CHAPTER 3
RESULTS

3.1 Preparation of proteases from papaya peels

In order to increase storage time, fresh papaya peels were dried in a tray-dryer until 10% w/w of the fresh was obtained. Papaya peel crude extract was prepared from dried papaya peels with 3 different methods. The best method was chosen to study the effect of cysteine and EDTA on efficiency of protease extraction. The crude extract possessing the highest proteolytic activity was then used to precipitate out the proteases.

3.1.1 Preparation of papaya peel crude extract

Papaya peel proteases were extracted from dried papaya peels by 3 different methods. For all 3 methods, the dried peels must be soaked with equal amount of water which was removed during drying the peels (90% w/w of the fresh peels) to refresh and to be easy to extract the enzymes. **Table 3.1** shows the first method of arid blending followed by soaking with 180 mL water resulted as the best efficacy for proteases extraction. For 20 g of dried papaya peels, it consumed only 15 min to provide the highest proteolytic activity of 260 units, whereas the last two methods needed 185 min for soaking and blending the peels and gave proteolytic activity of 115 units and 175 units for blending with and without water, respectively. In case of the last procedure, this was carried out followed by the method of Kanasawud *et al.* [2001] which found that blending the peels with water at the ratio of 2:1 (fresh peels : water) provided the highest proteolytic activity. Because 400 mL of water was used during blending the peels, the method gave the larger volume of crude extract (about 3.3-3.4 times) than that of the first two methods. As shown in **Table 3.1**, due to the time minimisation and highest proteolytic activity obtained, the first method, arid blending and then soaking of dried papaya peels, was chosen for extraction of proteases.

Table 3.1 Comparison of extracting methods for papaya peel proteases extraction from 20 g dried (200 g fresh) papaya peels.

Method	Soaking		Blending		Volume of crude extract (mL)	Protease activity (units)
	Water used (mL)	Time consumed (min)	Water used (mL)	Time consumed (min)		
1. Arid blending followed by soaking	180	10	0	5	152	260
2. Soaking followed by blending without water	180	180	0	5	145	175
3. Soaking followed by blending with water	180	180	400	5	500	115

3.1.2 Effect of cysteine and EDTA on proteases extraction

As the activities of papaya cysteine proteases from latex are activated by addition of small reducing agent such as cysteine and a chelating agent like EDTA. Therefore, the effects of cysteine and EDTA on proteases extraction from papaya peels and on their stabilization were investigated. Two solutions, 40 mM cysteine and 40 mM cysteine-20 mM Na_2·EDTA, were used in comparison with the water as control. The proteolytic activity of papaya peel crude extracts was checked during storage at -20°C, 4°C and room temperature (around 30°C) for 14 days. Results showed the crude solutions contained proteolytic activities of 8.90, 8.85 and 8.77 units per gram dried papaya peels for extraction with water, cysteine solution and cysteine-EDTA solution, respectively. The three extractions provided approximately the same enzyme activities. This indicated that neither cysteine nor EDTA had any significant effect on extraction of papaya peel protease.

All the three crude solutions were stored at -20°C, 4°C and room temperature for examination of the enzyme stability. As results shown in **Figure 3.1-3.3**, the profiles of enzyme stability were quite similar in all storage temperatures. The stability of proteases at 3 temperatures was satisfying within 4 days and after that, it was rapidly decreased. The proteases in the crude extract stored at -20°C were a bit more stable than those stored at 4°C and room temperature, respectively (**Figure 3.1-3.3**). It is notable that even

cysteine or cysteine-EDTA solution were used, the enzyme stability was not different from the water extraction. Therefore, water was the most suitable to extract proteases from papaya peels and the crude solution should be kept at -20°C within 4 days.

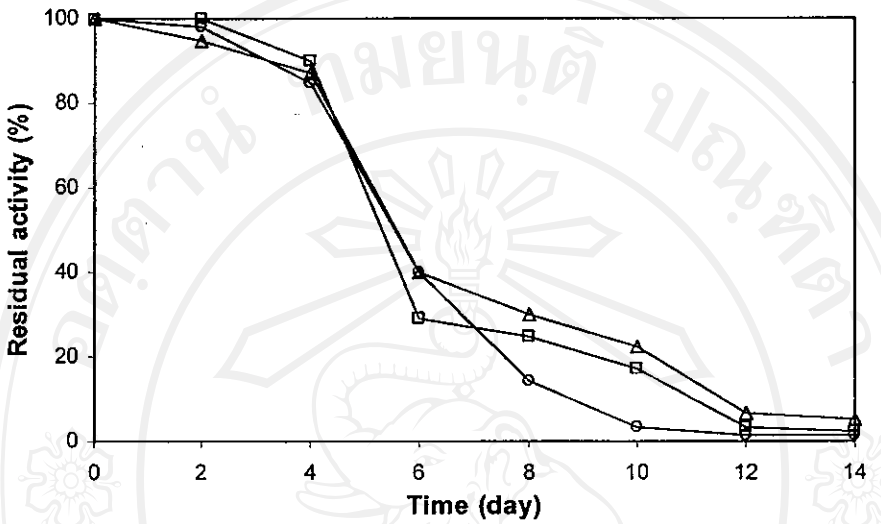


Figure 3.1 Residual protease activity of papaya peel crude extract at room temperature. The peels were extracted by water (○), 40 mM cysteine (□) and 40 mM cysteine-20 mM Na_2·EDTA (△).

