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Contributed Paper

A Comparative Study on Properties and Proteolytic Components of Papaya Peel and Latex Proteases

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ABSTRACT

Proteases from papaya peels were extracted with water followed by precipitation with ethanol and 57.6% yield was obtained. Their maximum hydrolysis of casein comparing to proteases from latex were similar in temperature but different in pH. Both of papaya proteases were fully activated by 5 mM cysteine, the peel enzymes were activated 1.6 times higher than latex enzymes. The peel proteases are also more stable in pH ≥ 8 and at 80°C than the latex proteases. Cathodic polyacrylamide gel electrophoresis and *in situ* proteolysis verified that papaya peel proteases are composed of papain as a major component, chymopapain, and possible two proteases which are absent in crude papain. Separation by anodic polyacrylamide gel electrophoresis and *in situ* proteolysis illustrated that proteases from papaya peels contained a protease with pI less than 8.3. Anion-exchange chromatography indicated that papaya peel proteases consisted of a number of proteins and proteases different from those found in papaya proteases.

Keywords: papaya latex, papaya peel, proteases, properties, proteolytic component.

1. INTRODUCTION

Dried *Carica papaya* latex proteases are commercially known as crude papain [1]. According to its broad specificity and thermostability [2], the enzyme has been used in several industries such as meat tenderization, beer chill-proofing, pharmaceutical applications, leather, textiles, and animal feed [3-4]. Industrially, crude papain is produced from papaya latex tapping from green fruits, which yield the maximum of latex. However, the collection of latex is laborious and time consuming. Therefore, the green fruit peels [5-6], leaves, petioles, stems, and bark [7-9] have been investigated as alternative sources of crude papain production.

Papaya latex proteases are composed of four cysteine proteases which contribute 69-89% of total protein: less than 10% papain, 26-30% chymopapain, 23-28% glycyl endopeptidase, and 14-26% caricain [2]. These four proteases have similar molecular weight of approximately 23 kDa. Therefore, it is very difficult to identify them by using SDS-PAGE [10-11]. Because all are basic proteins, native gel of cathodic polyacrylamide electrophoresis has been well recognized as a mean to investigate papaya cysteine proteases [eg. 12-14]. Their amino acid sequences have been determined both at the protein level and through sequencing corresponding cDNA

clones [15]. Their proteolytic activities are activated by additions of small reducing agent such as cysteine and a chelating agent like EDTA [1, 3]. It has been shown that proteases from latex of the fruit differ from those of the non-fruit parts [9, 16] and even from latex of newly wounded fruits [17-18]. The major component in the non-fruit enzymes is chymopapain and the proportions of other enzymes are greatly reduced from the latex proteases [9, 16]. A series of low molecular weight proteins are found in the latex obtained from newly wounded fruits [17]. Whereas, repeatedly wounded fruits accumulate and/or activate several enzymes including papain, chymopapain and caricain [18]. Recently, new cysteine protease with high hydrophobicity which appeared in the first trapped latex has been discovered [18]. Hence, diversities of papaya proteases will be achieved according to different sources.

Generally, papaya peels are discarded from home, restaurants, and industries. An accumulation of this waste can become environmental problem. However, the presence of their proteolytic activities has also been observed and crude papain could be produced from the dried peels [5-6]. Arimura studied different methods of preparation of papaya peels by drying, while Espin and Islam studied effects of adding various stabilizers before drying. Both groups refer to ground peels after drying as crude papain [5-6]. However, further enzyme extraction, property and protein component of proteases from the peels have not been studied. Each year more than 1000 tons of papaya peels from Thailand's pickle industries are discarded as waste (personal communication). Therefore, it is our interest to transform this waste into a valuable product like latex proteases. In this study their properties and protein components were determined and compared to those of dried papaya latex enzymes, which is commonly acceptable for many industrial uses.

2. MATERIALS AND METHODS

2.1 Materials

Carica papaya fruits were harvested from 70-100 days maturation of papaya trees planted in Chiang Mai, Thailand. Standard purified papain (95% purity) and chymopapain (90% purity) were purchased from Sigma (USA). Polyacrylamide, bis-acrylamide, ammonium persulfate, and tetramethylene-diamide (TEMED) were obtained from Sigma-Aldrich (USA). Ethanol, iso-propanol, methanol and ammonium sulfate were supplied by Fluka (USA).

