protease (1:1, v/v) or kiwifruit protease (1:1, v/v) or both fig and kiwifruit proteases (1:0.5:0.5, v/v/v). All purified proteases in combination were within protein concentrations of 0.4, 1, 2, and 4 mg/mL.

Proteolytic activity toward myofibrillar proteins For myofibrillar protein extraction, round top of beef (Longissimus dorsi, 30 g) was obtained from retail stores and immediately minced in a meat chopper after removal of fat and connective tissues. The minced meat was frozen by liquid nitrogen and ground in a mortar to obtain a fine powder. Following the method of Ma *et al.* (19), powdered meat was mixed with 5 volumes of extraction solution (0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 2 mM $Na_4P_2O_7$, 0.01 M Tris-HCl, pH 6.5) and left to stand at 4°C for 12 h. The mixture was centrifuged at 1,000 $\times g$ for 20 min at 4°C, and pellets were resuspended in the five volumes of extraction buffer and the operation repeated 5 times. After the last centrifugation at 12,000 $\times g$ for 20 min at 4°C, the pellets were resuspended in five volumes of 10 mM sodium phosphate buffer (pH 6.5) and further adjusted to 10 mg/mL for protease treatment after protein quantification using the protein determination kit.

Myofibrillar proteins (10 mg/mL) were treated with liquid and dry PPED (1 mg/mL) for 0, 1, 6, 12, and 24 h at 37°C. Further, various concentrations of PPED (0, 0.2, 0.4, 1, 2, and 4 mg/mL) were added to myofibrillar protein extract at 37°C for 12 h. For optimum combination of proteases (1 mg/mL), PPED was mixed with fig protease (1:1, v/v) or kiwifruit protease (1:1, v/v) or both fig and kiwifruit proteases (1:0.5:0.5, v/v/v) and incubated at 37°C for 12 h. Proteases treated myofibrillar protein (20 μ g) were subjected to 12% SDS-PAGE and visualized by staining in Coomassie Brilliant Blue R-250 and destaining.

Statistical Analysis All experiments were carried out in triplicate with three replicates, and results were expressed as mean $\pm$ standard deviation. The data were analyzed by one-way ANOVA, and the means of different groups were compared with the Duncan's multiple range test (DMRT) using the SPSS version 21.0 statistical software package. Values of $p < 0.05$ were considered as significant in all cases.

Results and Discussion

Protease extraction from pear, fig, or kiwifruit Cysteine proteases from pear, fig, and kiwifruit powder were extracted with extraction buffer (10 mM cysteine, 1 mM EDTA, 50 mM sodium phosphate, pH 6.5) followed by a 60% ethanol precipitation as described previously (14, 16). Here, cysteine in the extraction buffer is important to keep the activity of cysteine proteases because these proteases use a catalytic cysteine residue as a nucleophile during proteolysis and are activated by thiol compounds (20). Many reports on cysteine protease purification from pear, kiwifruit, or pineapple used various concentration of cysteine (1 to 100 mM) in the extraction buffer (11, 21).

The specific activities of fruit proteases were 4.4, 9.6, and 13.0 $\Delta A/min/mg$ protein in pear, fig, and kiwifruit, respectively. SDS-PAGE

Fig. 1. SDS-PAGE analysis of extracted proteases from pear, fig, or kiwifruit. Lane M is a protein standard marker. Proteases were fractionated from freeze-dried powder by extraction buffer (10 mM cysteine, 1 mM EDTA, 50 mM sodium phosphate, pH 6.5) and 60% ethanol precipitation. Arrows indicate expected molecular masses of cysteine protease isoforms in each fruit. There are protein bands with expecting molecular masses of 36 and 38 (pear proteases), and 17, 24, and 28 kDa (actinidin), 23 and 25 (ficin).

analysis is shown in Fig. 1. Pear protease showed two major bands at 36 and 38 kDa, while fig (ficin) showed two bands at 23 and 25 kDa, and kiwifruit (actinidin) showed three major bands at 17, 24, and 28 kDa. Cysteine proteases from the fruits were confirmed by in-gel activity staining or comparison to a commercial standard. These results are in agreement with previous reports regarding molecular weights of cysteine proteases in pear, fig, and kiwifruit. Pear showed two bands with expected molecular weights of 30 and 36 kDa, depending on cultivar (12,14). Fig had one or two distinct cysteine protease with molecular masses of 23–25 kDa (22) while actinidin from kiwifruit existed in multiple isoforms with molecular masses of 17, 24, 28, and 30 kDa (23).