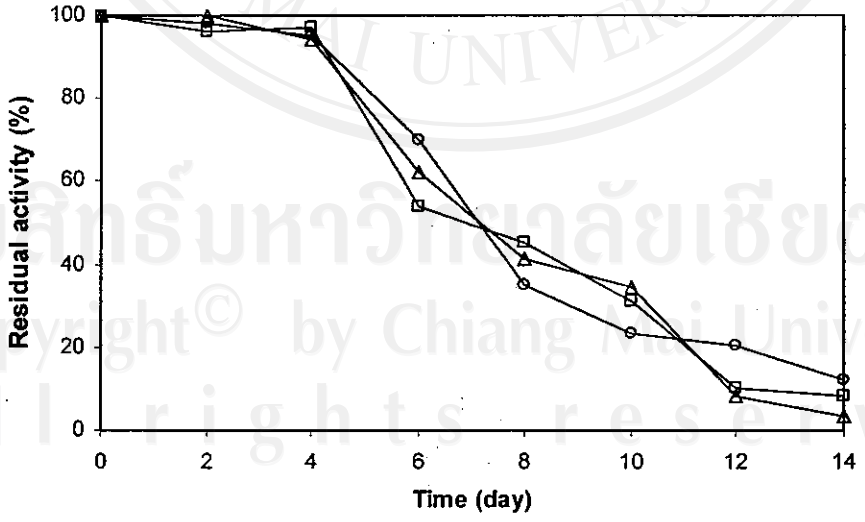


Figure 3.2 Residual protease activity of papaya peel crude extract at 4°C. The peels were extracted by water (○), 40 mM cysteine (□) and 40 mM cysteine-20 mM Na_2·EDTA (△).

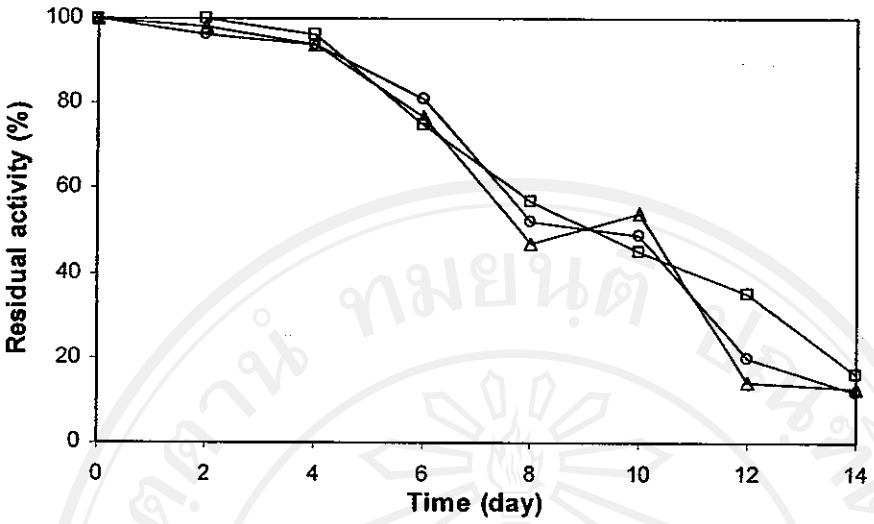


Figure 3.3 Residual protease activity of papaya peel crude extract at -20°C. The peels were extracted by water (○), 40 mM cysteine (□) and 40 mM cysteine-20 mM Na_2·EDTA (△).

3.1.3 Yield of papaya peel protease preparation

Papaya peel proteases were prepared by four different precipitations or spray drying. Each 38 mL of the extract from 5 g dried papaya peels contained 44 units of proteolytic enzyme activity and 41 mg of protein. This activity was assigned as 100% yield. Results in **Table 3.2** showed that the highest proteolytic activity yield of 57.61% was obtained by precipitation with 70% (v/v) ethanol. Among the three alcohols used, 70% ethanol possessed the highest efficiency for proteases separation, followed by 67% (v/v) 2-propanol and 75% (v/v) methanol which provided the yield 52.75% and 50.47%, respectively. All three precipitants have been previously used for precipitation of cysteine proteases from papaya latex at the same of their concentrations [Lesuk, 1986]. The precipitation with 60% saturated ammonium sulphate resulted as the lower yield of proteases (37.04%) than those using organic solvents (50.47-57.61%). From initial study, 60% saturated ammonium sulfate was able to separate efficiently most of proteases from papaya latex (86.2% yield). It is notable that all protease powders contained high ratio of non-proteolytic protein to proteases that reflected rather low of specific activities. Furthermore, the yields per gram of papaya proteases powder obtained from the peels were approximately 10 times lower than the dried latex enzyme (1623

units/g). Because of the highest yield obtained, papaya peel proteases precipitated by 70% ethanol was thus used for further experiments.

Table 3.2. Separation of papaya peel proteases from 38 mL of crude extract obtained from 5 g of dried papaya peels.

