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Contributed Paper

Antioxidant and Gelatinolytic Activities of Papain from Papaya Latex and Bromelain from Pineapple Fruits

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ABSTRACT

Papain and bromelain, the protease enzymes from papaya (*Carica papaya*) latex and pineapple (*Ananas comosus*) fruits were extracted and purified by the simple precipitation method, with the percentage yields of 16.76 and 0.97%w/w and the molecular weight of 23 and 25 kDa as same as their standards, respectively. The standard papain exhibited higher free radical scavenging activity than the standard bromelain of 400 times. The extracted papain gave lower free radical scavenging activity than the standard papain of 560 times. Both the standard and extracted papain inhibited the lipid peroxidation similar to the standard vitamin C and E, while both the standard and extracted bromelain did not show this activity. All protease enzymes at 25 µg/ml did not only show any cytotoxicity by the sulforhodamine B assay, but exhibited an interesting relative MMP-2 stimulation by zymography on human skin fibroblasts. The extracted papain gave the highest MMP-2 stimulatory activity at 2.10±0.1 folds of the control which were higher than the standard papain, the standard and extracted bromelain of 1.04, 1.31 and 1.24 times, respectively. This study has suggested that the extracted papain from plant can be further developed for the treatment of keloids and hypertrophic scars.

Keywords: papain, bromelain, antioxidant activity, cytotoxicity, MMP-2 stimulation

1. INTRODUCTION

Proteases are the group of enzymes that can break the long chain molecules of proteins into shorter fragments (peptides) and eventually into amino acids. Their applications are used to degum silk goods, manufacture liquid glue, prepare cosmetics and detergents and use as growth promoters. Also, they are widely used as meat tenderization and defibrinating agents. The major source of proteases are microorganisms such

as *Bacillus subtilis megatherium* [1] *Pennicillium chrysogenum* [2] and *Tannerella forsythia* [3]. Another source of proteases is from plants which governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. However, protease from plants are lack of hypersensitivity which is different from that of microorganisms. The protease from plants which has been supported by several researches are papain from latex of the papaya fruit peels and bromelain from the flesh of pineapple fruit. These enzymes have been used in several therapeutic properties including cleaning wounds from necrotized tissue, accelerating post-operation scar tissue resorption and debriding small residual traces (hypertrophic and keloid scars) after plastic operations, preventing aggregation of human blood platelets and proteolysis of collagen [4]. Thus, protease enzymes which have antioxidative and collagenolytic activity are widely used in dermatology for wound treatment. Hyperproliferative diseases of the skin such as keloids and hypertrophic scars are from skin alteration due to injuries. They are cutaneous abnormalities that can be characterized by the excessive accumulation of extracellular matrix (ECM) protein, especially collagen. Moreover, there is evidence that immunological and biochemical changes are associated with thermal injury, including pyridinoline crosslinks which are present in large quantities in hypertrophic scar. It has been reported that free radicals are assosciated with the formation of pyridinoline [5]. The antioxidant would be beneficial for treating scarring by free radical scavenging. Collagen degradation will result in the flatten and reducible size of the collagenous nodules. The protease enzymes which are the family of matrix metalloproteinase (MMPs) and involved in extracellular matrix (ECM) degradation have been shown to play the key role in collagenolytic activity. The hypertrophic scars and keloids are associated with the reduction of MMPs. The expression of MMPs was reduced in psoriatic keratinocytes with hyperproliferative keratinocytes [6]. Among MMPs, MMP-2 has been described to play an important role in the final degradation of the fibrillar collagens after the initial cleavage by collagenase. Purification of papain from papaya latex has traditionally been achieved by the simple precipitation method which is the cost- and time-saving extraction process. The aim of this study was to investigate and approve the biological activities including antioxidant, cytotoxicity on human skin fibroblast and gelatinolytic activity on MMP-2 of the extracted papain and bromelain by this method, but not to modify the extraction methods of the enzymes. The results from this study can be used to evaluate these protease enzymes for hypertrophic scar or keloid treatment.

2. MATERIALS AND METHODS

2.1 Materials

Papaya (*Carica papaya*) fruits and pineapple (*Ananas comosus*) fruits were harvested in Chiang Mai, Thailand. The standard papain (catalog #P3375, 1.5-3.5 U/mg, in the form of the crude latex granules), bromelain, acrylamide (minimum 99%) and glycerol were purchased from Sigma Chemicals Co., St Louis, MO, USA. Ammonium sulfate and glycine from BDH Limited Poole, England and trifluoroacetic acid and sodium chloride from Merck, Germany were used. Tris (hydroxymethyl) methylamine was purchased from Fisher Scientific UK Ltd., UK. N, N'-methylenebisacrylamide and TEMED (N,N,N',N'-tetramethyl ethylenediamine)

from Fluka, USA, sodium dodecyl sulfate and coomassie brilliant blue R-250 from Bio-Rad Laboratories, UK, and bromophenol blue dye and ammonium persulfate from Amersco Inc., USA were used. Ethanol, methanol, acetone, gracial acetic acid and acetronitrile were analytical grade solvents.