Cryoprotectors for pear protease extract (PPE) PPE was cryopreserved using 5 different cryoprotectors before the lyophilization in order to maintain the enzyme activity (Fig. 2A). The efficiencies of cryoprotectors by type and their concentration were confirmed by proteolytic activity using succinylated casein, and this is shown in Fig. 2A, 2B, and 2C. It was found that the lyophilization process resulted in the loss of 48.8% of the initial enzyme activity (Fig. 2A, control). Cryoprotectors examined were glucose, sucrose, dextrin, glycerol, and PEG 4000 at 10% concentration (w/v). During lyophilization, the water around a protein can freeze and denature the protein (24). To prevent activity loss during the freezing and lyophilization process, several cryoprotectors were investigated (25,26). The best cryoprotector for PPE was found for 10% (w/v) glucose and dextrin in a phosphate buffer (pH 6.5), which kept for 84.1 or 87.4% recovery of initial activity, respectively. In the presence of glycerol or PEG 4000, the loss of enzyme activity was greater than 55.0% for both, suggesting that they were not effective at cryopreserving PPE. Several studies reported that PEGs act as chaperones, which drive the folding of polypeptide chains into their native structures (26). It is assumed

that pear protease did not interact with PEG 4000 during the freezing/lyophilization process, resulting in the loss of enzymatic activity. In pear protease with glycerol, when temperatures reach below 0°C, viscosity start increasing that allows more water to stay unfrozen than in a solution without glycerol (24).

The best cryoprotectors for PPE (dry and liquid), dextrin, and glucose were investigated at 0, 2, 5, 7, and 10% concentrations (w/v) to determine their optimal concentration for freezing/lyophilization and concentration via a rotary evaporator at 65–70°C (Fig. 2B and 2C). Dry PPE exhibited 22–42% higher enzyme activity than that of liquid PPE since the freeze drying process may be more protective to thermal or oxidative inactivation than evaporating concentration at 65–70°C (Fig. 2B and 2C). Among the treatments, activity yield was high with 5% glucose and dextrin (w/v) with above 95 (dry) or 32% (liquid) activity. Dextrin was more effective than glucose in maintaining activity. Interestingly, with liquid PPE, 7% dextrin showed a three-fold higher activity (61% activity) than 7% glucose (22% activity) while 7% dextrin had a slightly higher yield than 7% glucose in the dry PPE (Fig. 2B and 2C). Overall, 5 or 7% dextrin is the best cryoprotector for pear protease activity during freezing/lyophilization process or concentration process. Lyophilized PPE showed a significantly increased activity with dextrin or glucose, regardless of its concentration while concentrated pear protease showed a slightly higher yield with dextrin or glucose, but 7% dextrin (three folds increased activity) was optimal. This finding of the best cryopreserved pear protease with 10% glucose agrees with previous reports about the best cryopreserved bromelain (pineapple protease) with 10% glucose (24) or crude kiwi extract with 5% dextrin (27). Dextrin (5%) was also added to the fig and kiwi protease extract during the freezing/lyophilization process and was used as a cryoprotectant for dry and liquid PPED for all other studies.

Functional characterization of PPED Dry and liquid PPED were characterized functionally with respect to pH, thermal inactivation, and enzyme kinetics. Enzyme activity of PPED was determined at various pH values (3–10) (Fig. 3A). The total activity of PPED was measured by hydrolysis of succinylated casein and expressed as $\Delta A/\text{min}$ for relative activity. Pear protease activity was low below pH 5 and above pH 8 but higher at pH values of 5 to 8 as shown in Fig. 2A. Both dry and liquid pear proteases showed a similar activity pattern at various pH conditions. This result is consistent with data from Bai and Rho (11) in which the pear protease was stable at pH 5–8 with an optimum pH of 6.5. Other proteases from fig, kiwifruit, and pineapple showed similar pH pattern for casein degradation, but kiwifruit protease had also another enzyme activity peak point at pH 3 and rapidly decreased at pH 4. Other studies (2,10) also showed high activity of pear protease on chicken actomyosin at pH 5–7 with a rapid decrease above pH 8.