Protease	Proteolytic activity (u)	Proteolytic yield (%)	Protein (mg)	Specific activity (u/mg)
Papaya peel crude extract	44.00	100	41.00	1.07
Precipitation by				
methanol 75% (v/v)	22.21	50.47	15.42	1.44
ethanol 70% (v/v)	25.35	57.61	15.09	1.68
2-propanol 67% (v/v)	23.21	52.75	17.32	1.34
60% sat. AS	16.30	37.04	14.17	1.15

3.1.4 Spray dried papaya peel proteases

Papaya peel proteases were also prepared by drying the crude extract with the built spray dryer. Because the papaya peel crude solution was much diluted, it resulted as very low yield of both spray dried powder (4.84%) and proteases activity (4.0%). Therefore, 20% w/v of ammonium sulfate was added to obtain the higher solid yield. Result showed increasing of the powder yield to 33.8% with protease activity up to 21.6%. Nevertheless, the yields obtained were still rather low when compared with that of the solution before drying. One can observe from the experiment that most of crude solution that dispersed by nebuliser was stuck inside the wall of drying chamber. Assuming the whole papaya peel solution was completely spray dried that retained all of the solid content, the result was consequently interpreted in **Table 3.3**. The proteolytic yield was relatively high at 91.2% with specific activity of 1.18 u/mg. However, a substantial amount of ammonium sulfate consisting in this spray dried proteases may be limited their further applications.

Figure 3.4 shows appearance of papaya peel proteases powders obtained from various precipitations above and from spray drying comparing with dried papaya latex (crude papain). The proteases precipitated by methanol and ammonium sulfate were

more yellow dark than those obtained from ethanol and 2-propanol. Spray dried proteases appeared the most whitening powder and quite higher containing moisture. From the observation, the proteases obtained by ammonium sulfate precipitation and spray drying were easier water-soluble than the other was. This was suggested that the presence of ammonium sulfate increased solubility of the enzymes as salting-in effect.

Table 3.3 Spray dried proteases from 38 mL of papaya peel crude extract (AS was added giving 20% w/v of final concentration before spray drying).

Sample	Solid content (% w/v)	Proteolytic activity (u)	Yield (%)	Protein (mg)	Specific activity (u/mg)
Crude extract	8.84*	42.0	100	40.5	1.04
Spray dried proteases	-	38.3	91.2	32.4	1.18

* obtained from drying the crude extract in an oven at 100°C until the solid content was stable. This was assumed as 100% yield of spray dried powder.

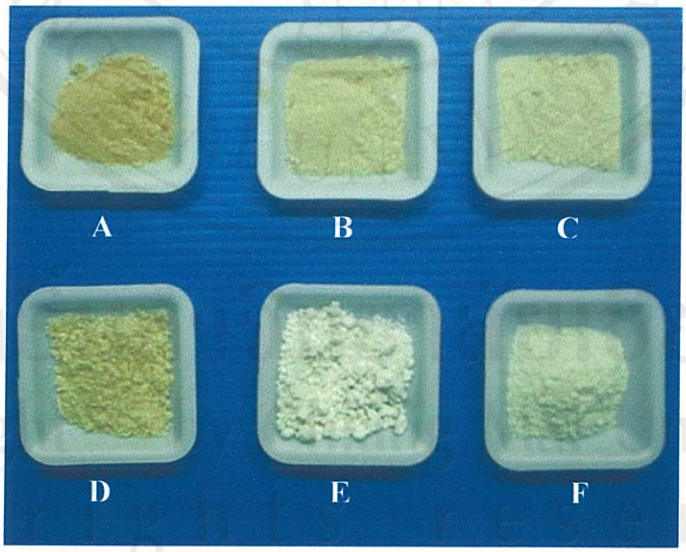


Figure 3.4 Typical papaya peel proteases in comparison with dried papaya latex (F). The proteases obtained from precipitation with 75% methanol (A), 70% ethanol (B), 67% 2-propanol (C) and 60% sat. ammonium sulfate (D), and spray drying with addition of 20% ammonium sulfate (E).

3.2 Comparison of enzyme catalysis between proteases from papaya peel and latex

Latex proteases, referring as crude papain or spray dried papain forms, have been well studied for their optimal catalysis properties, while the peel proteases have not been reported. Therefore, proteolytic properties of papaya peel enzymes, including optimal pH and temperature, stability in various pHs and at various temperatures and cysteine activation were investigated by comparison to the latex proteases. The assays of proteolytic activity were carried out by casein hydrolysis method.

3.2.1 Optimal pH

Results of caseinolytic activity at various pHs of proteases from papaya peels and latex are shown in **Figure 3.5**. Papaya peel proteases maximally hydrolysed casein at pH 8.0 and remained 62% at pH 7.0 which was an optimum activity of proteases from the latex. Higher or lower pH of their optimum decreased the activity of both enzymes. Their activities were nearly 0% at pH 2.0. Difference in optimal pH seems likely to imply that unequivalent components in both papaya enzymes.