2.2 Preparation of the Crude Extracts Containing Protease Enzymes

The crude extract containing papain, papaya latex was obtained from the peel of the unripe papaya fruits by making 1-2 mm deep longitudinal incusions on the fruit surfaces using a stainless steel knife. The exuded latex was collected into a glass container and stored at -20°C until use.

For the crude extract containing bromelain, the stalk (central core) of the ripe pineapple fruit was separated from the fleshy fruits. The flesh of the fruit portion was then cut into small pieces and crushed with a blender. The juice was filtered through a cloth to remove the fibrous materials. The filtrate was centrifuged (Hettich : Universal 32 R, UK) at 10,000 × g for 10 min to remove insoluble materials. The obtained clear supernatant was filtered again through the Whatman filter paper No.5 and stored at 4°C until use.

2.3 Partial Purification of the Protease Enzymes by Precipitation

Papain was separated from the papaya latex by precipitation with the solution containing 95% ethanol (papaya latex/ethanol = 1:3 weight ratio), sodium chloride (papaya latex/NaCl = 3:1 weight ratio) and saturated ammonium sulfate (papaya latex/$(NH_4)_2SO_4$ = 2.5:1 weight ratio) [7]. For bromelain, it was seperated by the slow addition of 95% ethanol, cooled (4°C) acetone and the saturated ammonium sulfate at the volume ratio of 1:1 with constant stirring. The stirring was continued for 30 min to allow the equilibration between the dissolved and aggregated protein. Then, the precipitated enzymes were collected by centrifugation at 10,000×g for 15 min. The resulting precipitate was lyophilized to get the dry powder. The percentage yields as well as the physicochemical properties of the extracted enzymes including solubility and chemical stability comparing to the standard enzymes were investigated. The solubility tests of the protease extracts were performed by a modified method of European Pharmacopoeia. Seven kinds of solvents were examined which were hot water (80±2°C), cold water (25±2°C), ethanol, methanol, propylene glycol, glycerol and mineral oil. The word "soluble" indicated the substance dissolved in the solvent with clear solution but no cloudy and precipitate. The chemical solutions including strong acid (hydrochloric acid: HCl), weak acid (acetic acid: CH_3COOH), strong base (sodium hydroxide: NaOH), weak base (ammonium hydroxide: NH_4OH), reducing agent (ferric chloride: $FeCl_3$), oxidizing agent (hydrogen peroxide: H_2O_2) and acid salt (sodium acetate: CH_3COONa) were added drop by drop until the color change or precipitate was observed in the enzyme solution. The enzymes were stored at 4°C.

2.4 Qualitative and Quantitative Analysis of the Protease Enzymes

High performance liquid chromatography (HPLC) analysis. The qualitative and quantitative analysis of the protease enzymes were performed by HPLC (AS 1000, Thermo Finigan, USA) using the reversed-phase column (Gemini-NX, 5μ C18 110A, 4.6×250 mm, Phenomenax, USA). The mobile phase for papain was acetonitrile/

distilled water (7:3, v/v) containing 0.05% trifluoroacetic acid, whereas that for bromelain was acetonitrile/distilled water (55:45 v/v) containing 0.1% trifluoroacetic acid delivered at 1 ml/min. An amount of 20 µl of the injection volume of the enzyme in distilled water (autosample, AS300, Thermo Finnigan, USA) was eluted in the column and monitored at 230 and 280 nm UV-detector for papain and bromelain, respectively. All samples were filtered through the 0.45-µm membrane filter prior to injection onto the HPLC column.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Various concentrations of the protease enzymes dissolved in electrophoresis loading-buffer (60 mM Tris-HCl, pH 6.8, with 10% glycerol and 2% SDS containing 25% bromophenol blue solution) were loaded on the gel consisting of the 15%w/v separating gel and 4%w/v stacking gel. The electrophoresis buffer was composed of 25 mM Tris base, 192 mM glycine and 0.1%SDS at pH 8.3. Electrophoresis was performed in the Mini-PROTEAN® 3Cell (Bio-Rad Laboratories, UK) at 80 mV constant voltage until the bromophenol dye front reached the bottom of the gel. The gel was stained with coomassie blue G-250 solution for 30 min and put in the destaining solution overnight at room temperature (27±2°C) to visualize the peptide bands. Electrophoretic data were documented by a gel documentation system (Bio-Rad Laboratories,UK) and analysed by Quantity 1-D analysis software. The bands of the enzymes which appeared in the gel were compared at the molecular weight (MW) with the protein marker and the standard enzymes. The area and intensity of all bands were calculated and the percentage purity of the enzymes was determined from the standard enzyme concentrations.