The thermal stability of PPED (dry and liquid) was determined using an Arrhenius plot (Fig. 3B). Dry PPED became inactive at 60, 70, and 80°C with incubation times of 107, 82, or 78 min, respectively. Liquid PPED required longer incubation time to be inactive with

Fig. 2. Cryoprotector effects on the enzymatic activity of pear protease extract (PPE). (A) Various cryoprotectors were added before freeze-drying PPE. (B) Dextrin and (C) glucose at various concentrations (0-10%, w/v) were added before freeze-drying (dry form) or evaporating (liquid form) of PPE and their enzymatic activities were determined before and after the process. Control is no cryoprotectant in pear protease solution. Values are means $\pm$ SD ($n=3$). Different letters (a-f) of each indicate significant difference at $p<0.05$ by DMR test.

times of 200, 150, or 118 min at 60, 70, or 80°C, respectively. These results indicate that dry PPED has higher enzyme activity, but is less stable at elevated temperatures than liquid PPED. Both enzyme samples incubated at 45°C lost more than 40% activity, whereas at 25 to 37°C, more than 60-70% of their activities remained after 60 min incubation.

With a 60 min incubation, dry and liquid PPED were stable at 4-60°C with 75-47 and 79-53% residual activities, respectively. However, they became unstable at 70°C with 34-44% residual activity and at 80°C with 25% residual activity. These findings are in agreement with previous reports in that crude pear protease showed a progressive increase in activity from 40-70°C and a sharp decrease above 70°C with casein, indicating an optimum at 70°C (11). Interestingly, crude

Fig. 3. Functional characterization of dry or liquid PPED with respect to pH, thermodynamics, and enzyme kinetics. (A) The pH vs. activity (pH 3-10) of PPED with succinylated casein substrate. (B) Thermodynamics of PPED as determined by Arrhenius plot. (C) The enzyme kinetics of dry or liquid PPED were measured using various concentrations of succinylated casein substrate (0.02-20.00 μ M) in 10 mM sodium phosphate at pH 6.5. Values are means $\pm$ SD ($n=3$).

pear extract has different temperature optimums which are substrate specific. Crude pear extract showed activity from 30-70°C with optimum activity at 60 or 65°C on chicken (12) and beef actomyosin (28) yet with the chemical substrate, CBZ-lys-Onp, the optimum temperature was 40°C.

From the slopes of the lines at each temperature, inactivation rate constants were calculated and presented in an Arrhenius plot (Fig. 3B). Equations for dry and liquid enzymes are $y = -2.239x + 1.932$ ($R^2=0.97$) and $y = -2.827x + 3.547$ ($R^2=0.94$). The temperature dependence of the thermal inactivation of PPED, activity expressed by the activation energy value, was calculated from Arrhenius equation. Their activation energies were 18.62 and 23.49 kJ/mol for dry or liquid samples, respectively. The high activation energy implies that the rate of this process is strongly temperature dependent, and thus this rate becomes insignificant at lower temperatures.

Both dry and liquid PPED (100 μ g protein) were analyzed for

kinetic parameters using succinylated casein substrate in 10 mM sodium phosphate at pH 6.5. Trypsin (20 µg protein) was also used as control (data not shown). Lineweaver-Burk plots of protease hydrolysis of succinylated casein (0.02–20 µM concentration) are shown in Fig. 3C. The specific activity of trypsin, dry and liquid PPED was 10.6, 0.54, and 0.31 ΔA/min/mg, respectively. The K_m , substrate binding indicator, was 0.09 µM with liquid PPED which was lower than that of dry PPED (0.11 µM). This implies that the liquid PPED has a slightly higher affinity for the casein substrate. Catalytic activity of the dry PPED (kcat) was 0.32/min, which was higher than that of liquid PPED (0.23/min). Overall, the kcat/ K_m value, representing the catalytic efficiency of enzyme, was similar with 2.9 and 2.7 µM/min for dry and liquid PPED, respectively.

In addition to dry PPED, fig (100 µg protein) and kiwifruit (100 µg protein) proteases were also evaluated for kinetic values with succinylated casein (data not shown). The specific activity of fig (ficin) and kiwifruit protease (actinidin) was 1.09 and 1.47 ΔA/min/mg which was 2–3 times higher than that of PPED. The kcat/ K_m values of fig and kiwifruit proteases were 4.1 and 10.0 µM/min which were 1.4 and 3.4 times higher than that of PPED samples, respectively. Since the kcat/ K_m value is the most reliable measure of enzyme specificity, fig and kiwifruit proteases show 1.4 and 3.4 times higher catalytic efficiency respectively than PPED towards the casein substrate. This pattern is similar with previous studies in that proteolytic activity of fig and kiwifruit extracts which were 1.1 and 3 times higher than that of crude pear extract on casein (11,13).