2.2 Preparation of Papaya Peel Crude Extract

Papaya peels were prepared by peeling the fruits and cutting the peels into suitable small pieces. To increase storage time, the peels were dried at 55°C in a tray-dryer until 10% w/w was obtained. The dried peels were ground in a blender and approximately 16 mesh size of the ground were obtained. Twenty gram of the ground peels were soaked in 180 ml distilled water for 10 min. After filtration through gauze, the filtrate was centrifuged at 9000xg, 4°C for 30 min (Kubota, 6800) to obtain a clear solution of papaya peel crude extract.

2.3 Separation of Papaya Peel Proteases

Papaya peels extract contains various compounds, including cysteine proteases. These enzymes were separated out from the extract by precipitation using a method described in Lesuk [8]. Four portions of 38 ml crude extract were pre-chilled to 4°C. Each precipitants; methanol, ethanol, and 2-propanol were slowly added to obtain to final concentration 75% (v/v), 70% (v/v) and 67% (v/v), respectively. Ammonium sulfate was also added to the last portion giving concentration of 60% saturated of salt (26.2 g/100 ml solution). The solutions were stirred at 4°C for 30 min. The precipitate of proteases wereproteases dded.nol, ude extract were pre-chilled to 4able product like crude

papain. the dried peels [5-6]. separated by centrifugation at 9000xg, 4°C for 5 min and then dialyzed 6 times against deionized water. After lyophilization, the protease powders were stored at -20°C until use.

2.4 Preparation of Papaya Latex Proteases

Fresh latex was collected from 70-100 days maturation of locally grown *Carica papaya* (Hang Dong District, Chiang Mai, Thailand) and stored at -20°C until use. The latex was thawed and dried at 55°C in a tray-dryer for 1 h to obtain latex proteases [19].

2.5 Assay for Proteolytic Activity

The procedure was modified from that of Arnon [20]. Papaya proteases from the peels and latex were investigated for their optimal pH and temperature, effect of cysteine on catalytic reaction, and their stability by using casein hydrolysis. The reaction mixture containing 0.10 ml of enzyme solution, 0.30 ml of buffer solution and 0.10 ml of activating agent (40 mM cysteine - 20 mM EDTA disodium salts) was incubated at constant temperature for 5 min. The reaction was initiated by adding 0.50 ml of 1% (w/v) casein solution. After 10 min, 1.50 ml of 5% cold trichloroacetic acid was added to terminate the reaction. The supernatant of the mixture was separated by centrifugation at 9000xg for 20 min. The absorbance was measured at 275 nm.

To determine the optimal pH, the reaction was performed at 37°C by using a number of buffers at concentration of 50 mM in the range of pH 2-11: phosphate buffer pH 2, 7 and 11, citrate buffer pH 3 and 6, acetate buffer pH 4 and 5, Tris-HCl buffer pH 8 and borate buffer pH 9 and 10. Optimal temperature of enzymatic activity was also determined at various temperatures between 50°C and 90°C in buffer pH 8.

The effect of cysteine on enzyme catalysis was investigated by performing reaction at 37°C in buffer pH 8. One hundred microlitles of 20 mM EDTA disodium salts with various concentrations of cysteine were used instead of the normal activating agent.

Stability of the proteases was analyzed by incubating the enzyme at constant temperature of 37°C in 50 mM of the buffers pH 6-10 or in 50 mM of buffer pH 8 at temperatures ranging from 20°C to 80°C for 10 min. The incubated enzymes were then assayed for proteolytic activity in pH 8 at 37°C.

One unit (u) of proteolytic activity was defined as the amount of enzyme releasing the product equivalent to 1 mmole tyrosine min^{-1} at assay conditions.

2.6 Determination of Protein Content

Protein content in the samples were determined by Bradford method [21].

