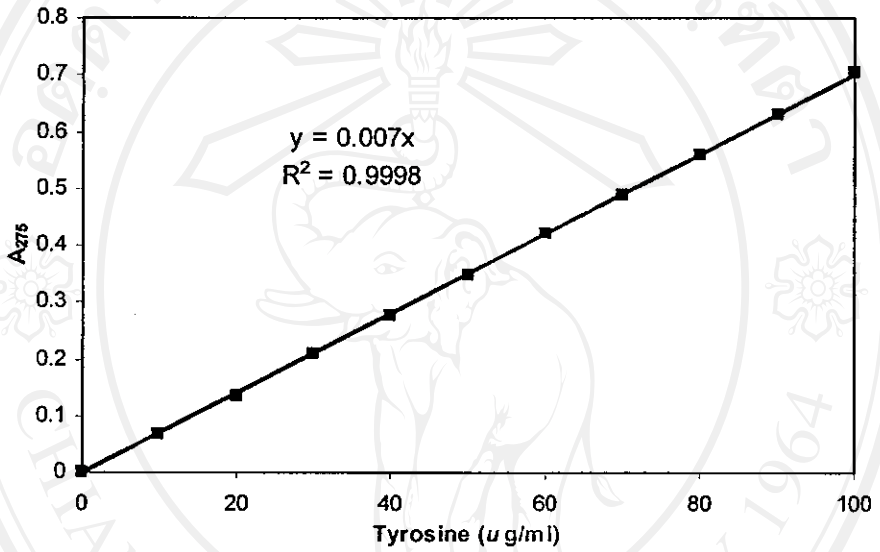
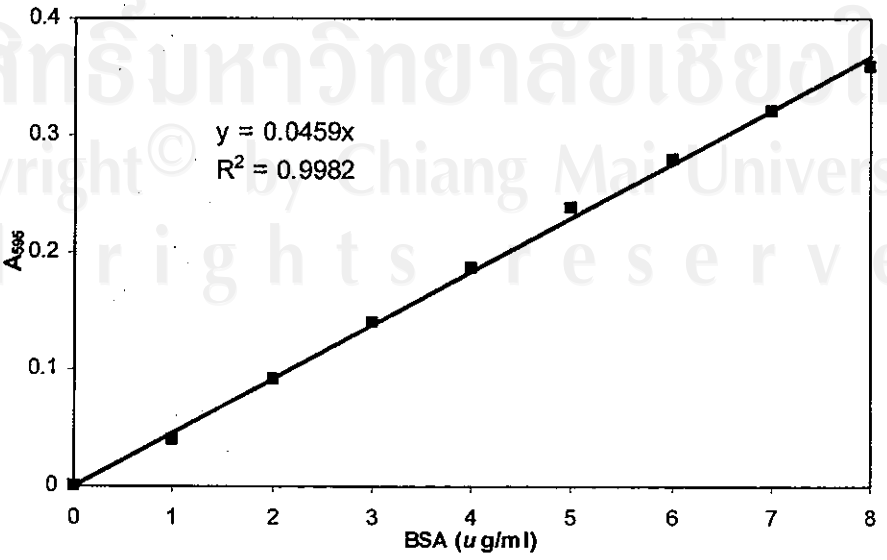
APPENDIX

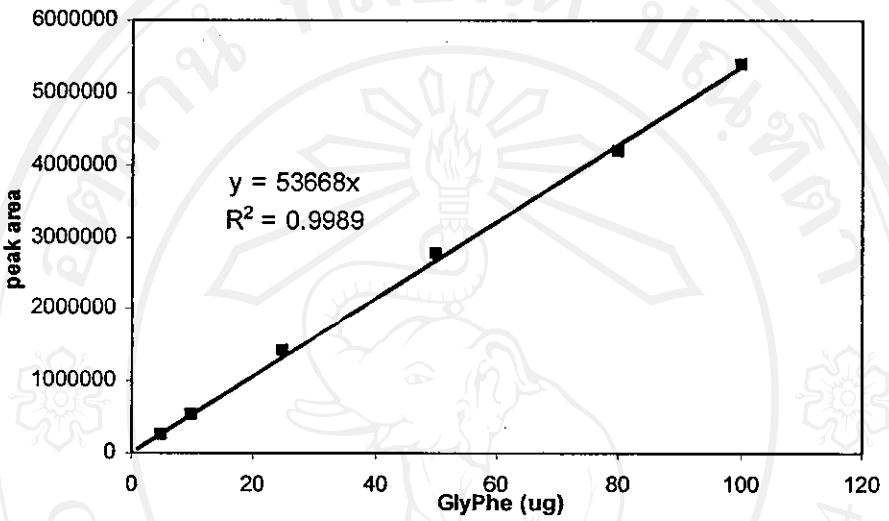
Appendix A: Standard curve of L-Tyrosine for calculating proteolytic activity.



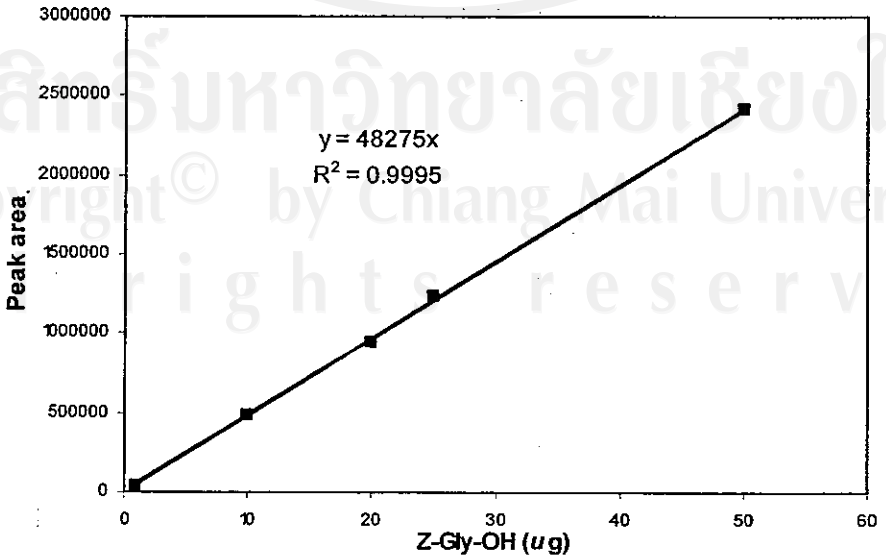
Appendix B: Standard curve of BSA for calculating protein content.



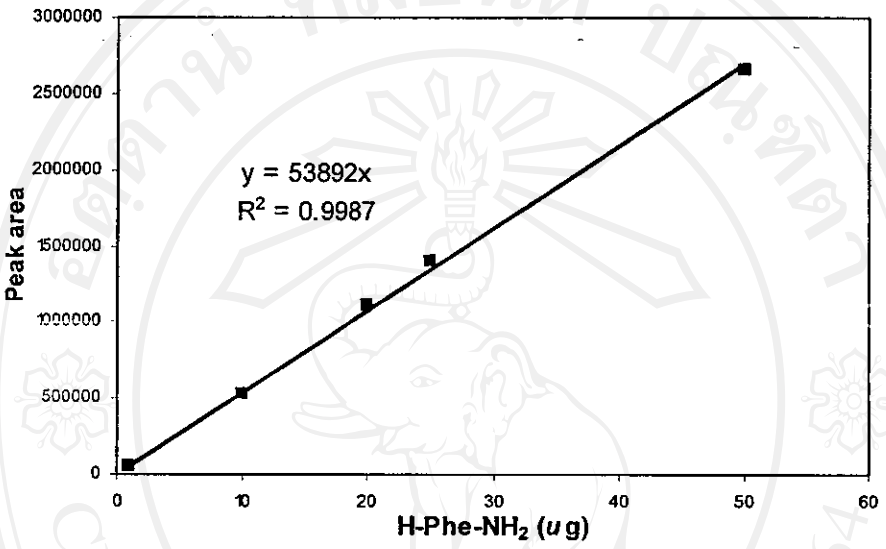
Appendix C: Standard curve of Z-Gly-Phe-NH_2 for calculating % peptide conversion



Appendix D: Standard curve of Z-Gly-OH for calculating amount of Z-Gly-OH in reaction mixture



Appendix E: Standard curve of H-Phe-NH_2 for calculating amount of H-Phe-NH_2 in reaction mixture



CURRICULUM VITAE

Name Mr. Phanuphong Chaiwut

Date of Birth 30th July 1978

Education

1997-2001 B.Sc. (Biochemistry and Biochemical Technology), Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

2001-2006 Ph.D. (Biotechnology), Division of Biotechnology, The Graduate School, Chiang Mai University, Chiang Mai, Thailand.