2.5 Biological Activities

2.5.1 Free radical scavenging assay

Free radical scavenging activities of the extracted enzymes, the standard antioxidant (vitamin C) and the standard enzymes were determined by a modified DPPH assay. Briefly, 50 µl of five serial concentrations of the extracted enzymes or the standard enzymes (0.001-10 mg/ml) dissolved in distilled water and 50 µl of ethanol solution of DPPH were put into each well of a 96-well microplate (Nalge Nunc International, NY, USA). The reaction mixtures were allowed to stand for 30 min at 27±2°C, and the absorbance was measured at 515 nm by a well reader (Bio-Rad, model 680 microplate reader, USA) against the blank (95%v/v ethanol). Vitamin C (0.001-10 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of radical scavenging activity were calculated as follows: scavenging (%) = $[(A-B)/A]\times100$, where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% of scavenging (SC_{50}) were calculated from the graph plotted between the percentages of scavenging and sample concentrations.

2.5.2 Lipid peroxidation inhibition activity

The antioxidant activity of the extracted enzymes was assayed by the modified Ferric-thiocyanate method. An amount of 50 µl of five serial concentrations of the extracted enzymes and the standard protease enzymes (0.01-100 mg/ml) dissolved in distilled water was added to 50 µl of linoleic acid in 50%v/v DMSO. The reaction was initiated by the addition of 50 µl of NH_4SCN (5 mM) and 50 µl of $FeCl_2$ (2 mM). The

mixture was incubated at 37±2°C in a 96-well microplate for 1 h. During the oxidation of linoleic acid, peroxides are formed leading to the oxidation of Fe^{2+} to Fe^{3+}. The latter ions forming a complex with thiocyanate can be detected at 490 nm. The solution without the sample was used as a negative control. Vitamin C and E (0.001-10 mg/ml) were used as positive controls. All determinations were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated by the following equation: Inhibition of lipid peroxidation (%) = [(A-B)/A] ×100, where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% inhibition of lipid peroxidation (IPC_{50}) were calculated from the graph plotted between the percentages of lipid peroxidation inhibition and sample concentrations.

2.5.3 Normal human fibroblast cytotoxicity by SRB assay

2.5.3.1 Cell cultures

The human skin fibroblast was obtained from the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand. The cells were maintained as adherent cells in T75 culture flasks at 37°C in a humidified incubator containing 5%CO_2 (Shel Lab. Model 2123TC, USA). The Dulbecco's modified Eagle's medium (DMEM) which was supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). For subculturing, cells were rinsed with phosphate buffer saline (pH 7.4) and finally detached with 0.25% trypsin-EDTA.

2.5.3.2 Cytotoxicity assay

Cells were seeded in 96-well plates at an amount of 1×10^4 cells/well and allowed to incubate overnight. Then, cells were exposed to various concentrations of the enzyme for 24 h. After incubation, the adherent cells were fixed by adding 50 µl of cold 50%w/v trichloroacetic acid and incubated for further 1 h at 4°C. Then, the cells were rinsed five times with distilled water, air-dried and stained with 50 µl of 0.4% SRB in 1% glacial acetic acid for 30 min at room temperature (27±2 °C). The unbound SRB was removed by washing with 1% glacial acetic acid solution for four times. After air-drying, 100 µl per well of 10 mM Tris base were added to dissolve the bound dye. After mixing, the absorbance was measured at 540 nm with a microplate reader (Biorad, Milan, Italy). The cells with no treatment were used as the negative control. The assays were done in three independent separate experiments. Cell viability (%) was calculated using the following equation

$$\text{Cell viability (\%)} = (\text{Absorbance}_{\text{treated}}/\text{Absorbance}_{\text{control}}) \times 100$$

2.5.4 Gelatinolytic activity (zymography) on MMP-2

The samples (25 µg/ml) which showed no toxicity on human skin fibroblasts (from section 2.5.3.2) were tested for gelatinolytic activity of MMP-2 stimulation in comparing to concanavalin A. The cells were seeded in 6-well plates at an amount of 5×10^5 cells/well. The monolayer of cells was maintained in the culture medium without FBS for 24 h, treated with the samples and incubated for 72 h. The culture supernatants were collected to assess the gelanolytic activities of MMP-2 in the culture media.