2.7 Electrophoresis and *in situ* Proteolytic Activity Assay

Cathodic polyacrylamide gel electrophoresis was carried out on a slab gel using Hoefer miniVE electrophoresis system (Amersham Biosciences) following a method described by Nitsawang and Kanasawud [22] which was modified from Reisfeld et al. [23]. A slab gel consisted of a resolving gel (pH 4.3, 15% w/v acrylamide) and a stacking gel of 4% acrylamide (pH 6.7). The upper and lower chamber electrode buffer consisted of 0.36 ²-alanine-0.14 M acetic acid (pH 4.5). Electrophoresis was run at a constant current 40 mA, 300 V for 1.5 h that the protein samples migrated from anode toward cathode. Anodic polyacrylamide gel electrophoresis was run at a constant 40 mA, 300 V for 60 min by using 0.025 M Tris-0.192 M Glycine buffer pH 8.3 as electrode buffer [24]. The protein samples moved towards the anode during electrophoresis.

After electrophoresis, the gel was cut into two equal parts. The first half was stained with Coomassie Brilliant Blue in a solution of acetic acid/methanol/water (1:5:4 v/v) and then destained by the same solution with a different solvent ratio of 2:3:1. The *in situ* proteolytic activity was determined in the second half by

using a method modified from that of Moutim et al. [11]. The gel was rinsed twice with buffer pH 8.0. The same buffer containing 0.5% (w/v) agarose and 1.8% (w/v) casein was then applied on the gel surface. After incubation at 37°C for 24 h, casein hydrolysis on the gel was observed.

2.8 N-terminal Analysis

Identity of proteases on the cathodic gel was determined up to the sixth residue from the N-terminal by automated Edman degradation (Callaghan, NSW, Australia). The phenylthiohydrantoin (PTH) derivatives of amino acids were separated using high performance liquid chromatography equipped with a 220 mm PTH C18 column.

2.9 Anion-exchange Chromatography

Twenty-five microliter of enzyme solution (50 μg protein) was loaded on to a Mono Q HR 5/5 column (1 ml) attached to Fast Protein Liquid Chromatography (FPLC system, Upsala, Sweden). The column was pre-equilibrated with 20 mM Glycine-NaOH buffer pH 10.6. The elution of the bound protein was performed with a linear concentration gradient of NaCl from 0-0.5 M at pH 10.6 (total volume 35 ml, flow rate 1 ml/min) followed by an isocratic elution with 1 M NaCl for 5 min. The absorbance at 280 nm of chromatographic fractions (1 ml/fraction) and their proteolytic activities were determined. The proteolytic activity of each fraction was interpreted by comparison to starting enzymatic activity before loading.

2.10 Ninhydrin Test

Ninhydrin test [25] was performed in a test tube by adding 1 ml of ninhydrin solution (0.35 g ninhydrin in 100 ml ethanol) to 2 ml of sample. After covering with paraffin, the tube was placed in a water bath 100°C for 5 min. The tube was then cooled to room temperature in a cold water bath. Cysteine, glycine, phenylalanine, tryptophan and tyrosine were used as standard amino acids.

3. RESULTS AND DISCUSSION

3.1 Separation of Papaya Peel Proteases

The results of protease separations from 5 g dried papaya peels by precipitation of proteins are shown in table 1. The precipitation of protease with 70% (v/v) ethanol provided the highest proteolytic activity of 57.61% with purification fold of 1.57. Both the proteolytic yield and purification fold were a bit decreased when the other two alcohols, 75% v/v methanol and 67% v/v 2-propanol, were used. All three precipitants have been previously used for precipitation of cysteine

Table 1. Separation of proteases from crude extract of 5 g dried papaya peels, the protease activities and proteins were compared to 1 g of dried papaya latex.