Oral Presentation

1. Chaiwut, P., Halling, P. J. and Kanasawud, P. 2005. Solid-to-solid peptide synthesis. RGJ-Ph.D. Congress VI, Pattaya, Thailand. p. 181.

Poster Presentations

1. Jaivoot, P., Palivanich, M., Theppakorn, T., Nitsawang, S. and Kanasawud, P. 2002. Separation of proteases from papaya peels. The 14th Annual Meeting of the Thai Society for Biotechnology, Khon Kaen, Thailand. pp. 164.
2. Jaivoot, P. and Kanasawud, P. 2004. Properties of proteases from *Carica papaya* fruits peel. The 15th Annual Meeting of the Thai Society for Biotechnology, Chiang Mai, Thailand. pp. 26.
3. Jaivoot, P., Theppakorn, T. and Kanasawud, P. 2004. Formation of reversibly inactive form of papaya cysteine proteases. The 16th Annual Meeting of the Thai Society for Biotechnology, Phitsanulok, Thailand. pp. 95.

Publications

1. Chaiwut, P., Kanasawud, P. and Halling, P. J. 2006. Solid-to-solid peptide synthesis by glycyl endopeptidase. *Enzyme Microb. Technol.*, *in press*.
2. Chaiwut, P., Nitsawang, S., Shank, L. and Kanasawud, P. 2007. Comparative study on properties and proteolytic components of papaya peel and latex proteases. *Chiang Mai J. Sci.*, *in press*.

Scholarships

2002 Dr. Bruno Weldermann Foundation

2003-2006 The Royal Golden Jubilee Ph.D. Program from the Thailand Research Fund (TRF)

SUPPORTING PAPERS

1. Chaiwut, P., Kanasawud, P. and Halling, P. J. 2006. Solid-to-solid peptide synthesis by glycyl endopeptidase. *Enzyme Microb. Technol., in press.*
2. Chaiwut, P., Nitsawang, S., Shank, L. and Kanasawud, P. 2007. Comparative study on properties and proteolytic components of papaya peel and latex proteases. *Chiang Mai J. Sci., in press.*

Model
MT-7410; No. of Pages 7
ARTICLE IN PRESS

Available online at www.sciencedirect.com
ScienceDirect

Enzyme and Microbial Technology xxx (2006) xxx–xxx

ENZYME AND MICROBIAL TECHNOLOGY

www.elsevier.com/locate/emt

Solid-to-solid peptide synthesis by glycyl endopeptidase

Phanuphong Chaiwut [a], Pawinee Kanasawud [b], Peter J. Halling [c,*]

[a] *Division of Biotechnology, Graduate School, Chiang Mai University 50200, Thailand*
[b] *Department of Chemistry, Faculty of Science, Chiang Mai University 50200, Thailand*
[c] *WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, Thomas Graham Bldg, 285 Cathedral Street, Glasgow G1 1XL, UK*

Received 6 July 2006; accepted 21 July 2006

ostract

Glycyl endopeptidase catalysed solid-to-solid synthesis can be carried out efficiently for the model peptides Z-Gly-Phe-NH_2, Z-Gly-Leu-NH_2, Z-y-Tyr-NH_2 and Z-Gly-Tyr-OEt. A small excess of acyl donor Z-Gly improved both the initial rate and final yield, whereas an excess of nucleophile e-NH_2 prevented reaction. The highest conversion was achieved when using a substrate molar ratio (Z-Gly:Phe-NH_2) of 2:1. Including solid steine in the reaction mixture improved both initial rate and final conversion, probably by protecting the enzyme from oxidation. The reasons why nversion to Z-Gly-Phe-NH_2 stopped at around 83% were investigated. Entrapment of residual solid starting material did not seem significant, ile autolysis and inactivation of glycyl endopeptidase in the reaction mixture during catalysis was important. The role of chemical equilibrium sition was less clear.

words: Peptide synthesis; Papaya enzymes; Mainly solid system; Glycine specific

Introduction

Enzymatic solid-to-solid peptide synthesis is an attractive ernative for biologically active peptide preparation. This proach clearly combines good rates similar to those in aque- s solution, with the high yields typical of reaction in organic lvents. Furthermore, the high substrate concentration leads a very high ratio of product to reactor volume [1,2]. The sence of organic solvents makes this strategy more environ- ntally friendly and offers important advantages for the food d drugs industries. It also avoids possible enzyme deactiva- n by organic solvents [3]. In addition, the process offers other nefits including: fewer steps for purifying product (often only shing), only one equivalent of water produced as a waste and v temperature operation. All of these positive features make s enzymatic synthesis system an attractive green chemistry ion [4].

In the last decade, solid-to-solid peptide synthesis has been ll developed for a wide range of substrates and enzymes 5–9] and scaled up from mmol to multi mol by several groups [10–12]. The reaction takes place in a saturated liquid phase that makes up around 10% of the reaction mixture, of which the overall appearance is a solid or highly viscous suspension or paste [11]. Because precipitation of the product provides a driving force for the very good yield obtained, the term 'precipitation driven biocatalysis' has also been used to describe this reaction system [2]. Erbeldinger et al. have reported the main parameters affecting the reaction, including water concentration, enzyme concentration, mixing conditions and substrate molar ratio [1,6]. They achieved the solid-to-solid synthesis of Z-Gln-Leu-NH_2; and showed that an excess of nucleophile, Leu-NH_2, dramatically increased the reaction rate and yield, suggesting an effect via the pH of the liquid phase [6].

Glycyl endopeptidase (EC 3.4.22.25) is one of the four papaya cysteine proteinases. It is a major component which constitutes almost 30% of total protein in the latex of *Carica papaya* [13–14], whereas the most extensively studied enzyme papain appears as a minor component at 8% [15]. Glycyl endopeptidase has high specificity to cleave only peptide bonds with Gly at P_1 [16]. Unlike other papaya cysteine proteinases, glycyl endopeptidase fails to hydrolyse the usual synthetic substrate, DL-Bz-Arg-pNA (BAPNA), and is not inhibited by cystatin [17–19]. Because of the strict specificity, the enzyme is widely used in analysis of protein primary structure (e.g., [20–22]). It

Corresponding author. Tel.: +44 141 548 2683
E-mail address: p.j.halling@strath.ac.uk (P.J. Halling).

1-0229/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
10.1016/j.enzmictec.2006.07.042

lease cite this article as: Phanuphong Chaiwut et al., Solid-to-solid peptide synthesis by glycyl endopeptidase, Enzyme and Microbial Technology (2006), doi:10.1016/j.enzmictec.2006.07.042

so used to identify disulfide bridges because it appears to e efficiently in the vicinity of disulfide bonds in the protein 23].

everal enzymes have been used in peptide synthesis with ly undissolved reactants—thermolysin, subtilisin, chypsin, papain and chymopapain [24–25]. In this report, yl endopeptidase was chosen as a different enzyme, and for the solid-to-solid synthesis of several Z-Gly peptides, ding Z-Gly-Phe-NH_2, which has been reported as a sub- of thermolysin [26]. The influence of substrate molar ratio investigated, and the factors limiting final conversion were ed.

aterials and methods

Chemicals

bstrates, *N*-benzyloxycarbonyl-glycine (Z-Gly-OH), L-phenylalanine (H-Phe-NH_2), L-tyrosine amide (H-Tyr-NH_2), L-leucine amide (H-Leu- and L-tyrosine ethyl ester (H-Tyr-OEt) were purchased from Bachem Z-Gly-OH from Novabiochem gave rather lower reaction rates, but this has not been investigated in detail. The activating agents, DL-cysteine and disodium salt (Analar) were obtained from Aldrich and Merck, respec- Acetonitrile, trifluoroacetic acid and sodium chloroacetate were obtained Aldrich. The specific substrate for glycyl endopeptidase, Boc-Ala-Ala- NA, was purchased from Bachem. Standard protein markers were obtained Sigma.

Purification of glycyl endopeptidase

ycyl endopeptidase was purified from fresh latex of *C. papaya* by an us-two-phase system following by salt precipitation, as presented in out- eviously [27]. One hundred grams of fresh papaya latex was agitated with water and then centrifuged to separate the insoluble material. The clear on was adjusted to pH 5.0 and then papain was removed by an aqueous-two- system of 6% (w/w) PEG and 15% ammonium sulfate [28]. Chymopapain ricain were separated from the salt-rich bottom phase by precipitation with (w/v) ammonium sulfate. After centrifugation, the supernatant protein ntration was adjusted to 8 mg/mL and then NaCl was added to 20% (w/v) cipitate out chitinase. Further, NaCl was added to the supernatant giving w/v) to precipitate glycyl endopeptidase. The enzyme was re-dissolved in sed water and dialysed six times against deionised water. After lyophilisa- nzyme powder was stored at −4 °C. The purity of the enzyme was verified hodic polyacrylamide gel electrophoresis and its ability to hydrolyse the ic substrate Boc-Ala-Ala-Gly-pNA but not DL-BAPNA.