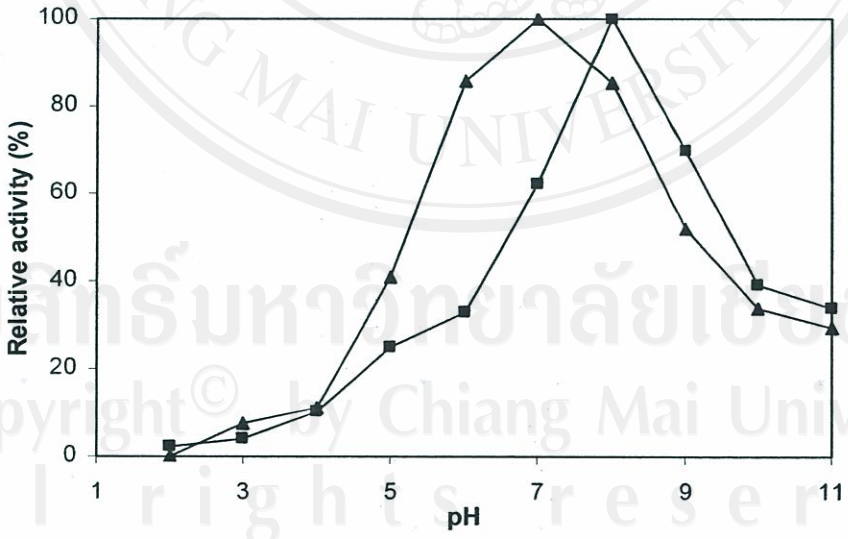


Figure 3.5 Optimal pH at 37°C on casein hydrolyses of papaya peel proteases (■) and papaya latex proteases (▲).

3.2.2 Optimal temperature

Optimal temperature of proteases from papaya peels and latex were also investigated. The enzymes were allowed to hydrolyze casein at various temperatures for 10 min. As result shown in **Figure 3.6**, both papaya peel and latex proteases showed the highest casein hydrolyses at 75°C. Moreover, they also possessed the same activity patterns at all ranging of temperatures tested. Their activities were increased when raising the temperatures from 30 to 75°C which possessed the optimum hydrolysis. The enzyme activities were sharply decreased after 75°C and almost inexistence at 90°C.

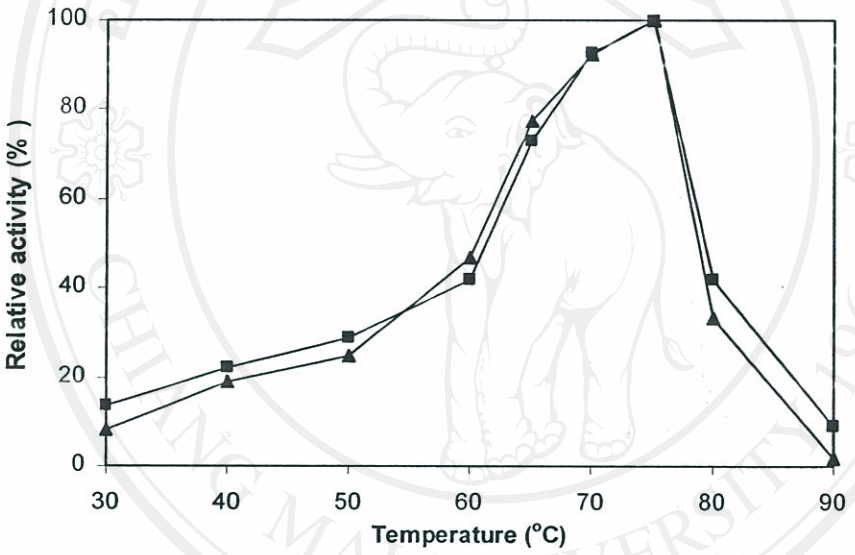


Figure 3.6 Optimal temperature on casein hydrolyses in Tris-HCl buffer pH 8.0 of papaya peel proteases (■) and papaya latex proteases (▲).

3.2.3 Stability at various pHs

The stability of proteases from papaya peels and latex were determined by incubation them in various pH buffers for 10 min before measuring their activities in buffer pH 8.0 at 37°C. Results clearly shown in **Figure 3.7** that proteases from the peels were more stable in pH 8.0-10.0 than those from the latex, especially at pH 10.0 which papaya peel proteases possessed 32% higher stability than that of latex proteases. On the other hand at pH lower than 7.0, stability of papaya peel proteases was substantial lower than those of the latex enzymes.