Proteases	Proteolytic activity			u/g of dried peels	Total protein (mg)	Specific activity (u/mg)
	u/g of enzyme	Total activity (u)	Proteolytic yield (%)			
Papaya peel crude extract	-	44.00	100	8.80	41.00	1.07
Precipitation by						
Methanol 75% (v/v)	152.65	22.21	50.47	4.44	15.42	1.44
Ethanol 70% (v/v)	169.79	25.35	57.61	5.07	15.09	1.68
2-Propanol 67% (v/v)	152.40	23.21	52.75	4.64	17.32	1.34
60% sat. Ammonium sulfate	116.07	16.30	37.04	3.26	14.17	1.15
Dried papaya latex (latex proteases)	1623	1623	-	-	327.7	4.95

proteases from papaya latex [8]. The precipitation with 60% saturated ammonium sulphate resulted as the lowest yield of proteases at 37.04%, although our preliminary studied showed that it efficiently separated most of proteases from clarified papaya latex (data not shown). The explanation is that papaya peels contain enzymes that are less hydrophobic on their surface. This made the salting out method less effective on these enzymes. As shown in table 1, the crude extract and enzymes from the peels contained higher ratio of non-proteolytic proteins to proteases than that of the latex; this corresponds with previous report [26]. As a consequence, specific activities of all papaya peel proteases from the four precipitations were lower than that of the latex. Furthermore, the activity per gram of proteases powder obtained from the peels were approximately 10 times lower than that of the latex enzyme (1623 u/g).

3.2 Optimal Activity of Papaya Proteases

Because of the highest yield obtained, papaya peel proteases precipitated by 70% ethanol were used for further investigation in comparison to latex proteases. Proteases from papaya peels showed maximum casein hydrolysis in buffer pH 8 (figure 1A) and at 75°C (figure 1B) which is similar to that from the latex. The difference in optimal pH between these two proteases indicates their unequivalent protein compositions. It has been reported that papain, chymopapain and caricain hydrolyzed casein at the optimal pH of 8, 7, and 8, respectively [27]. The difference in the optimal pH between proteases from papaya peels and latex is likely the result of their different enzymatic contents.

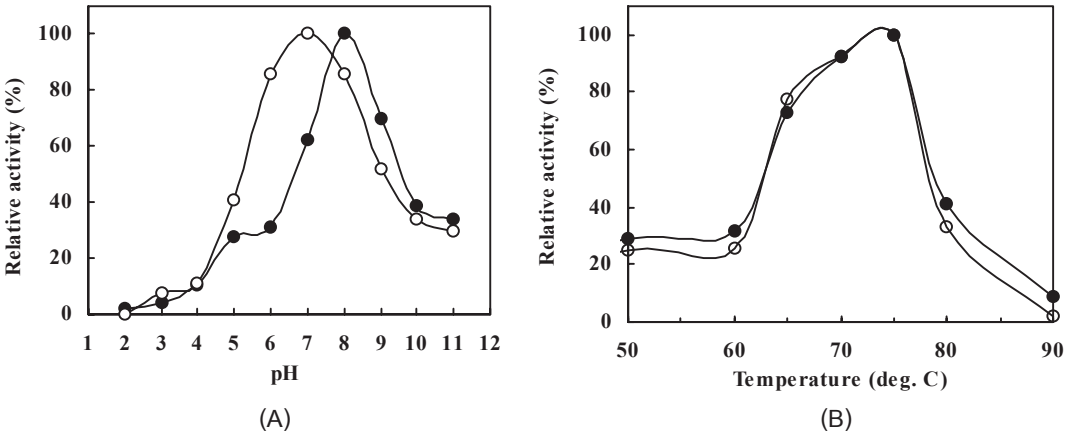

Figure 1. Optimal pH at 37°C (A) and optimal temperature in buffer pH 8.0 (B) on casein hydrolyses of papaya peel proteases (●) and latex proteases (○).

Proteases in *Carica papaya* are cysteine proteases which need small reducing agents such as cysteine to activate them before catalysis of the reaction. These reducing agents convert reversibly inactive forms of enzymes to the active forms and protect their catalyzed-essential thiol group from oxidation [1, 3]. Figure 2 shows that both proteases were maximally activated by 5 mM cysteine. The activation in the papaya peel proteases appeared to be 1.6 times greater than that of the latex enzyme, illustrating the existence of higher amount of reversibly inactive form in papaya peel proteases. The constant effect at cysteine concentration higher than 5 mM indicates that full activation of enzymes.