Solid-to-solid synthesis of Z-Gly-Phe-NH_2 catalysed by endopeptidase

rious substrate molar ratios were obtained by mixing $(1+x)$ mmol of Z- H and $(1-x)$ mmol of H-Phe-NH_2 with 20 mg solid cysteine in 10 mL lycyl endopeptidase was used at 20 mg per reaction. (Preliminary studies d that larger amounts gave no further increase in initial rate.) The enzyme st dissolved in activating agent (sodium phosphate buffer pH 7.5 contain- mM cysteine and 20 mM Na_2-EDTA) and incubated at 40 °C for 5 min. nzyme solution was then added to the solid substrates giving a final water t of 10% (w/w). The reaction mixture was then mixed well and separated dividual Eppendorf tubes, each with around 7% of the total. These sealed were immersed in a waterbath at 40 °C.

HPLC analysis

the required time intervals, individual portions of reaction mixtures were ed in 3.5 mL of acetonitrile/water/TFA 30:66.5:3.5 (v/v/v) to terminate the reaction. After removing solid particles by filtration (through 0.2 μm nylon, Whatman), the samples were analysed by HPLC on a Water Alliance system with C-18 reverse phase column and detection at 254 nm. The mobile phase consisted of 30% acetonitrile in 0.01% aqueous TFA at a flow rate of 1.0 mL min^{-1}. The retention times of Phe-NH_2, Z-Gly and Z-Gly-Phe-NH_2 were 2.9, 4.7 and 7.9 min, respectively.

2.5. Analysis of liquid phase equilibrated with solids

About 1 mL water was mixed into a total mass of 373 mg of Z-Gly-OH and H-Phe-NH_2, in mole ratios of 1:1, 1.5:1 or 2:1, and incubated at 40 °C for 30 h. The resulting mixture still had an extensive solid mass, but a visible liquid supernatant. The supernatant (70–260 μL) was taken, centrifuged to remove any remaining suspended solids, and analysed by HPLC.

2.6. Investigation of incomplete peptide conversion

These reactions were all performed with a substrate molar ratio 2:1, 20 mg of enzyme and 20 mg of solid cysteine.

2.6.1. Reaction tube size

As noted individual small portions of reaction mixture were incubated separately in order to monitor progress. In some cases, liquid droplets were observed to form in the upper part of the tube, away from the solid mass at the bottom. This might indicate separation of water from the reaction mixture, although it is not clear what might be the driving force for this, given the uniform temperature expected from complete immersion of the tube. In order to eliminate any effect of this separation on the reaction progress, we switched to using smaller Eppendorf tubes (0.5 mL), and these were centrifuged when liquid separation was observed, forcing it back down into the rest of the mixture. These treatments slightly increased the final conversion of the peptide synthesis reaction.

2.6.2. Grinding of substances

Substrate particles of smaller size might be more available for reaction. Substrates were ground by hand in a mortar (previously dried at 100 °C and stored in a dessicator until used).

2.6.3. Alteration of water amount in reaction mixture

Possible effects on reaction equilibrium were investigated. After reaction progress had stopped, more water was added in the same quantity as initially (10%, w/w), and mixed thoroughly. Other additions studied were buffer pH 7.5, activating agent and enzyme solution. Water was also removed by drying the reaction mixture over molecular sieve in a dessicator at 40 °C. Removal of water was monitored by periodically weighing, until half that added originally had been removed (5%, w/w). The reaction mixture was then re-incubated with and without addition of new-activated enzyme solution.

2.6.4. Analysis of solid phase of the reaction mixture

The mixture was suspended in 1 mL deionised water, shaken for a few seconds and then the liquid phase was removed. The solid phase remaining in the tube was dissolved with 2 mL of acetronitrile/water/TFA 30:66.5:3.5 (v/v/v). Both portions were analysed by HPLC as described (Section 2.4).

2.7. Determination of glycyl endopeptidase activity in the reaction mixture

The mixture in the Eppendorf tube was suspended in 1 mL deionised water. Insoluble material was removed using filter paper and the enzyme activity in solution was determined by following the method, modified from that of Buttle [13].

The enzyme solution sample (200 μL) was mixed with 500 μL of activating agent (40 mM cysteine/20 mM Na_2-EDTA in phosphate buffer pH 7.5). The volume was made up to 975 μL with the same buffer and the mixture was incubated at 40 °C for 5 min to give fully active enzyme. The reaction was then started by adding 25 μL of substrate stock solution (50 mM Boc-Ala-Ala-Gly-pNA in dimethylsulfoxide). After exactly 8 min, 1 mL of stopping reagent

se cite this article as: Phanuphong Chaiwut et al., Solid-to-solid peptide synthesis by glycyl endopeptidase, Enzyme and Microbial Technogy (2006), doi:10.1016/j.enzmictec.2006.07.042

le 1
cyl endopeptidase catalysed solid-to-solid synthesis of various peptides

leophile	Product	Initial rate (nmol min^{-1} mg $enzyme^{-1}$)	Highest conversion (%)
$he-NH_2$	Z-Gly-Phe-NH_2	23	54
$yr-NH_2$	Z-Gly-Tyr-NH_2	21	52
$eu-NH_2$	Z-Gly-Leu-NH_2	100	48
yr-OEt	Z-Gly-Tyr-OEt	125	70

leophiles were used at 1.1 mol per mol acyl donor Z-Gly-OH, with 20 mg lycyl endopeptidase and 20 mg of solid cysteine. Highest conversions were dy levels observed after reaction for 10–30 h.

)mM sodium chloroacetate/30 mM sodium acetate/70 mM acetic acid, pH was added and A_{410} was measured ($\varepsilon_{410\,nm} = 8800\ M^{-1}\ cm^{-1}$).

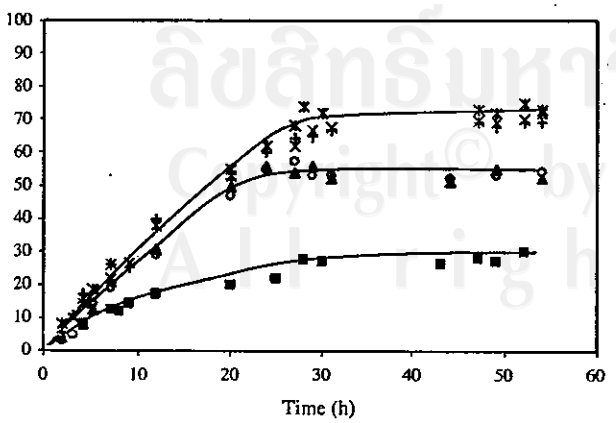
SDS-PAGE analysis

Autolysis of glycyl endopeptidase in the reaction mixture was investigated DS-PAGE, using a slab gel in a Biorad Mini-Protein II unit. The experiment carried out according to the method of Laemmli [29], with the separating 15% (w/v) acrylamide and the stacking gel 4%. The buffer in both electrode nbers was 0.124 M Tris–0.959 M glycine. Electrophoresis was run at a con- t 200 V and bromophenol blue was used as a dye marker. The protein bands e obtained by staining with Coomassie Brilliant Blue. Molecular weight stan- ls were bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), it muscle glyceraldehyde phosphate dehydrogenase (36 kDa), bovine ery- cytes carbonic anhydrase (29 kDa), bovine pancrease trypsinogen (24 kDa), ean trypsin inhibitor (20 kDa), bovine milk α-lactalbumin (14.2 kDa) and ne lung aprotinin (6.5 kDa).

Results and discussion

Successful peptide synthesis under solid-to-solid ditions

Table 1 shows that glycyl endopeptidase successfully catal- d solid-to-solid synthesis of four different peptides, with sonable initial rates. All reactions stopped with incomplete versions (typical progress curves may be seen in Fig. 1). It be seen that the enzyme has significantly different activity h the different nucleophiles. In all cases, the reaction mix-



1. Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of y-Phe-NH_2, with the substrate molar ratios (Z-Gly-OH:H-Phe-NH_2) at 1:1 1.05:1 (○), 1.1:1 (▲), 1.3:1 (+), 1.5:1 (×) and 2:1 (*). Lines just illustrate end.

tures with 10% water appeared as mainly solid pastes. Synthesis of Z-Gly-Phe-NH_2 was chosen as a model for most subsequent studies.