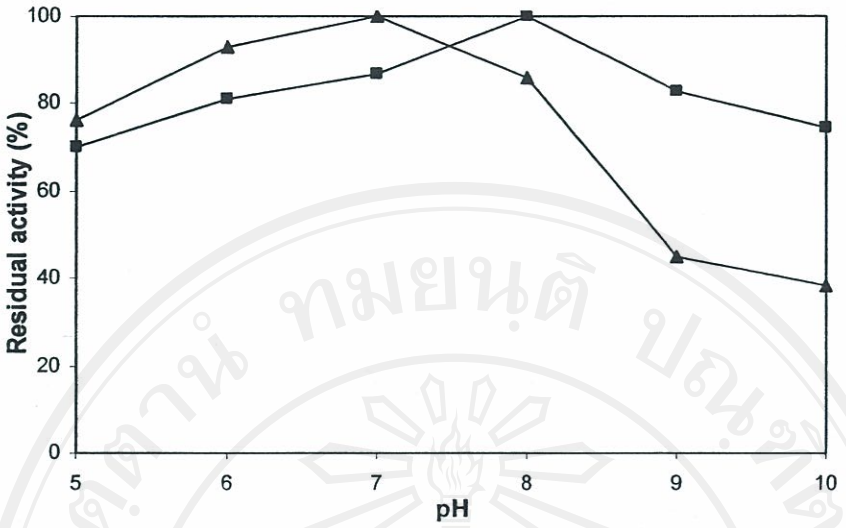


Figure 3.7 Stability of papaya peel proteases (■) and latex proteases (▲). The enzymes were incubated in various pH buffers before determining their proteolytic activities in pH 8.0 at 37°C.

3.2.4 Stability at various temperatures

Enzyme stability at various temperatures was also investigated. Papaya peel and latex proteases were incubated at temperature ranging 20-80°C for 10 min before examining their activities in buffer pH 8.0 at 37°C. **Figure 3.8** demonstrates both proteases showed good stability up to 60°C and their activities slightly declined at 70°C. Papaya peel proteases remained 60% of starting activities at 80°C, whereas latex proteases rapidly lost stability which remained 28% of their starting point. The result clearly showed that papaya peel proteases can resist the high temperature greater than those of the latex enzymes.

3.2.5 Effect of cysteine

Reducing agent such as cysteine functions to convert reversibly inactive forms of enzymes to the active forms and protects their catalysed-essential thiol group from oxidation. Thus, the effect of cysteine concentration on enzyme catalysis was investigated. **Figure 3.9** shows that both papaya peel and latex proteases were maximally activated by 5 mM cysteine. The activation in the papaya peel proteases appeared to be 1.6 times greater than those of the latex enzymes, illustrating the existence of higher amount of reversibly inactive form in papaya peel proteases. The constant effect at

cysteine concentration higher than 5 mM indicated the full activation of enzymes at this concentration.

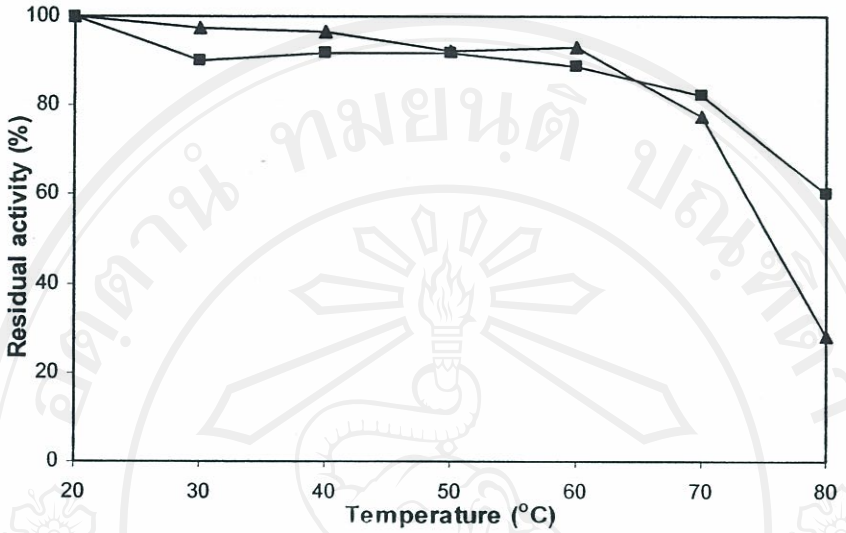


Figure 3.8 Stability of papaya peel proteases (■) and latex proteases (▲). The enzymes were incubated in various temperatures before determining their proteolytic activities at pH 8.0 at 37°C.

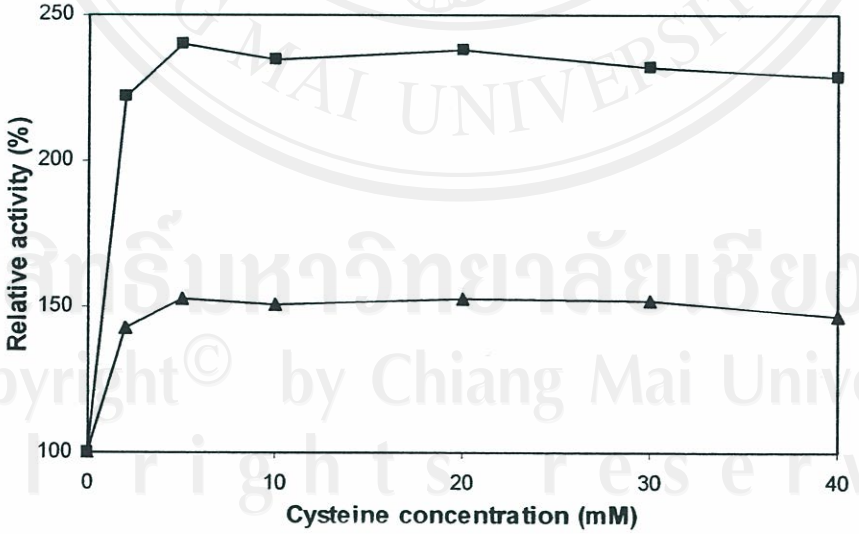


Figure 3.9 Effect of cysteine on caseinolytic activities of proteases from papaya peels (■) and latex (▲) in buffer pH 8.0 at 37°C. The activities of the two reactions without cysteine were given as 100% relative activity.