SDS-PAGE zymography using gelatin as a substrate was performed according to the method previously described with some

modification [8]. Briefly, the cell culture supernatant was suspended in the loading buffer (0.125 M Tris (pH 6.8), 4% SDS and 0.04% bromophenol blue, without prior denaturation) and run on the 10% SDS polyacrylamide gel with the presence of 0.1%w/v gelatin. After electrophoresis, gels were washed to remove SDS and incubated for 20 min in the renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, 2.5% Triton X-100). The gels were then incubated for 24 h at 37°C in the developing buffer (50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100). Gels were subsequently stained with 0.5% Coomassie brilliant blue G-250 and de-stained in 30% methanol and 10% v/v acetic acid at room temperature (27±2 °C) to visualize the bands, and the gelanolytic activity was detected as a white band against a blue background. Electrophoretic data was determined by gel documentation system (Bio-Rad Laboratories,UK) and analysed by the Quantity 1-D analysis software. The area multiplied by intensity (mm^2) of the bands on the gel was determined as the relative MMP-2 content. The MMP-2 stimulation in comparing to the control (the untreated systems) was calculated by the following equation:

$$\text{Relative MMP-2 stimulation} = \text{MMP-2 content}_{sample} / \text{MMP-2 content}_{control}$$

The assays were done in three independent experiments. The potency of MMP-2 stimulation of the samples was compared with concanavalin A.

2.6 Statistical Analysis

The results were presented as the mean ±SD of three independent experiments. ANOVA was used for the analysis of the test results (LSD test) at the significance level of p-value < 0.05.

3. RESULTS AND DISCUSSION

3.1 Characteristics of the Enzymes

Although the purification of the protease enzyme by the traditional precipitation gave the purified enzyme with the contamination of other proteases, it was the easy and low cost method. In this study, the papain and bromelain were extracted by the simple precipitation with 95%ethanol and the saturated ammonium sulfate which gave the percentage yield of 16.76 and 0.97%, respectively. The low percentage yield of the extracted bromelain may be due to the high water contents (87%) of the pineapple juice. After freeze dried, the extracted papain and bromelain were in white and yellow powder, respectively. The MW of the extracted enzymes were compared with their standards and the protein marker by SDS-PAGE as shown in Figure 1. Figure 1A indicated the molecular weight of the papain at about 23 kDa [7]. The lower bands might be from other proteases such as chymopapain, caricain and glycyl endopeptidase [7]. The band of papain (upper band) showed smaller area than other proteases (lower band). It has been reported that papain is a minor constituent among the papaya proteases [4]. For bromelain, its molecular weight was about 25 kDa (Figure 1B) [9]. From the band of the enzyme on the SDS-PAGE, the purity of papain and bromelain was 77.68 and 44.95%, respectively. The tentative identification of the enzyme can be determined by the comparison of the retention times between the standard and the extract chromatograms [10]. The retention times of the extracted enzyme were similar to those of the standard enzymes (Figure 2). The extracted papain and bromelain demonstrated the major peaks with the retention times at 1.660 and 1.583 min, while their standard enzymes were at 1.658 and 1.600 min, respectively.

The major peaks of papain and bromelain demonstrated the retention times at 1.660 and 1.583 min, while the minor peaks of the retention times at more than 2 min were of other proteases from the extracted enzymes. The purity of papain and bromelain of the extracted enzymes determined by HPLC were 82.31 and 38.03%, respectively which were close to those determined by the SDS PAGE method.

The physicochemical properties including solubility and stability in chemicals (strong acid, weak acid, strong base, weak base, reducing agent, oxidizing agent and acid salt) of the extracted enzymes were similar to their standards. All enzymes were soluble in distilled water but insoluble in alcohol and organic compounds such as glycerin and propylene glycol. The color change or precipitate in the enzyme solution indicated the chemical instability of the extracted enzymes. The extracted enzymes were less chemical stable than the standard enzymes. For papain, the extracted enzyme was unstable to strong acid, weak acid, reducing agent and acid salt, whereas the standard enzyme was stable to all reagents except the strong acid. For bromelain, the extracted enzyme was unstable to strong acid, strong base and reducing agent, whereas the standard enzyme was unstable to strong base and reducing agent. This may be due to the effects from several other compounds in the extracted enzymes in accordance with many protein bands on the SDS-PAGE gel and the multiple peaks on the HPLC chromatogram. However, papain and bromelain are expected to be stable in the HPLC and SDS-PAGE conditions which have the pH values of 3.2-3.5 and 6.8, respectively. In fact, papain has been reported to be stable at pH 5.0 and becomes unstable at pH lower than 3.0 and above 11 [11]. Bromelain is stable at pH 3.0-6.5 [12].