3.2. Effect of substrate molar ratios

Fig. 1 shows that the reaction rate and yield of Z-Gly-Phe-NH_2 synthesis were very dependent on the substrate molar ratios. Even a very slight excess of Z-Gly leads to a substantial increase in the rate of synthesis, and in the final conversion achieved. A similar effect was observed for the other three nucleophiles listed in Table 1 if the substrate ratio was reduced to 1:1. At the substrate ratio 1.3:1, 1.5:1 and 2:1, there was a further increase in the final conversion (Fig. 1), although the initial rate is slightly lower in nmol min^{-1} mg $enzyme^{-1}$ terms. The ratio of 2:1 seems to give the highest conversion, and was consequently used in further experiments. Note that the scatter in the experimental points is greater than for a typical reaction in solution, which is normal for these heterogeneous mainly solid systems.

Whereas an excess of Z-Gly improved the reaction, excess of Phe-NH_2 completely prevented reaction (data not shown). This is in contrast to the previous observations [6] with thermolysin-catalysed Z-Gln-Leu-NH_2 synthesis, where a very large increase in the initial rate was achieved when using an excess of Leu-NH_2, but the opposite effect was found with excess Z-Gln. Erbeldinger et al. suggested that slightly unequal substrate molar ratio can have a large effect on reaction rate, because the composition of the liquid phase in which the reaction takes place may be greatly dependent on this ratio [6]. They suggested that acid–base conditions were critical, and supported this theory by showing greatly increased reaction rate after adding $KHCO_3$ or K_2CO_3 to the reaction mixture [7]. We analysed liquid phases equilibrated with our reaction substrates in various molar ratios. With excess solid Z-Gly-OH, its total concentration in the liquid phase was found to be 0.24 M, higher than the total concentration of H-Phe-NH_2 (0.18 M). Electroneutrality requires that the ionised forms of these two must be present in equal concentrations, so there must be about 0.06 M Z-Gly-OH in the neutral acidic form. This would give rise to relatively acidic conditions in the liquid phase (around pH 5 in the equivalent dilute system). Where equimolar solids were used, the liquid phase concentrations were more variable, probably because of differing slight departures from an exactly 1:1 ratio. The total H-Phe-NH_2 concentration was similar to or higher than that of Z-Gly-OH, which would suggest somewhat alkaline conditions. It seems clear that mildly acidic conditions are optimal for solid-to-solid peptide synthesis catalysed by glycyl endopeptidase. We did try to add HCl (100% by mol of substrates) to the reaction mixture, but no conversion was observed, probably because conditions were too strongly acidic.

It should be noted that if the solid phases present are Z-Gly-OH and H-Phe-NH_2, the liquid concentrations should be the same, mutually saturated, whatever the relative amounts of solid. The behaviour noted above is probably because the two reactants form a combined solid phase, most likely a salt with ionised amino and carboxyl groups. Such behaviour has been suggested with other protected amino-acid reactants [6].

ease cite this article as: Phanuphong Chaiwut et al., Solid-to-solid peptide synthesis by glycyl endopeptidase, Enzyme and Microbial Technology (2006), doi:10.1016/j.enzmictec.2006.07.042

2

rate and conversion% of glycyl endopeptidase catalysed solid-to-solid Z-Gly-Phe-NH_2 synthesis in the presence and absence of cysteine

ce of cysteine in		Initial rate (nmol min^{-1} mg $enzyme^{-1}$)	Highest conversion (%)
ting solution (mM)	Reaction mixture (%, w/w of enzyme)		
0	0	6	59
100	100	7	67
0	0	11	63
50	50	12	74
100	100	16	76
200	200	16	76

ons were performed at substrate molar ratio 2:1 and using 20 mg enzyme per reaction. Activating solution is used to pre-treat the enzyme before mixing with id substrates.

the reaction with equimolar substrates (but not those with s Z-Gly-OH), a byproduct was also observed. Accurate and UV absorption spectrum identified this as Z-Gly-Phe- and also confirmed the identity of the Z-Gly-Phe-NH_2). e early stages of reaction, the byproduct was a very small ortion of the products, but it eventually rose to about 26% e total. It is presumably formed by hydrolysis of Z-Gly- NH_2 synthesised earlier.

Effect of cysteine on glycyl endopeptidase catalysis

aqueous media, cysteine proteinases, including glycyl peptidase, need to be activated by a reducing agent, such steine for maximum activity. The effect of cysteine on l endopeptidase-catalysed peptide synthesis in the solid-to- system was investigated, with it present both in the initial ne solution and as solid in the reaction mixture. Table 2 s that the initial rate was doubled when dissolving glycyl peptidase in a buffer containing cysteine (activating agent). lition, the presence of solid cysteine in the reaction mixture substantial effect as the reaction proceeds, with the highest rsion approximately 10% higher than in its absence. The al addition of solid cysteine was 100% (w/w) relative to the ne amount (Table 2). It has also been reported that added ine increased the conversion of peptide synthesis in *tert*- ol catalysed by immobilised papain that was prepared by ropanol-rinsed method [30]. Addition of solid Na_2-EDTA) was also investigated, but this activator had no significant on the progress of the reaction (data not shown). EDTA ions to eliminate free metal ions, and that present in the ting solution is presumably sufficient.

Effect of reactant particle size

key feature of enzymatic solid-to-solid synthesis is the fact ne reaction occurs in a liquid phase, normally saturated with substrates [11]. The particle size of the solid substrates may ne a key factor if mass transfer to all parts of the liquid is ng, with smaller particles more easily dissolved. We stud- e effect of grinding the two solid substrates, but neither l rate nor final conversion were significantly different, with f the four nucleophiles listed in Table 1 (data not shown). s probably because both substrates were already in powder , which were small enough to avoid mass transfer limitation. However, grinding of solid cysteine before adding to the reaction mixture had a substantial effect on the reaction progress (Fig. 2). The unground solid cysteine was observed to be in the form of larger crystals, which presumably dissolved only slowly in the reaction mixture to protect the enzyme during catalysis.

3.5. Factors affecting final conversion yield and its improvement

As the results (Figs. 1 and 2) show, the reaction progress in the solid-to-solid synthesis of Z-Gly-Phe-NH_2 stops at around 75% conversion. Various factors have been investigated in order to understand why the glycyl endopeptidase reaction is incomplete.

3.5.1. Separation of reaction mixture

As detailed in the Section 2.6.1, some apparent separation of the reaction mixture was observed at longer incubation times. This could be prevented by reducing the size of tubes used, and periodically centrifuging. Fig. 3 shows that this increased the final peptide conversion from 75 to around 83%. However, the conversion was still well below 100%.

3.5.2. Entrapment of solid substrates

The solid-to-solid reaction system is mainly solid and very viscous. When the reaction proceeds, the product may precipitate around the particles of substrates. This may prevent the

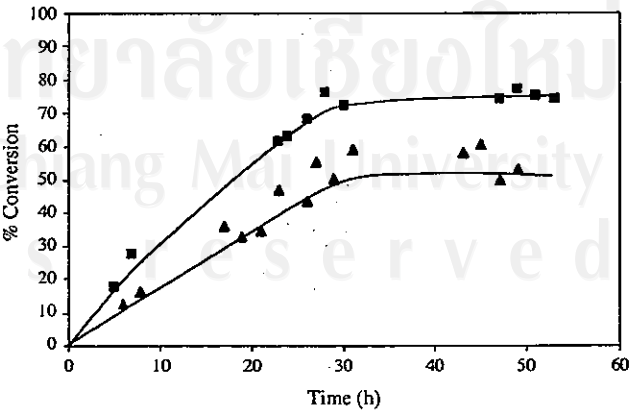
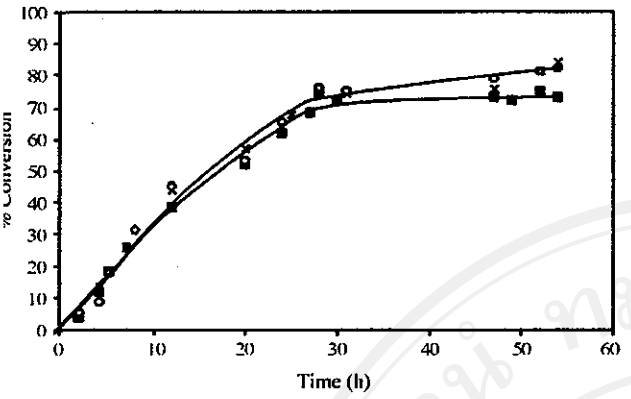


Fig. 2. Effect of grinding solid cysteine on glycyl endopeptidase catalysed peptide synthesis. Substrate molar ratio (Z-Gly-OH:H-Phe-NH_2) of 2:1, added solid cysteine was ground (■) or unground (▲).

se cite this article as: Phanuphong Chaiwut et al., Solid-to-solid peptide synthesis by glycyl endopeptidase, Enzyme and Microbial Technology (2006), doi:10.1016/j.enzmictec.2006.07.042



g. 3. Effect of tube size and centrifugation on glycyl endopeptidase catalysed lid-to-solid synthesis of Z-Gly-Phe-NH_2. Substrate molar ratio (Z-Gly-OH:H-e-NH_2) was 2:1, substrates were ground. The reaction was carried out in the es of 1.5 mL (■) and 0.5 mL with (○) or without (×) centrifugation.