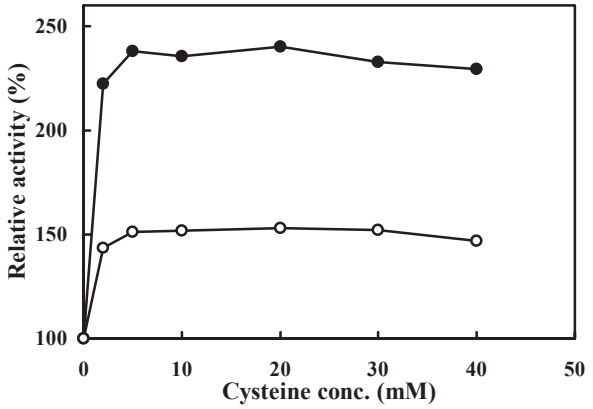

Figure 2. Effect of cysteine on caseinolytic activities of papaya peel proteases (●) and latex proteases (○) in buffer pH 8 at 37°C. The activities of the two reactions without cysteine were given as 100% relative activity.

The stability of proteases from papaya peels and latex were determined by incubation at various pH and temperatures for 10 min before measuring their activities in buffer pH 8 at 37°C. Results clearly show that the proteases from the peels were more stable in pH 9-10 than that from the latex (figure 3A). Both of proteases showed good stability up to 60°C and their activities slightly declined at 70°C, whereas 80°C, latex proteases rapidly lost their activities more than the peel proteases (figure 3B). As previous study shown chymopapain was more stable than papain and caricain, respectively [27]. Therefore, the difference in stability profiles of these two papaya proteases suggests once again that their protease compositions are unequivalent.

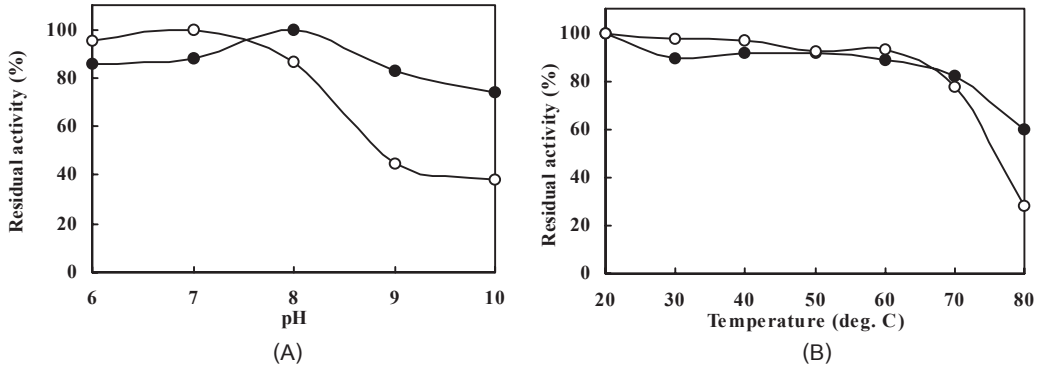

Figure 3. Stability of papaya proteases from the peels (●) and latex (○). The enzymes were incubated at various pH (A) and at various temperatures (B) for 10 min before determining their proteolytic activities in pH 8 at 37°C.

3.3 Composition of Papaya Peel Proteases

Cathodic and anodic polyacrylamide gel electrophoresis and anion-exchange FPLC were used to study the component of papaya peel proteases in comparison with the latex proteases and purified standard papain.

Mobilities of papain, chymopapain, glycyl endopeptidase, and caricain in the latex proteases separated by cathodic electrophoresis (figure 4A, lane 2) correspond to those of previous reports [9, 12-14, 22, 27-29]. It is notable that the least basic enzyme

papain was present in small quantity in figure 4A, lane 2. This is possible because this latex protease sample was obtained from the first time incised fruits. It has been reported that the newly wounded papaya fruits contain only small amount of papain comparing to those obtained from the repeatedly wound fruits [17-18]. The first six N-terminal residues of the protein bands in figure 4A were determined via amino acid sequencing for identification. Result showed papain, chymopapain, glycyl endopeptidase, caricain and chitinase possessing their N-terminus as IPEYVD, YPWSID, LPESVD, LPENVD and GIEKII, respectively. This corresponds to those previously reported [14-15]. Result from cathodic gel electrophoresis revealed that papain is a major protein component of papaya peel proteases. In addition, glycyl endopeptidase and caricain were not observed (figure 4A, lane 4 and 5). Papaya peel proteases also contained three major proteins; one located above and two below papain. Based on the pH 4.5 of electrode buffer used, the protein band above papain should have pI lower than 8.75 of papain, while the two located below should have higher pI than that of papain.