Table 1. Caseinolytic activity of dry or liquid PPED

Protein content (mg/mL)	Commercial ¹⁾ (ΔA/min)	Dry PPED ²⁾ (ΔA/min)	Liquid PPED (ΔA/min)
0.1	0.35±0.02 ³⁾⁴⁾	0.06±0.01 ^b	0.07±0.00 ^c
0.2	0.75±0.03 ^e	0.40±0.04 ^b	0.24±0.02 ^b
0.4	1.21±0.02 ^d	0.51±0.05 ^a	0.26±0.05 ^{ab}
1.0	1.75±0.09 ^c	0.53±0.04 ^a	0.31±0.04 ^a
2.0	1.97±0.12 ^b	0.55±0.01 ^a	0.30±0.01 ^a
4.0	2.21±0.08 ^a	0.51±0.01 ^a	0.21±0.01 ^b

¹⁾Commercial meat tenderizer was composed of crude pineapple extract and phosphate salts.

²⁾Proteolytic activity of PPED was determined using the substrate succinylated casein (100 µg protein).

³⁾Value are means±SD ($n=3$).

⁴⁾Within columns, different letters (a–f) of each indicate significant difference at $p<0.05$ by DMR test.

Proteolytic activity of PPED: Casein and myofibrillar proteins The activities of dry and liquid PPED (0.1–4 mg protein/mL) on casein and beef myofibrillar proteins is shown in Table 1. As a positive control, common commercial meat tenderizer was applied that contained pineapple bromelain, potassium phosphate, and pyrophosphate.

For proteolytic activity with casein, the total activity of proteases or commercial tenderizer was expressed as ΔA/min. PPED showed a progressive increased in activities at 0.1–1 mg concentration, then remained similar at 1–4 mg concentrations, indicating an optimum at 1 mg concentration (Table 1). However, commercial tenderizer had a tendency to increase in activity up to 4 mg. At 1 mg of enzyme

Fig. 4. SDS-PAGE analysis of myofibrillar proteins treated by dry or liquid PPED with different incubation times (A) and concentrations (B). (A) Myofibrillar protein extracts (10 mg/mL) were treated with PPED for different incubation times (0–24 h, 1 mg/mL) or (B) with different concentrations (0–4 mg/mL) for 12 h at 37°C. Lane M is a protein standard marker. MHC, myosin heavy chain; α, α-actinin; A, actin; Tn.T, Troponin; Tm, tropomyosin; LC1 and LC2, myosin light chains. Commercial product is bromelain.

Fig. 5. Proteolytic activities of dry PPED, fig, and kiwifruit proteases on casein and myofibrillar protein. (A) PPED, fig or kiwifruit protease, and commercial meat tenderizer were analyzed at various concentrations in 10 mM phosphate buffer with succinylated casein (pH 6.5). (B) PPED was mixed with fig protease (1:1, v/v) or kiwifruit protease (1:1, v/v) or both fig and kiwifruit proteases (1:0.5:0.5, v/v/v) and assayed with succinylated casein. Values are means $\pm$ SD ($n=3$). Different letters (a-f) of each indicate significant differences within concentrations of each protease (A) and within all samples (B) at $p<0.05$ by DMR test. (C) SDS-PAGE analysis of myofibrillar proteins (10 mg/mL) treated by proteases (1 mg/mL) individually or in combination. Lane M is a protein standard marker. Lane N is myofibrillar proteins without treatment, lanes CM, P, F, and K are myofibrillar proteins treated with commercial tenderizer, pear, fig, or kiwifruit protease, respectively. Lanes P/F, P/K, or P/F/K are myofibrillar proteins treated with a combination of proteases, pear and fig (1:1), pear and kiwifruit (1:1), or pear, fig, and kiwifruit (1:0.5:0.5), respectively.

treatment, dry protease showed a 1.7 times higher proteolytic activity than liquid protease, while it had a 3 times lower activity than commercial tenderizer. Here, the commercial tenderizer containing bromelian exhibited continuous hydrolysis which may be due to the presence of pyrophosphate which has been suggested to causes loosening and dissociating of the myofibrillar structure (26).