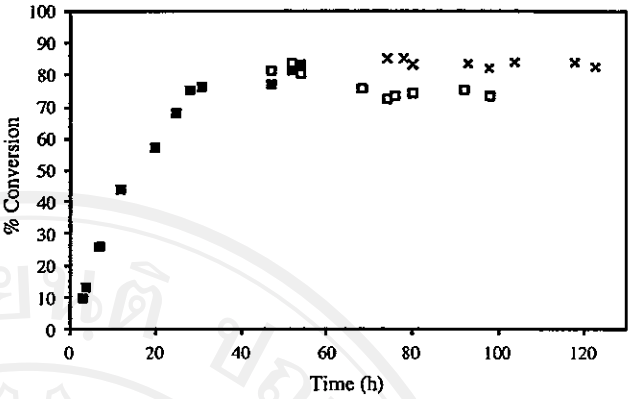
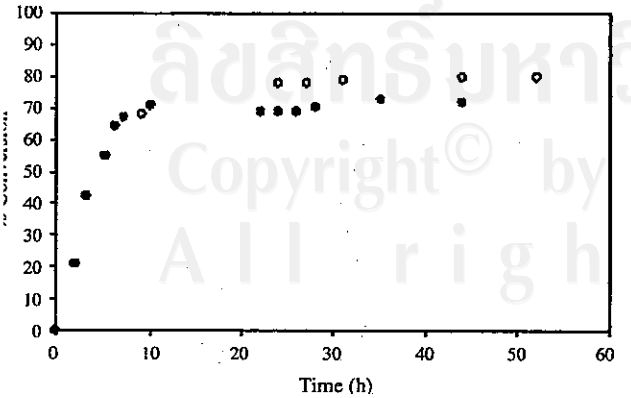


Fig. 5. Effect of adding fresh enzyme after conversion had stopped in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2. Substrate molar ratio (Z-Gly-OH:H-Phe-NH_2) of 2:1. After normal reaction (■) stopped (48 h), more enzyme solution was added to the reaction mixture with (×) and without drying (□).

bstrates dissolving in the liquid phase, so their concentrations ere fall and they are less available to the enzyme. Eventually eir concentrations may become low enough so that further syn-esis is not favourable. At this point, re-mixing of the reaction ixture might help the reaction continue further, by re-exposing e substrate particles. Such an effect was observed in reactions ing H-Tyr-OEt as nucleophile (Fig. 4), but with H-Phe-NH_2 or -Leu-NH_2 (data not shown) there was no change in conversion i further incubation after re-mixing of the reaction mixture. iis suggests that in these reactions there was no entrapment of lid substrates by peptide product.

3.3. Reaction reaches its equilibrium position

In thermodynamically controlled peptide synthesis, another portant factor is the reaction equilibrium, as clearly conver-on cannot continue as equilibrium is approached. When fresh zyme solution was added to the reaction mixture (in a first test possible enzyme inactivation), a progressive decline in prod-t peptide was observed (Fig. 5). A similar result was found th the Z-Gly-Tyr-OEt synthesis reaction (data not shown).



. 4. Effect of re-mixing of reaction mixture on glycyl endopeptidase catal-d solid-to-solid synthesis of Z-Gly-Tyr-OEt. Reaction mixture with substrate lar ratio 1.1:1 was mixed once at starting time (•) or re-mixed after 9 h reac-n (○).

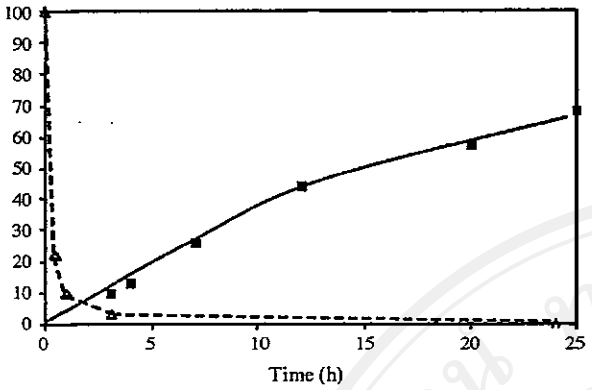
This suggested that the observed conversion might be equilibrium controlled, with the increase in water content from 10 to 20% (w/w) making hydrolysis more favoured. Therefore, the reaction mixture after 48 h was completely dried before adding new-activated enzyme solution. Unfortunately, the peptide conversion was still stable at the same level as the normal reaction (Fig. 5). This would support the idea that the reaction mixture is already near equilibrium for the 10% water level.

In theory, these solid-to-solid reactions can only reach equilibrium when one (or both) of the solid substrates is exhausted from the reaction mixture [31]. However, HPLC analysis of the solid phase after brief washing (see Section 2.6) clearly showed that both Z-Gly and Phe-NH_2 are still present (data not shown). In a further test, water was removed from the reaction mixture after 48 h, to see if this would shift the equilibrium towards peptide product. However, no change in conversion was observed. We also tested adding water, buffer or activating agent in place of the enzyme solution (in an experiment analogous to Fig. 5). None of these led to any significant change in conversion (data not shown). This difference from the result where enzyme was added (Fig. 5) indicates that loss of enzyme activity is probably a factor, as well as any possible influence of equilibrium position.

3.6. Loss of glycyl endopeptidase activity in the reaction mixture

The results described above suggest that enzyme inactivation during the synthesis reaction is significant, so its residual activity was investigated. The glycyl endopeptidase assay revealed that activity was dramatically decreased within 3 h of incubation and after that it seems to decline further until 24 h (Fig. 6; Table 3). It is note worthy that the reaction is still proceeding well when only a few percents of enzyme activity remain. Table 3 also shows the activity profile of glycyl endopeptidase after a second addition of enzyme to the reaction mixture. The fresh activated enzyme was added to the completely dried mixture after the reaction stopped, but the new glycyl endopeptidase was very rapidly inactivated. This would explain the result in Fig. 5 that there was no change

Please cite this article as: Phanuphong Chaiwut et al., Solid-to-solid peptide synthesis by glycyl endopeptidase, Enzyme and Microbial Technology (2006), doi:10.1016/j.enzmictec.2006.07.042



Inactivation of glycyl endopeptidase while catalysing solid-to-solid syn-
f Z-Gly-Phe-NH_2. Substrate molar of 2:1. Progress of synthesis (■), and
l amidase activity of enzyme (Δ).

version. Therefore, it seems that loss of enzyme activity is
ificant factor causing the solid-to-solid peptide synthesis
) at around 83% conversion.

o possibilities that could cause loss of glycyl endopep-
activity were considered. The first is oxidation of the
:atalytic site group to become a sulfinic acid or sulfonic
However, the reaction mixture contains sufficient reactive
ne that should prevent oxidation. Another reason is the
sis of glycyl endopeptidase itself during catalysis. This
ıalysed by running SDS-PAGE of enzyme recovered from
ıction mixture after 5 min, 30 min, 1 h, 3 h and 24 h (Fig. 7).
tensity of the glycyl endopeptidase band decreased rapidly
ncubation time. The recovered enzyme solution was also
ed by HPLC on a C_4 column to confirm its autolysis. The
(not shown) was similar to that of SDS-PAGE, with the
n peak disappearing over time. Therefore, it was concluded
ıe rapid autolysis of glycyl endopeptidase in the mixture
ı important factor in causing incomplete conversion in this
o-solid synthesis.

of glycyl endopeptidase in reaction mixture for Z-Gly-Phe-NH_2
is

endopeptidase	Amidase activity (U/mg)	Residual activity (%)
mixing with solid substrates	2710	–
lard reaction mixture at		
ı	2530	100
in	1210	47.6
in	560	22.1
	252	10.0
	85.7	3.39
	4.49	0.18
:cond addition of enzyme[a]		
ı	533	21.0
in	20.3	0.80
	13.5	0.54

substrate used was Boc-Ala-Ala-Gly-pNA and one unit is defined as
product released within 1 min at pH 7.5 and 37 °C.
r 48 h standard reaction, mixture was dried for 24 h, then fresh enzyme
ı mixed in.