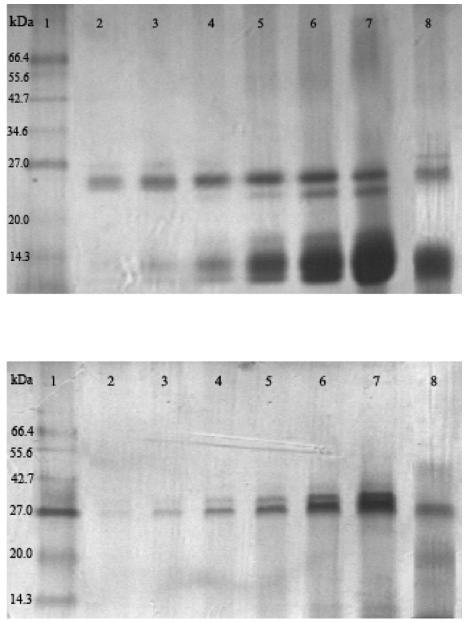

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the extracted papain (a), extracted bromelain (b) in comparing to the molecular weight marker (lane 1) and their standards at 1 mg/ml (lane 8); lanes 2-7 were the enzymes at 0.156, 0.312, 0.625, 1.25, 2.5 and 5 mg/ml, respectively.

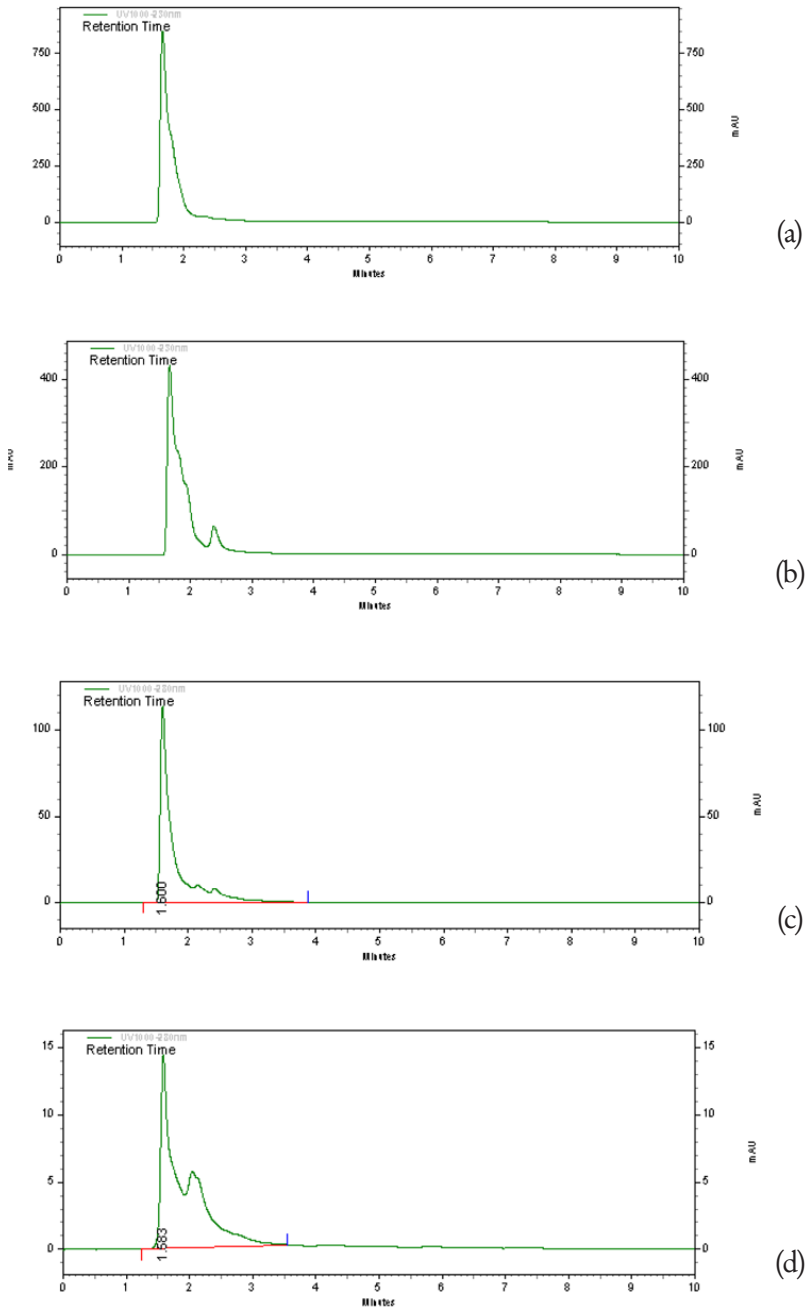

Figure 2. The HPLC chromatograms of the standard papain (a), extracted papain (b), standard bromelain (c) and extracted bromelain (d).