Hydrolysis of meat myofibrillar and collagen proteins has been considered as the principle basis of meat tenderization and can be used as an indicator for meat tenderness (26). In this study,

myofibrillar proteins were extracted from beef round, and time-course and dose dependent hydrolysis was conducted with PPED and commercial meat tenderizer (Fig. 4). Based on apparent molecular masses reported in the literature (19), the major bands in the myofibrillar proteins were myosin heavy chain (223 kDa), α -actinin (103 kDa), actin (42 kDa), tropomyosin (33 kDa), troponin T (28-35 kDa), and myosin light chains (23-25 kDa). Myofibrillar protein extracts (10 mg/mL) were treated with PPED for different incubation times (0-24 h, 1 mg/mL) or with different concentrations (0-4 mg/

mL) for 12 h at 37°C. Myofibrillar proteins exhibited significant hydrolysis at 12 h incubation by both dry and liquid PPED, resulting in degradation of myosin heavy (223 kDa) and light chains (23-25 kDa). Most of the myofibrillar proteins were hydrolyzed at 24 h as shown in Fig. 4A. This finding with 12 h incubation time is longer than that of another report with a 6-12 h incubation time for optimum hydrolysis, which may have been due to pH differences during hydrolysis (10).

For protease concentrations to achieve desirable tenderness level, 1 mg of pear protease was effective at hydrolyzing myofibrillar proteins, specifically myosin and actin. With a commercial meat tenderizer, myofibrillar proteins were drastically hydrolyzed at 0.4 mg concentration and broken down indiscriminately (Fig. 4B). The indiscriminate hydrolysis of myofibrillar proteins by bromelain with pyrophosphate in the tenderizer may be continuous as presented with casein previously (Table 1).

Optimum combination of PPED with other proteases PPED (dry), fig, and kiwifruit proteases (0.1-4 mg/mL) were assayed with casein or beef myofibrillar proteins individually or combined to assess the proteolytic activity (Fig. 5). In respect to proteolytic activity with casein, all proteases, except the commercial, showed increased activities with increased concentrations up to 1 mg/mL, with no significant differences greater than 1 mg/mL, suggesting an optimum concentration at 1 mg (Fig. 5A). Among proteases at 1 mg concentration, PPED showed 46, 36, and 30% lower proteolytic activity than fig protease, kiwifruit protease, and commercial tenderizer, respectively. These findings are in agreement with previous reports that crude pear extract (20 µM) showed a lower hydrolytic activity than fig (22 µM) and kiwifruit protease (59 µM) on casein (11,13).

Recently, pear protease was reported as the ideal meat tenderizer to prevent indiscriminate break down or mushy spots in meat compared to fig and kiwifruit proteases (10,29). In this study, pear protease, which exhibits a mild tenderizing effect, was mixed with highly active proteases to investigate concentrations that produce desirable tenderization. PPED was mixed with fig protease (1:1, v/v) or kiwifruit protease (1:1, v/v) or both fig and kiwifruit proteases (1:0.5:0.5, v/v/v) and assayed with casein (Fig. 5B). Among the combinations of proteases, the PPED and kiwifruit mixture showed higher proteolytic activity than the PPED and fig mixture or the three proteases mixture. At optimum concentration (1 mg), PPED and kiwifruit combination exhibited 158 or 120% higher activity than PPED and fig combination or the three proteases combination, respectively (Fig. 5B). Specifically, the PPED and kiwifruit combination showed almost similar caseinolytic activity as with kiwifruit protease only.

Myofibrillar proteins (10 mg/mL) treated by purified proteases (1 mg/mL) individually or together and their proteolytic patterns are shown in the SDS-PAGE analysis (Fig. 5C). Among the individual proteases, kiwifruit protease showed almost complete degradation of myosin heavy and light chains while PPED was specific for the myosin heavy chain. For hydrolysis of myofibrillar proteins with

combined proteases, the PPED and kiwifruit mixture was the most effective protease with hydrolyzed myosin and actin (Fig. 5C). Consistent with casein hydrolysis data, the three proteases combination was more effective at degrading myofibrillar proteins than the PPED and fig combination but not as effective as the PPED and kiwifruit mixture. This data was similar to results found by Kim *et al.* (10), in which pear extract and kiwifruit extract combination (1:1, w/w), showed similar actomyosin degradation extent compared to kiwifruit extract alone.

In conclusion, purified pear protease was efficiently stabilized by the addition of 5% dextrin and further functionally characterized with respect to pH activity, thermodynamics, and enzyme kinetics. Purified and stabilized pear protease (PPED) was found to have very good proteolytic activity on casein and beef myofibrillar proteins, especially in combination with purified kiwifruit protease. It is suggested that PPED and kiwifruit proteases could be complementary to be a desirable combination for meat tenderizing applications.

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