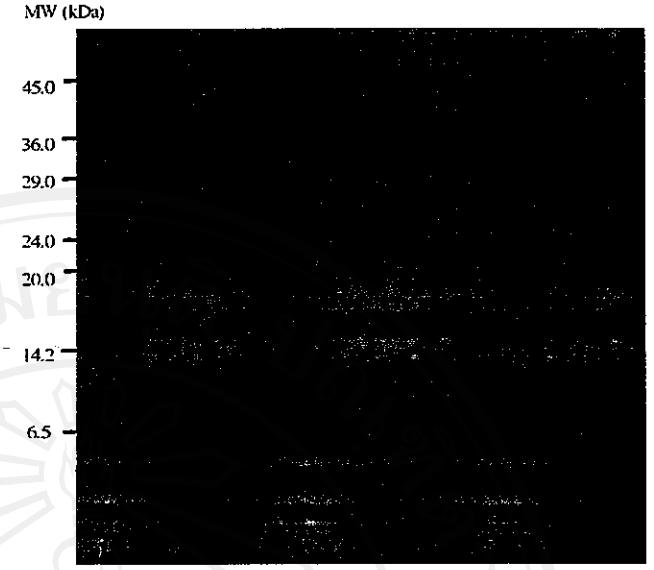


Fig. 7. SDS-PAGE of glycyl endopeptidase recovered from the reaction mixture. After incubation for 5 min (lane 3), 30 min (lane 4), 1 h (lane 5), 3 h (lane 6) and 24 h (lane 7), comparing to the enzyme before addition to the reaction mixture (lane 2).

4. Conclusion

Glycyl endopeptidase works well as a catalyst for solid-to-solid synthesis of Z-Gly-Phe-NH_2 and other Z-Gly peptides. The reaction rate and conversion were very dependent on the substrate molar ratios. The highest conversion at around 83% was achieved with a 2:1 Z-Gly to Phe-NH_2 ratio, and 20 mg enzyme and 20 mg solid cysteine per reaction. Grinding of the two substrates, re-mixing of the mixture after reaction stopped and adding of new-activated enzyme solution resulted in no further conversion. It was found that a main factor in synthesis stopping at 83% conversion is the rapid autolysis of glycyl endopeptidase. This needs to be investigated in the future in order to prevent enzyme autolysis and hence further improve the synthesis.

Acknowledgement

We thank the Thailand Research Fund for supporting Phanuphong Chaiwut via the Royal Golden Jubilee Ph.D. Program (2.B.CM/46/F.1).

References

[1] Erbeldinger M, Ni X, Halling PJ. Effect of water and enzyme concentration on thermolysin-catalysed solid-to-solid peptide synthesis. Biotechnol Bioeng 1998;59:68–72.
[2] Ulijn RV, Martin LD, Gardossi L, Halling PJ. Biocatalysis in reaction mixture with undissolved solid substrates and products. Curr Org Chem 2003;7:1333–46.
[3] Erbeldinger M, Ni X, Halling PJ. Enzymatic synthesis with mainly undissolved substrates at very high concentrations. Enzyme Microb Technol 1998;23:141–8.
[4] Ulijn RV, Halling PJ. Solid-to-solid biocatalysis: thermodynamic feasibility and energy efficiency. Green Chem 2004;6:488–96.

ıe cite this article as: Phanuphong Chaiwut et al., Solid-to-solid peptide synthesis by glycyl endopeptidase, Enzyme and Microbial Technology (2006), doi:10.1016/j.enzmictec.2006.07.042

] Krix G, Eichhorn U, Jakubke H-D, Kula M-R. Protease-catalyzed synthesis of new hydrophobic dipeptides containing non-proteinogenic amino acids. Enzyme Microb Technol 1997;21:252–7.
] Erbeldinger M, Ni X, Halling PJ. Kinetics of enzymatic solid-to-solid peptide synthesis: intersubstrate compound, substrate ratio, and mixing effects. Biotechnol Bioeng 1999;63:316–21.
] Erbeldinger M, Ni X, Halling PJ. Kinetics of enzymatic solid-to-solid peptide synthesis: synthesis of Z-aspartame and control of acid-basse conditions by using inorganic salts. Biotechnol Bioeng 2001;72: 69–76.
] Ulijn RV, Martin LD, Gardossi L, Janssen AEM, Moore BD, Halling PJ. Solvent selection for solid-to-solid synthesis. Biotechnol Bioeng 2002;80:509–15.
] Kim C, Lee IK, Ahn JE, Shin CS. Synthesis of kyotorphin precursor from eutectic mixtures catalysed by α-chymotrypsin. Biotechnol Lett 2001;23:1423–7.
] Eichhorn U, Bommarius AS, Drauz K, Jakubke H-D. Synthesis of dipeptides by suspension-to-suspension conversion via thermolysin catalysis: from analytical to preparative scale. J Pept Sci 1997;3:245–51.
] Erbeldinger M, Ni X, Halling PJ. Scale-up of enzymatic solid-to-solid peptide synthesis and enzyme recovery. AIChE J 2001;47:500–8.
] Gill I, Valivety R. Pilot-scale enzymatic synthesis of bioactive oligopeptide in eutectic-based media. Org Proc Res Dev 2002;6:684–91.
] Buttle DJ. Glycyl endopeptidase. Methods Enzymol 1994;224:531–55.
] Buttle DJ, Kembhavi AA, Sharp SL, Shute RE, Rich DH, Barrett AJ. Affinity purification of the novel cysteine proteinase papaya proteinase IV, and papain from papaya latex. Biochem J 1989;261:469–76.
] Azarkan M, Moussaoui AE, Wuytswinkel DV, Dehon G, Looze Y. Fractionation and purification of the enzymes stored in the latex of *Carica papaya*. J Chromatogr B 2003;790:229–38.
] Buttle DJ, Ritonja A, Pearl LH, Turk V, Barrett AJ. Selective cleavage of glycyl bonds by papaya proteinase IV. FEBS Lett 1990;260:195–7.
] Buttle DJ, Ritonja A, Dando PM, Abrahamson M, Shaw EN, Wikstrom P, et al. Interactions of papaya proteinase IV with inhibitors. FEBS Lett 1990;262:58–60.
] O'Hara BP, Hemmings AM, Buttle DJ, Pearl LH. Crystal structure of glycyl endopeptidase from *Carica papaya*: a cysteine endopeptidase of unusual substrate specificity. Biochemistry 1995;34:13190–5.
[19] Thomas MP, Verma C, Boyd SM, Brocklehurst K. The structural origins of the unusual specificities observed in the isolation of chymopapain M and actinidin by covalent chromatography and the lack of inhibition of chymopapain M by cystatin. Biochem J 1995;306:39–46.
[20] Brouwer M, Enghild J, Hoexum-Brouwer T, Thogersen I, Truncali A. Primary structure and tissue-specific expression of blue crab (*Callinectes sapidus*) metallothionein isoforms. Biochem J 1995;311:617–22.
[21] Ritonja A, Coetzer THT, Piket RN, Dennison C. The amino acid sequences, structure comparisons and inhibition kinetics of sheep cathepsin L and sheep stefin B. Compd Biochem Physiol 1996;114B:193–8.
[22] De La Cruz EM, Ostap EM, Brundage RA, Reddy KS, Sweeney HL, Safer D. Thymosin-β4 changes the conformation and dynamics of actin monomers. Biophys J 2000;78:2516–27.
[23] Bernard VD, Peanasky RJ. The serine protease inhibitor family from *Ascaris suum*: chemical determination of the five disulfide bridges. Arch Biochem Biophys 1993;303:367–76.
[24] Jorba X, Gill I, Vulfson EN. Enzymatic synthesis of the delicious peptide fragments in eutectic mixtures. J Agric Food Chem 1995;43:2536–41.
[25] Fandino-Lopez R, Gill I, Vulfson EN. Protease-catalyzed synthesis of oligopeptides in heterogenous substrate mixtures. Biotechnol Bioeng 1994;43:1024–30.
[26] Nagase H, Harris ED. Ovostatin: A novel proteinase inhibitor from chicken egg white. II. Mechanism of inhibition studied with collagenase and thermolysin. J Biol Chem 1983;258:7490–8.
[27] Nitsawang S, Kanasawud P. Fractionation and purification of cysteine proteinases and chitinase from *Carica papaya* latex. Biothailand. Bangkok, Thailand; 2005. p. 130.
[28] Nitsawang S, Hati-Kaul R, Kanasawud P. Purification of papain from Carica papaya latex: aqueous two-phase extraction versus two-step salt precipitation. Enzyme Microb Technol 2006;39:1103–7.
[29] Laemmli UK. Cleavage of structural proteins during the assembly the head of bacteriophage T4. Nature 1970;227:680–5.
[30] Theppakorn T, Kanasawud P, Halling PJ. Activity of immobilized papain dehydrated by *n*-propanol in low-water media. Biotechnol Lett 2004;26:133–6.
[31] Ulijn RV, Janssen AEM, Moore BD, Halling PJ. Predicting when precipitation-driven synthesis is feasible: application to biocatalysis. Chem Eur J 2001;7:2089–98.

lease cite this article as: Phanuphong Chaiwut et al., Solid-to-solid peptide synthesis by glycyl endopeptidase, Enzyme and Microbial Technology (2006), doi:10.1016/j.enzmictec.2006.07.042

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

Phanuphong Chaiwut (a), Sarote Nitsawang (a), Lalida Shank* (b) and Pawinee Kanasawud (b)

(a) Division of Biotechnology, Graduate School, Chiang Mai University, Chiang Mai 50200, Thailand.