Verification of proteolytic activities of protein bands is shown in figure 4B. It was observed that papaya peel proteases contained papain, chymopapain, and at least one additional protease which is absent in the latex (see protease I in lane 3-5 of figure 4B). As shown in the figure, papain proficiently hydrolyzed casein on the gel and resulted as clear zone due to its broad specificity of peptide bond cleavage [2]. On the other hand, other proteases with lower efficiency and/or higher specificity produced the darker area from precipitation of parts of the casein molecules. Naturally in solution, casein is found as micellar structure from hydrophobic interaction of individual molecule in the core of micelle. Its partial hydrolysis releases the hydrophilic part on the outside away. This

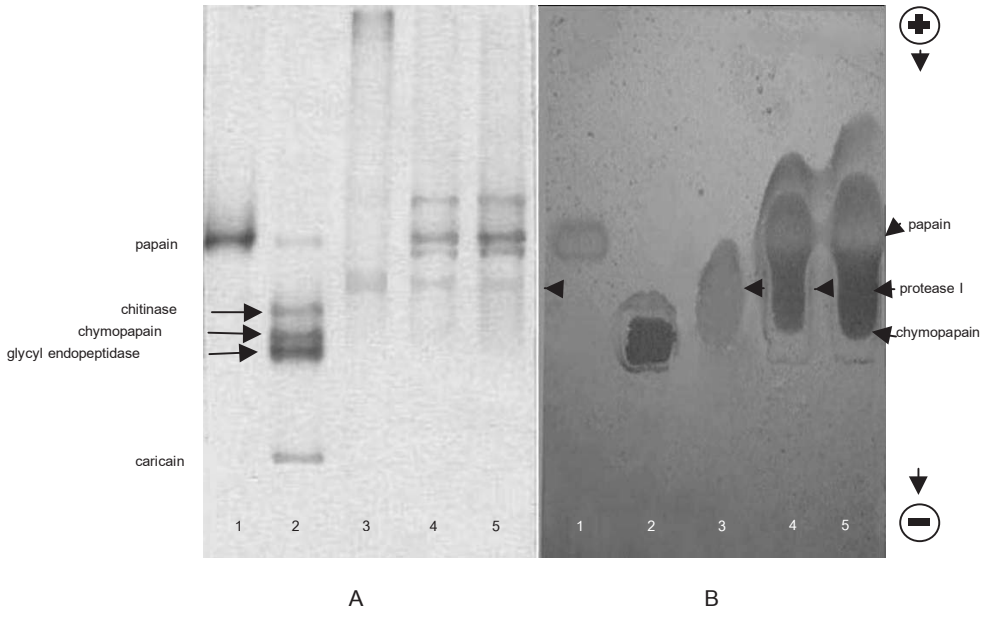

Figure 4. Separation of proteins by cathodic gel electrophoresis, stained with Coomassie Brilliant Blue (**A**) and *in situ* verifying their proteolytic activities (**B**). Lane 1; standard papain (3.7 μg protein), lane 2; latex proteases (2.9 μg protein), lane 3; papaya peel crude extract (20.6 μg protein), lane 4 & 5; papaya peel proteases; (5.3 & 15.9 μg protein, respectively).

exposes the hydrophobic part and results in the precipitation of casein. Further cleavage of casein by sufficient protease produces the complete hydrolysis [30]. This suggests that lower caseinolytic activities of papaya proteases except for papain could result in cloudy precipitation which appearing as dark band rather than the clear zone. In case of papain and caricain in lane 2 of figure 4B, their proteolytic activities could not be detected due to the low content of papain in the first time wounding fruit latex proteases and the instability of caricain on the acidic gel. Although *in situ* hydrolysis result was not sufficient for quantitative analysis of proteolytic activity, it suggested different patterns of protein bands between proteases obtained from the latex and the peels.