3.2 Biological Activities of Protease Enzymes

3.2.1 DPPH radical scavenging activity

Antioxidant activity of the enzymes determined by DPPH radical scavenging was shown in Table 1. The SC_{50} value of the standard papain (1.71±1.45 mg/ml) was lower indicating of higher activity than the extracted enzyme (956.10±62.06 mg/ml) of 560 times. The crude latex of the papaya fruit contains the mixture of the cysteine proteases including papain, chymopapain, glycyl endopeptidase and caricain, which has more than 80% of the whole enzyme fraction. Papain which is the major component of the papaya latex has been extensively studied for antioxidant activity [13]. The high purity of papain may give the high antioxidant activity. The previous study has reported that the papain extracts from Korean elk velvet antler showed antioxidative activity by scavenging free radicals [14]. The standard bromelain gave the SC_{50} value of 684.27±100.13 mg/ml, whereas the extracted bromelain showed no activity. The standard papain and bromelain enzymes gave lower activity than the standard vitamin C (SC_{50} = 0.0023±0.0001 mg/ml). The DPPH radical scavenging activity of the enzymes may be from the amino acid contents which can scavenge the DPPH radical by donation of the hydrogen atom to form a stable DPPH-H molecule. The standard papain indicated higher scavenging activity than the standard bromelain of 400 times owing to the contents of amino acids and peptides, that 20 amino acids are found in the enzymes have the potential to interact with the free radicals. Also, the amino acids in the enzymes which including Tryptophan (W), Tyrosine (Y), Methionine (M), Cysteine (C), Histidine (H) and Phenylalanine (F) have been proposed to contribute to antioxidant activity and be important free radical scavengers in the natural peptides [15-16]. The numbers of these amino acids in papain (39) was more than bromelain (35) of 4 (Figure 3) [17]. The hydrogen atom donation by the amino acids in papain may be more than that in bromelain resulting in high DPPH radical scavenging. The impurities of the extracted papain may cause lower scavenging activity than the standard enzymes [12]. The extracted bromelain which gave low percentage of purity (38-44%) did not show any scavenging activity.

Table 1. Antioxidant activities of the extracted enzymes in comparing to the standards.

Sample	%yield	%purity		SC_{50}[a] (mg/ml)	IPC_{50}[b] (mg/ml)
		SDS-PAGE	HPLC		
Standard papain	ND	ND	ND	1.71±1.45*	0.05±0.003
Extracted papain	16.76±5.74	77.68±4.56	82.31±5.22	956.10±62.06*	0.38±1.02
Standard bromelain	ND	ND	ND	684.27±100.13*	-
Extracted bromelain	0.97±0.16	44.95±3.88	38.03±2.04	-	-
Vitamin C	ND	ND	ND	0.0023±0.001	0.05±0.01
Vitamin E	ND	ND	ND	ND	0.32±0.10

Note : - = no activity, ND = not determine, values represent mean ± S.D. (n=4).

[a]SC_{50} = scavenging concentration at 50% activity (mg/ml).

[b]IPC_{50} = inhibition peroxidation concentration at 50% activity (mg/ml).

* significant difference ($p < 0.05$) in comparing to the standards

1 IPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGIIKIRTGNLNEYSEQEILDCDRRS 60
61 YGCNGGYPWSALQLVAQYGIHYRNTYPYEGVQRYCRSREKGPYAAKTDGVRQVQPYNEGA 120
121 LLYSIANQPVSVVLEAAGKDFQLYRGGIFVGPCGNKVDHAVAAVGYGPNYILIKNSWGTG 180
181 WGENGYIRIKRQTQNSYGVCGLYTSSFYPVKN 212

(a)

1 AVPQSIDWRDYGAVTSVKNQNPCGACWAFAAIATVESIYKIKKGILEPLSEQQVLDCAKG 60
61 YGCKGGWEFRAFEFIISNKGVASGAIYPYKAAKGTCKTDGVPNSAYITGYARVPRNNESS 120
121 MMYAVSKQPITVAVDANAAFQYYKSGVFNGPCGTSLNHAVTAIGYGQDSIIYPKKWGAK 180
181 WGEAGYIRMARDVSSSSGICGIAIDPLYPTLEE 212

(b)

Figure 3. The amino acid sequences of papain (a) and bromelain (b). The beginning and the end of every part of the sequence were numbered [38].