(b) Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.

*Author for correspondence (E-mail: lalida_shank@yahoo.com)

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 $\geq$ 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 tetramethylenediamide (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 were 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 μmole 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 β-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 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).

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

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 (fig. 1A) and at 75°C (fig. 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.

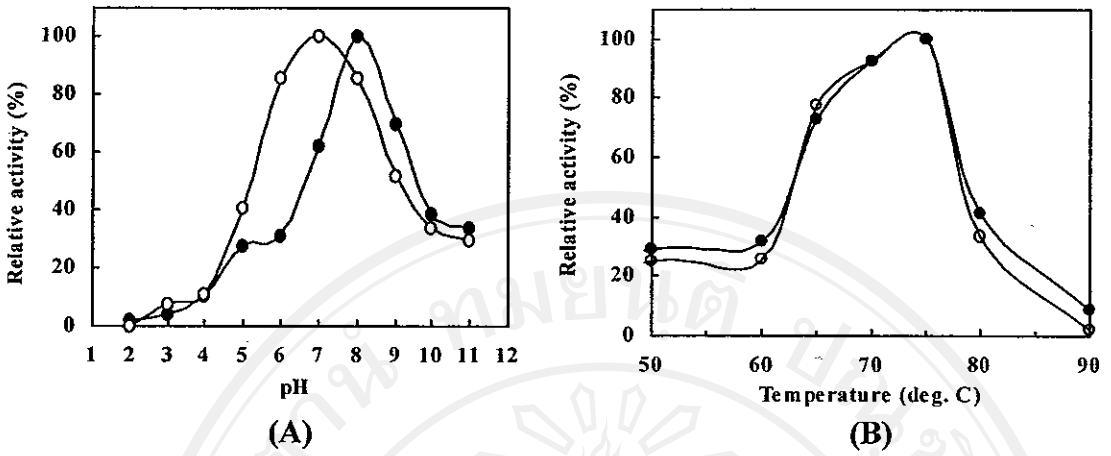


Figure 1. Optimal pH at 37°C (A) and optimal temperature in buffer pH 8.0 (B) on casein hydrolyses of papaya peel proteases (●) and latex proteases (○).

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.

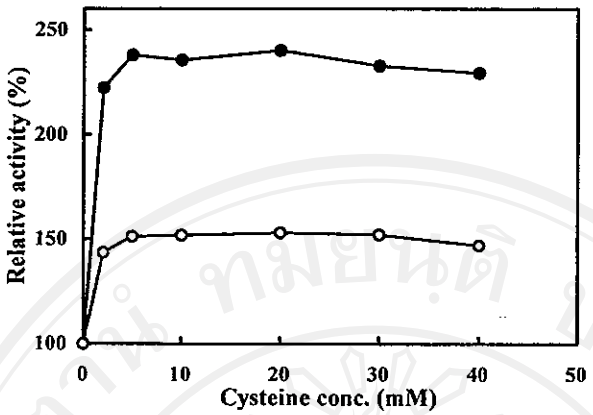


Figure 2. Effect of cysteine on caseinolytic activities of papaya peel proteases (●) and latex proteases (○) in buffer pH 8 at 37°C. The activities of the two reactions without cysteine were given as 100% relative activity.

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 (fig. 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 (fig. 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.

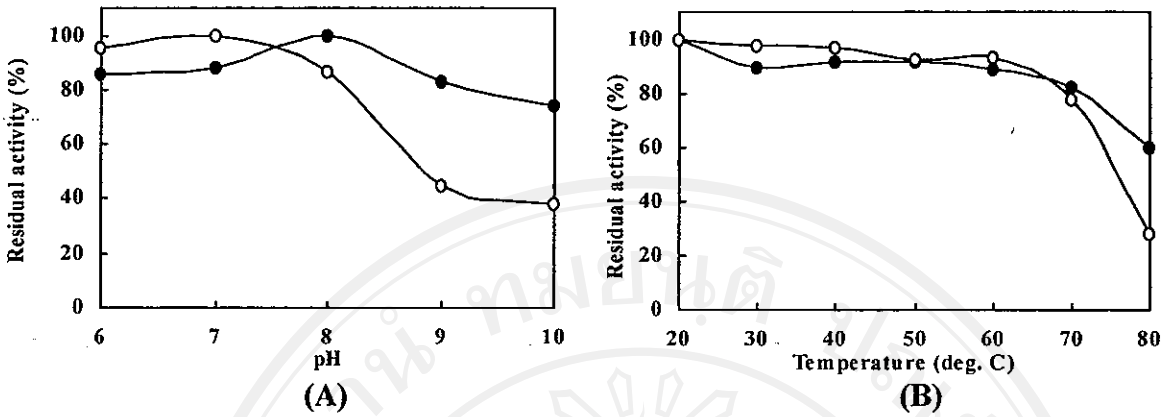


Figure 3. Stability of papaya proteases from the peels (●) and latex (○). The enzymes were incubated at various pH (A) and at various temperatures (B) for 10 min before determining their proteolytic activities in pH 8 at 37°C.

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.

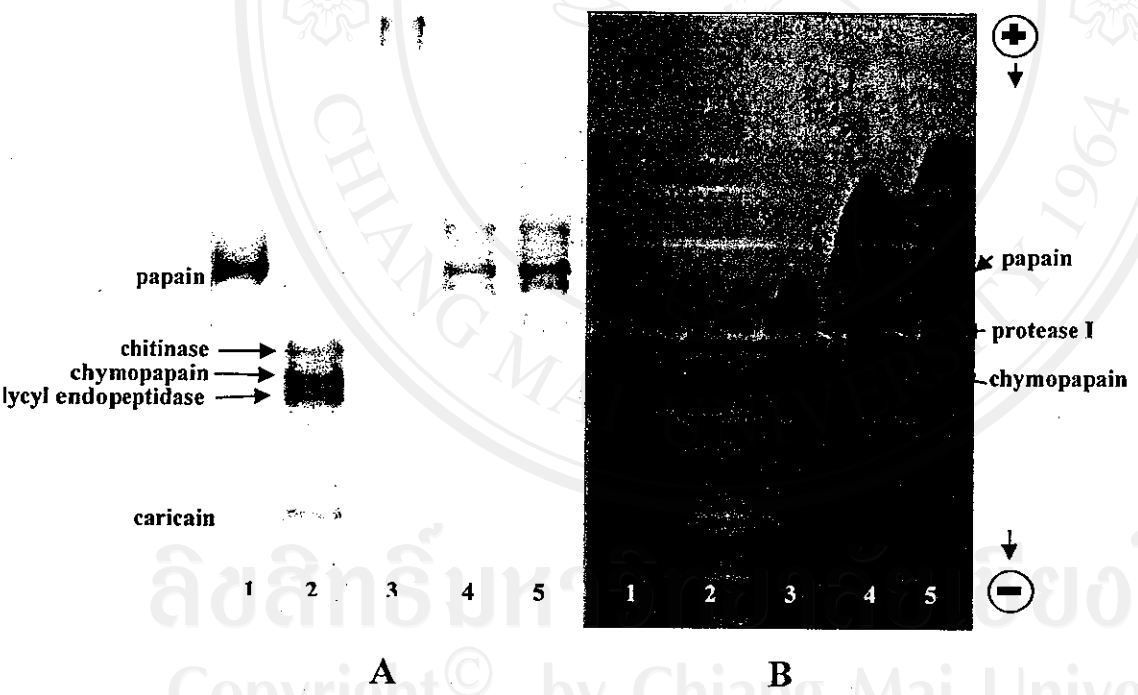
Mobilities of papain, chymopapain, glycyl endopeptidase, and caricain in the latex proteases separated by cathodic electrophoresis (fig. 4A, lane 2) correspond to those of previous reports [9, 12-14, 22, 27-29]. It is notable that the least basic enzyme papain was present in small quantity in fig. 4A,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

ıantitative analysis of proteolytic activity, it suggested different patterns of protein ınds between proteases obtained from the latex and the peels.