Preliminary anodic gel electrophoresis experiment of latex proteases and standard papain showed no band of proteins (data not shown). As previously known, the four cysteine proteases in papaya latex – papain, chymopapain, glycyl endopeptidase, and caricain possess pI greater than pH 8.3 of electrode buffer used, therefore, these positive net charge proteases should not moved toward anode. In contrast, migration of papaya peel proteases in the gel indicated that the proteins had pI less than 8.3 and one of them displayed the proteolytic activity (data not shown).

Figure 5 shows the elution profile of papaya peel proteases eluted from anion-exchange column. The result revealed that the peels are composed of more proteins than those of the latex which were completely eluted before fraction 20 (data not shown). The main components in papaya peel proteases are highly negative charged molecules (fraction 36-39). Their absorbances at 280 nm indicate that the molecules contain aromatic substances. Due to their negative results to Bradford reagent, they should not be protein but may be the hydrolyzed products of protein as previously described by Guo and Jiang [31]. In addition, positive result with ninhydrin confirms that they act or behave as amino acids or peptides from protein hydrolysis. In conclusion, the major components should be either aromatic peptides or aromatic amino acids. The first peak containing protein which unbound to the column and absent of caseinolytic activity (fig. 5), has been reported as a chitinase [28]. Determination of proteolytic activity in each fraction revealed that papaya peel proteases had the enzymatic activities in 2 fractions (d and e at fraction 21 and 32) which were absent in the latex proteases. This confirmed the result of *in situ* enzyme activity on the anodic gel (not shown) which suggested that the protease which pI less than papain are present in papaya peels, but not in the latex.

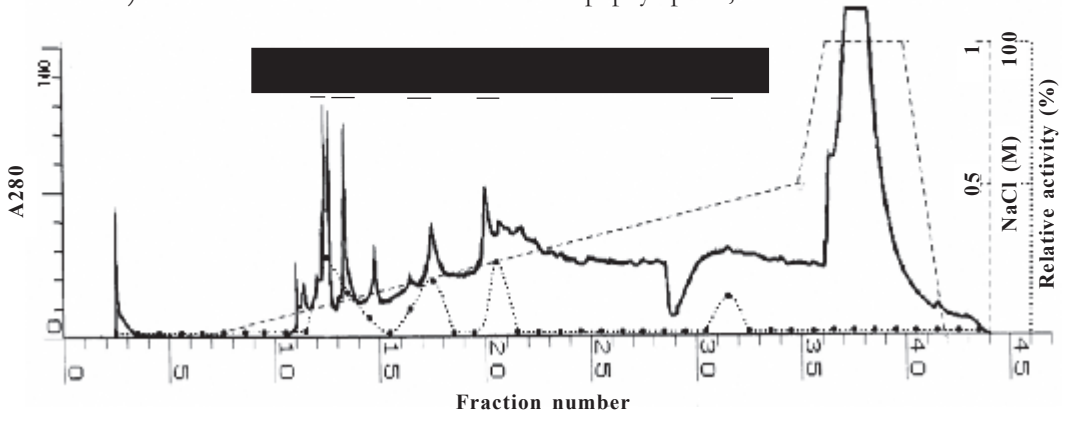

Figure 5. Anion-exchange chromatography of papaya peel proteases, eluted with a linear gradient of NaCl (—). Fractions were collected and analyzed by measurement at 280 nm (?) and proteolytic activity against casein (…).

Recently, a new cysteine protease from papaya latex was discovered [18]. It might correspond to one of these two new proteases from papaya peels. However, this study aims to elucidate the difference in the protein components between proteases from papaya peels and latex. Identification of each protein composition still requires further investigation.

4. CONCLUSION

Proteases from papaya peels were best precipitation by using 70% (v/v) ethanol. Their maximal casein hydrolysis occurred in the presence of 5 mM cysteine at 75°C similar to the latex proteases; however their optimal pH were different. The papaya peel enzymes were more stable in alkaline medium and at higher temperatures than the enzyme from the latex. They consisted of papain and chymopapain, similar to the latex proteases. Papaya peel enzymes most likely contain new proteases with pI greater than 8.75 (protease I), and also one with pI less than 8.3, and a large number of either small peptides or aromatic amino acids.

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