3.2.2 Lipid peroxidation inhibition activity

The standard papain and the extracted papain showed lipid peroxidation inhibition at the IPC_{50} values at 0.05±0.003 and 0.38±1.02 mg/ml, respectively (Table 1). The standard papain exhibited no significant differences ($p < 0.05$) lipid peroxidation inhibition activity in comparing with vitamin C (0.052±0.01 mg/ml), while the extracted papain showed similar activity to vitamin E (0.324±0.099 mg/ml). However, both standard bromelain and bromelain extract did not give any lipid peroxidation inhibition activity. Infact, this activity might be from the hydrophobic amino acids which can inhibit the peroxidation formation [18]. In the amino acid sequence of the enzymes (Figure 3), 35% hydrophobic amino acids in papain can exert high affinity to the linoleic acid. Thus, papain may interact with the lipid molecules and scavenge lipid peroxyl radical by donating proton to lipid-derived radicals. In addition, the position of the hydrophobic amino acids, Leucine (L) at the N-terminal and Proline (P), Histidine (H) or Tyrosine (Y) in the peptide sequences have been reported to have high potent lipid peroxidation inhibitory activity [19-20]. The numbers of these amino acids in papain (31) were 5 more than in bromelain (26) (Figure 3). Therefore, it is possible that these specific amino acid residues in the enzymes may play an important role in increasing the interaction between the peptides and the fatty acids (linoleic acid) in the lipid peroxidation inhibitory assay. The standard papain exhibited better lipid peroxidation inhibition activity than the extracted papain. Similar to free radical scavenging activity, the impurity of the extracted enzyme may cause this effect [12].

3.3 Cytotoxicity of Protease Enzymes

Various concentrations of the enzymes were investigated for normal human fibroblast cytotoxicity by the SRB assay. When the concentrations of the enzymes increased from 10^{-9} to 100 μg/ml, cell viability was decreased. All samples at the concentration range of 10^{-9} to 25 μg/ml gave cell viability of more than 85% with no significant difference at $p < 0.05$. At high concentration (100 μg/ml), the standard papain, extracted papain, standard bromelain

and extracted bromelain showed % cell viability at 16.17, 15.61, 19.24 and 29.95, respectively (Table 2). Since positively charges in the basic amino acids are also related to cytotoxicity, papain which has more number of basic amino acids (24) showed higher cytotoxicity than bromelain which has less number of basic amino acids (21). When human skin contacted with papain at 0.2%w/v for 24 hours, the large amount of the intercellular material in the stratum corneum was lost and most of the extracellular components were digested [21]. Nakauchi et al. [22] have reported that the basic amino acids including Lysine (K), Arginine (R) and Histidine (H) were apparently severe damage in human retinal pigment epithelial cells. The cytotoxic effects of the extracted enzymes were less than those of the standard enzymes. This was possibly from the protective effect of some compounds such as glycoprotein [23]. These compounds may have the protective effect on cell viability by the steric hindrance around the cells, thereby decreasing the membrane permeability and resulting in the reduced cytotoxicity of the extracted enzymes in comparison to the standard enzymes. The highest concentration of the standard papain, extracted papain, standard bromelain and extracted bromelain that gave cell viability of more than 85% which were 85.95±12.35, 95.14±7.39, 90.82±10.88 and 88.21±11.30%, respectively was observed at 25 μg/ml. Thus, this concentration of all enzyme samples was selected for gelatinolytic activity on the MMP-2 study.

Table 2. The percentages of cell viability on human skin fibroblast by the SRB assay of the protease enzymes.

Concentration of enzymes (μg/ml)	%cell viability			
	Standard papain	Extracted papain	Standard bromelain	Extracted bromelain
1×10^{-9}	104.48±0.61	110.08±2.73*	105.53±2.39	106.98±3.82
6.25	101.41±6.189	106.30±2.07	100.96±2.68	102.60±3.00
12.5	4.80±3.278	99.85±1.78	94.65±7.46	99.47±7.20
25	5.95±12.354	95.14±7.39*	90.82±10.88	88.21±11.30
50	8.96±8.971	65.10±8.27*	59.95±4.77	63.48±0.52
100	6.17±0.28	15.61±0.35	19.24±0.42	29.95±1.77*

Note : Cell viability (%) = ($absorbance_{test\ cell}$/ $absorbance_{control\ cell}$) ×100, * significant difference ($p < 0.05$) in comparing to their standards