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



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

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.

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.

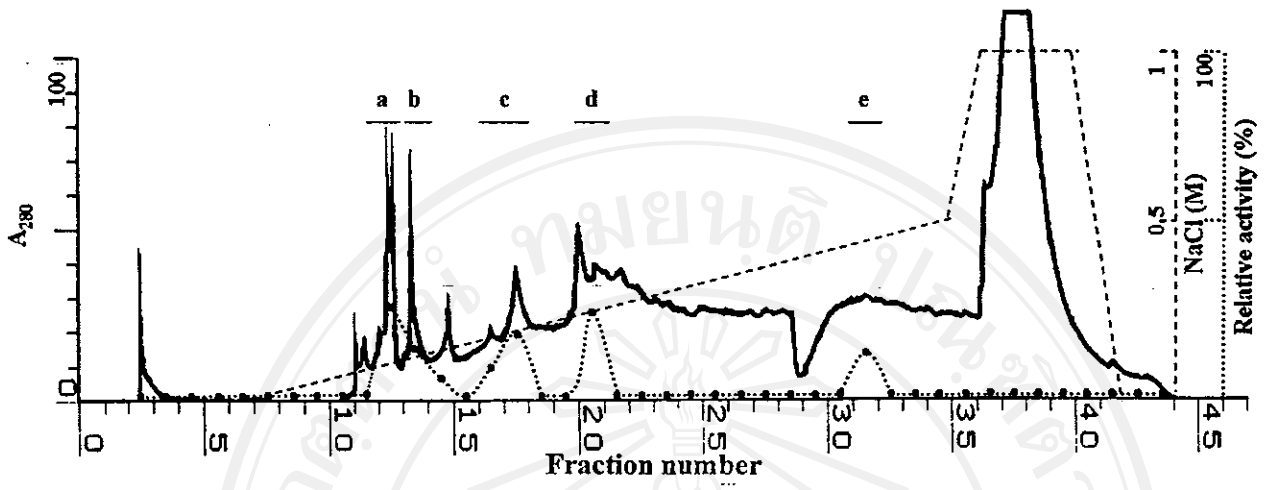


Figure 5. Anion-exchange chromatography of papaya peel proteases, eluted with a linear gradient of NaCl (---). Fractions were collected and analyzed by measurement at 280 nm (—) and proteolytic activity against casein (…).

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.

ACKNOWLEDGEMENTS

The authors gratefully thank the Thailand Research Fund for financial support and for the scholarship to Phanuphong Chaiwut via the Rayal Golden Jubilee Ph.D Program (2.B.CM/46/F.1). We appreciate Prof. Peter Halling for his insightful advice and Graduate School, Chiang Mai University for financial support.

REFERENCES

[1] Poulter N.H., and Caygill, J.C., Production and utilization of papain - a proteolytic enzyme from *Carica papaya* L., *Trop. Sci.*, 1985; 25: 123-137.

[2] Barrett A.J., Rawlings N.D., and Woessner J.F., Introduction: cysteine peptidases and their clans; in *Handbook of proteolytic enzyme*, San Diego: Academic Press, 1998: 545-566.

[3] Caygill J.C., Sulphydryl plant proteases, *Enzyme Microb. Technol.*, 1979; 1: 233-241.

[4] Finley J.W., Stanley W.L., and Watters G.G., Romoval of chill haze from beer with papain immobilized on chitin, *Biotechnol. Bioeng.*, 1977; XIX:1895-1897.

[5] Arimura N., Preparation of crude papain with green papaya fruit or its peel, *Japan Pat. No.* 01013995 (1989).

[6] Espin N., and Islam M.N., Stabilization of papain from papaya peels, *Food Sci. Technol. Inter.*, 1998; 4: 179-187.

[7] Balls A.K., and Thompson R.R., Crude papain: preparation and properties, *Ind. Eng. Chem.*, 1940; 32: 1144-1147.

[8] Lesuk A., Process for the purification of papain, *US Pat. No.* 3011952 (1961).

[9] Mckee R.A., and Smith H., Purification of proteinases from *Carica papaya*, *Phytochem.*, 1986; 25: 2283-2287.

[10] Silva L.G., Garcia' O., Lopes' M.T.P., and Salas C.E., Changes in protein profile during coagulation of latex from *Carica papaya*, *Braz. J. Med. Biol. Res.*, 1997; 30: 615-619.

[11] Moutim V., Silva L.G., Lopes M.T.P., Fernandes G.W., and Salas C.E., Spontaneous processing of peptides during coagulation of latex from *Carica papaya*, *Plant Sci.*, 1999; 142: 115-121.

[12] Buttle D.J., Kembhavi A.A., Sharp S.L., Shute R.E., Rich D.H., and Barrett A.J., Affinity purification of the novel cysteine proteinases papaya proteinase IV, and papain from papaya latex, *Biochem. J.*, 1989; 261: 469-476.

[13] Dekeyser P.M., Smedt S.D., Demeester J., and Lauwers A., Fractionation and purification of the thiol proteinases from papaya latex, *J. Chromatog. B*, 1994; 656: 203-208.

[14] Nitsawang S., and Kanasawud P., A rapid process for purification of chitinase from the latex of *Carica papaya*, *Chiang Mai J. Sci.*, 2006; 33: 3-8.

[15] Moussaoui A.E.I., Nijs M., Paul C., Wintjens R., Vincentelli J., Azarkan M., and Looze Y., Revisiting the enzymes stored in the laticifers of *Carica papaya* in the context of their possible participation in the plant defence mechanism, *Cell. Mol. Life Sci.*, 2001; 58: 556-570.

[16] Brocklehurst K., and Salih E., Fresh non-fruit latex of *Carica papaya* contains papain, multiple forms of chymopapain A and papaya proteinase Ω, *Biochem. J.*, 1985; 228: 525-526.

[17] Azarkan M., Wintjens R., Looze Y., and Baeyens-Volant D., Detection of threee wound-induced proteins in papaya latex, *Phytochem.*, 2004; 65: 525-534.

[18] Azarkan M., Dibiani R., Baulard C., and Baeyens-Volant D., Effects of mechanical wouding on *Carica papaya* cysteine endopeptidases accumulation and activity, *Inter. J. Biol. Macro.*, 2006; 38: 216-224.

[19] Theppakorn T., *Production, stability and behavior in non-conventional media of cysteine proteases from papaya (Carica papaya* L.*) latex*, PhD Thesis, Chiang Mai University, Thailand, 2003.

[20] Arnon R., Papain, *Methods Enzymol.*, 1970; 19: 226-242.

[21] Bradford M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, *Anal. Biochem.*, 1976; 72: 248-254.

[22] Nitsawang S., Hatti-Kaul R., and Kanasawud P., Purification of papain from *Carica papaya* latex: Aqueous two-phase extraction versus two-step precipitation, *Enzyme Microb. Technol.*, 2006; 2006; 39: 1103-1107.

[23] Reisfeld R.A., Lewis U.J., and Williams D.E., Disk electrophoresis of basic proteins and peptides on polyacrylamide gel, *Nature*, 1962; 195: 281-183.

[24] Hames B.D., *Gel electrophoresis of proteins: A practical approach*, Oxford and Washington D.C.: IRL Press, 1981: 130.

[25] Friedman M., Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences, *J. Agric. Food Chem.*, 2004; 52: 385-406.

[26] Kamaruzzaman M., Chowdhury S.D., Podder C.K., and Pramanik M.A.H., Dried papaya skin as a dietary ingredient for broiler chickens, *British Poultry Sci.*, 2005; 46: 390-393.

[27] Kang C.K., and Warner W.D., Tenderization of meat with papaya latex proteases, *J. Food Sci.*, 1974; 39: 812-818.

[28] Goodenough P.W., and Owen J., Chromatographic and electrophoretic analyses of papaya proteinases, *Phytochem.*, 1987; 26: 75-79.

[29] Sumner I.G., Vaughan A., Eisenthal R., Pickersgil R.W., Owen A.J., and Goodenough P.W., Kinetic analysis of papaya proteinase Ω, *Biochim. Biophys. Acta*, 1993; 1164: 243-251.

[30] Walsh G., *Proteins biochemistry and biotechnology: Casein biochemistry*, 1st Edn., England: John Wiley & Sons, 2002.

[31] Guo M.-L., and Jiang Y.-M., Monitoring the hydrolysis of protein using the Coomassie Brilliant Blue protein assay, *J. Biochem. Mol. Biol. & Biophys.*, 1998; 1: 295-298.