3.3 Comparison of protein composition between proteases from papaya peels and latex

The protein components in papaya peel proteases were investigated by comparing with the latex proteases and commercial standard papain. Three different methods were chosen for the study; cathodic and anodic polyacrylamide gel electrophoresis and anion-exchange FPLC.

3.3.1 Cathodic gel electrophoresis and *in situ* proteolysis

Native cathodic gel electrophoresis is a well-recognised method for investigation of papaya cysteine proteases from latex. According to difference in their charge and size, ability of the enzymes for moving forward to cathode pole in acidic condition is dissimilar. **Figure 3.10A** shows the mobility of papaya enzymes separated by cathodic electrophoresis. Papain, chymopapain, chitinase, glycyl endopeptidase and caricain in lane 2 correspond to electrophoresis patterns of previous reports [Buttle *et al.*, 1989; Dekeyser *et al.*, 1994; Monti *et al.*, 2000; Pendzhiev, 2002; Nitsawang *et al.*, 2006a; 2006b]. Caricain, the most basic enzyme migrated furthest in this acidic gel. Contrasting with the least basic enzyme papain moved nearest in the gel. Due to similarity in their size and charge [Dekeyser *et al.*, 1994], chymopapain and glycyl endopeptidase have practically almost the same electrophoretic properties. It is notable that papain was present in very small quantity in lane 2 due to fewer proportion of papain in this latex sample. Result from cathodic gel electrophoresis revealed that papaya peel proteases (**Figure 3.10A**, lane 4-5) consisted of papain presenting as a major component and heterogeneously separated proteins, one of them possibly displayed as chymopapain. They also contained some proteins locating above papain, implying that their pI values were lower than 8.75 of papain. In contrast, caricain and glycyl endopeptidase were absent in proteases from the peels. It should be noted that some quantity of papaya peel proteins in lane 3-5 of **Figure 3.10A** were unable to migrate and stuck at loading point of stacking gel.

Verification of proteolytic activities on electrophoresis bands of proteins is shown in **Figure 3.10B**. It was observed that papaya peel proteases contained papain, chymopapain, and at least one additional protease which was absent in the latex (see protease I in lane 3-5 of **Figure 3.10B**). As shown in this figure, papain proficiently hydrolysed casein on the gel and resulted as the clear zone due to its broad specificity of

peptide bond cleavage [Barrett *et el.*, 1998]. On the other hand, other proteases possess lower efficiency and/or higher specificity producing precipitation of parts of the casein, which were observed as the darker area. In case of papain and caricain in lane 2 of **Figure 3.10B**, their proteolytic activities could not be detected due to the low content of papain in this latex protease sample and instability of caricain on the acidic gel.

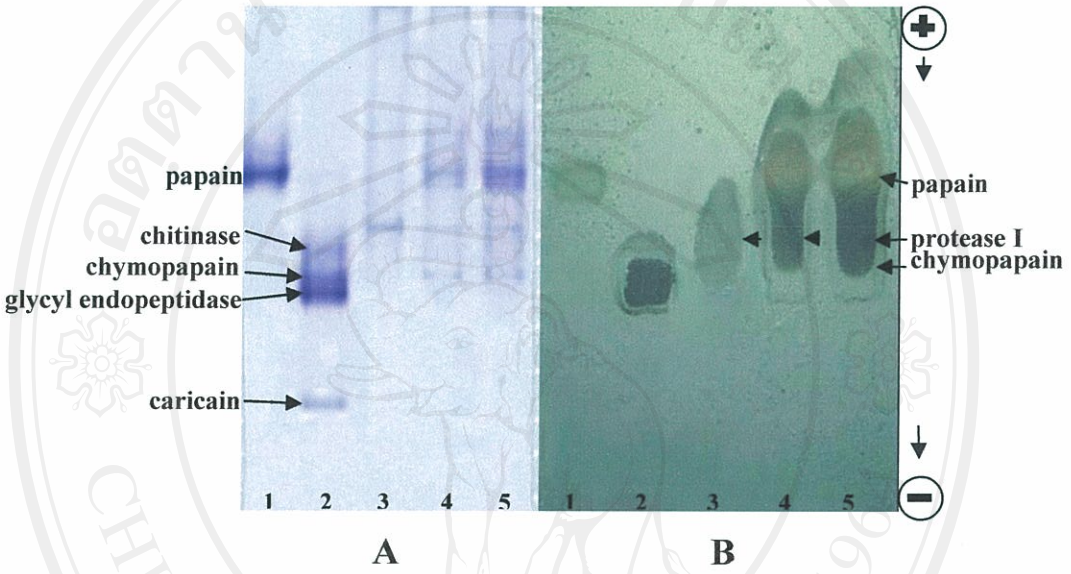


Figure 3.10 Separation of proteins by cathodic gel electrophoresis, stained with Coomassie Brilliant Blue (**A**) and *in situ* verifying their proteolytic activities (**B**). Lane 1; standard papain (3.7 µg protein), lane 2; crude papain (2.9 µg protein), lane 3; papaya peel crude extract (20.6 µg protein), lanes 4 & 5; papaya peel protease; (5.3 & 15.9 µg protein, respectively).