3.4 Gelatinolytic Activity (Zymography) on MMP-2

Figure 4 showed the relative MMP-2 stimulation by zymography of the enzymes at 25 μg/ml in comparing to the negative (untreated cells) and positive (concanavalin A) control systems. The cells treated with the standard papain, extracted papain, standard bromelain and extracted bromelain indicated the relative MMP-2 stimulation of 2.01±0.14, 2.10±0.14, 1.63±0.27 and 1.71±0.12, respectively. However, the MMP-2 stimulatory activity of all enzymes was lower than that of concanavalin A (2.59±0.22) of 1.29, 1.23, 1.59 and 1.51 times, respectively. Infact, it is still unknown for the protease enzyme mechanism on the MMP-2 stimulation. One possible mechanism is that the protease may induce collagenase secretion [24]. Werb et al. have

demonstrated that papain has higher proteolytic activity and gives more amount of the secreted collagenase in the fibroblast than bromelain. Thus, papain gave higher stimulatory activity than bromelain. Also, the pH of our system (pH 7.0) may affect the activity, as papain has an optimum activity at pH 5-8 [4], while bromelain has high activity at an optimum pH at 4.5-5.5 [25]. The pH of the system appeared to be favorable for papain. The extracted enzymes showed slightly higher stimulatory activity than the standard enzymes but no significant difference ($p > 0.05$), although the purity of both enzymes was different. This may be due to the addition effects from other protease enzymes existing in the extract such as chymopapain, glycyl endopeptidase and caricain in papaya latex [4, 7], whereas comosain and ananain were found in pineapple [26].

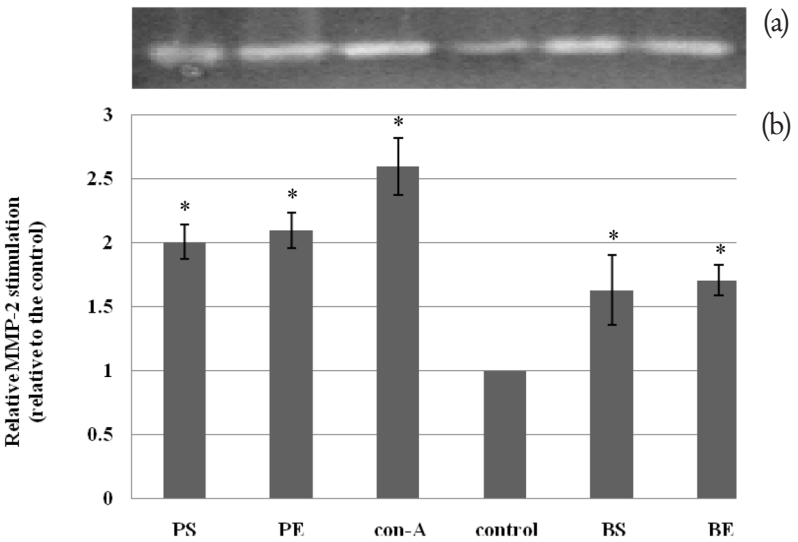

Figure 4. Gelatinolytic activity of the protease enzymes (25 μg/ml) in comparing to the control and concanavalin A (40 μg/ml). (a) zymograms and (b) MMP-2 stimulation relative to the control. PS = the standard papain, PE = the extracted papain, con-A = concanavalin A (positive control), BS = the standard bromelain and BE = the extracted bromelain.
* significant difference ($p < 0.05$) in comparing to the control.

4. CONCLUSION

This study has demonstrated that the protease enzymes (papain and bromelain) from papaya latex and pineapple fruits which gave similar collagenolytic activity to their standards can be extracted by the simple precipitation with 95%ethanol and saturated ammonium sulfate which gave the percentage yields of 16.76 and 0.97%w/w, respectively. The MW of the extracted papain and bromelain which estimated by the protein markers were 23 and 25 kDa, respectively which is the same as the MW of their standards. The purities of the extracted papain and bromelain determined by HPLC were 82.31 and 38.03% which were similar to those by the SDS-PAGE that gave 77.68 and 44.95%, respectively. The standard and extracted papain exhibited higher antioxidant activity than those of bromelain both in free radical scavenging (SC_{50}) and lipid peroxidation inhibition (IPC_{50}) activity. However, the extracted papain showed lower antioxidant

activity than the standard papain. All enzymes (standard papain, extracted papain, standard bromelain and extracted bromelain) gave lower SC_{50} values than the standard vitamin C, while the standard and extracted papain gave similar IPC_{50} values to the standard vitamins C and E. The cytotoxicity of all enzyme samples on human skin fibroblasts was dose dependent. All enzymes at the concentration range of 10^{-9} to 25 μg/ml gave cell viability of more than 85%. The extracted papain and bromelain showed the MMP-2 stimulatory activity on zymograms of 2.10 and 1.71 similar to their standards, but lower than concanavalin A. In conclusion, this study has indicated that the protease enzymes especially the extracted papain obtained from plants prepared by the simple precipitation method which is the cost- and time-saving extraction process exhibited the collagenolytic activity similar to their standards which can be further developed for keloid and hypertrophic scar treatment.

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