3.3.2 Anodic gel electrophoresis and *in situ* proteolysis

The enzyme components were examined by native anodic gel electrophoresis in association with assay proteolytic activity on the gel. As shown in **Figure 3.11A** and **B**, latex proteases and standard papain (lane 4 and 5, respectively) showed no band of proteins. As previously known, the four cysteine proteases in papaya latex–papain, chymopapain, glycyl endopeptidase, and caricain possess pI value more than pH 8.3 of electrode buffer used, therefore, these positive net charged proteases could not be moved toward anode. In contrast, the figure obviously shows proteases from papaya peels contained the proteins having pI value less than 8.3 (**Figure 3.11A**, lane 1, 2 and 3). The furthest migration protein (indicated by arrow) was present in proteases but disappeared

in the crude extract of papaya peels. On the contrary, this peel extract comprised of one major protein band unlike the peel proteases. Although the anodic gel electrophoresis showed the heterogeneous separation of papaya peel enzymes, one major protein displayed the proteolytic activity on the gel (protein I designated by arrow on lane 1 and 2 of **Figure 3.11B**). A casein digestion of papaya peel crude extract was not observed (**Figure 3.11B**, lane 3) possibly due to lower proportion of protease to non-proteolytic protein in the extract solution.

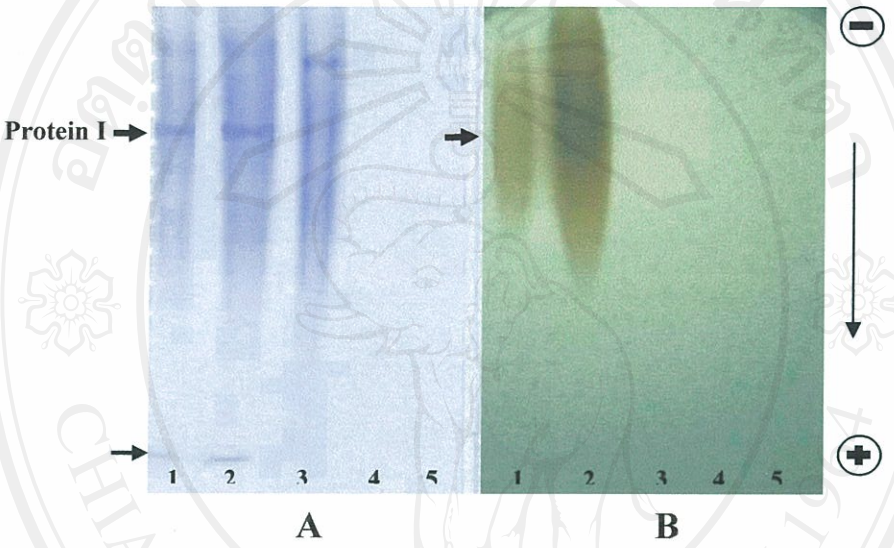
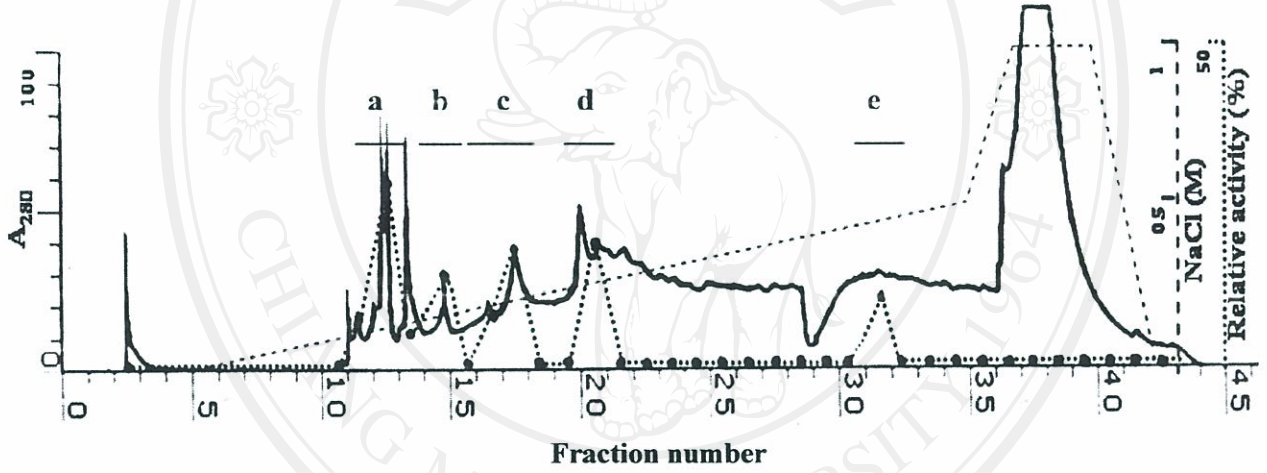


Figure 3.11 Separation of proteins by anodic gel electrophoresis, stained with Coomassie Brilliant Blue (**A**) and *in situ* verifying their proteolytic activities (**B**). Lane 1 & 2; papaya peel protease (6.4 & 12.7 μg protein, respectively), lane 3; papaya peel crude extract (20.6 μg protein), lanes 4; crude papain (5.8 μg protein) and lane 5; standard papain (7.4 μg protein).

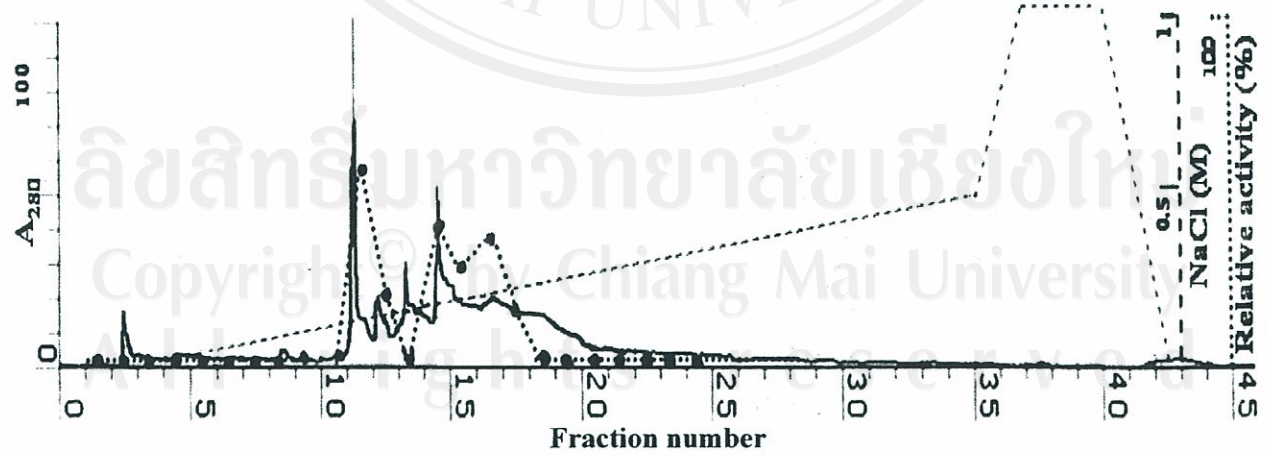
3.3.3 Mono Q column FPLC

Figure 3.12A shows the elution profile of papaya peel proteases eluted from Mono Q anion-exchange column attached to FPLC. The results revealed that the peels are composed of more proteins than those of the latex, which were completely eluted before fraction 20 (**Figure 3.12B**). The main components in papaya peel proteases are highly negative charged molecules, which were eluted by the highest ionic strength of NaCl (fraction 36-39). Their absorbances at 280 nm indicated that the molecules contain aromatic substances. Due to their negative results to Bradford reagent, they should not be

protein molecules but probably are the hydrolysed products of protein. The first peak containing protein which unbound to the column and absent of caseinolytic activity (**Figure 3.11A**, fraction 2-3), has been reported as a chitinase [Goodenough *et al.*, 1987]. Determination of proteolytic activity in each fraction revealed that papaya peel proteases had the enzymatic activities in 5 pools (a-e). The pools a and b were similar to elution profile of caricain, glycyl endopeptidase and chymopapain of papaya latex proteases, while pool c was identical to standard papain fraction. Papaya peel proteases also displayed the caseinolytic activity at pools d and e (fraction 21 and 32) which were absent in the latex proteases. This confirmed the result of *in situ* enzyme activity on the gel of **Figure 3.11**, illustrated that some proteases which pI values were less than papain presenting in papaya peels but not in the latex.



(A)



(B)

Figure 3.12 Anion-exchange FPLC of papaya peels (A) and latex (B) proteases, eluted with a linear gradient of NaCl (---). Fractions were collected and analysed by measurement the absorbance at 280 nm (—) and proteolytic activity toward casein (····).

3.4 Presence of glycyl endopeptidase in papaya peels and latex

The results of Section 3.3 showed the difference in protein components of proteases from papaya peels and latex. Because glycyl endopeptidase displays as a major composition in the latex, it should be interesting to investigate this enzyme in papaya peels. If it was found in adequate amount, papaya peels may be used as an alternative source of glycyl endopeptidase. The papaya proteases were separated by cathodic gel electrophoresis followed by measurement the protein bands by a densitometer. As **Figure 3.13** shown, latex proteases contain papain, chitinase, chymopapain, glycyl endopeptidase and caricain that were identified by N-terminal analysis (Nitsawang, personal communication) and possessed relative mobility (Rm) 0.34, 0.46, 0.51, 0.54 and 0.71, respectively. Protein bands in papaya peel proteases and the crude extract were quite different from the latex. As least 6 proteins (protein I to VI) were found in the peels, but not present in the latex. Moreover, glycyl endopeptidase was not observed in papaya peels, while it appeared in the largest proportion in the latex.

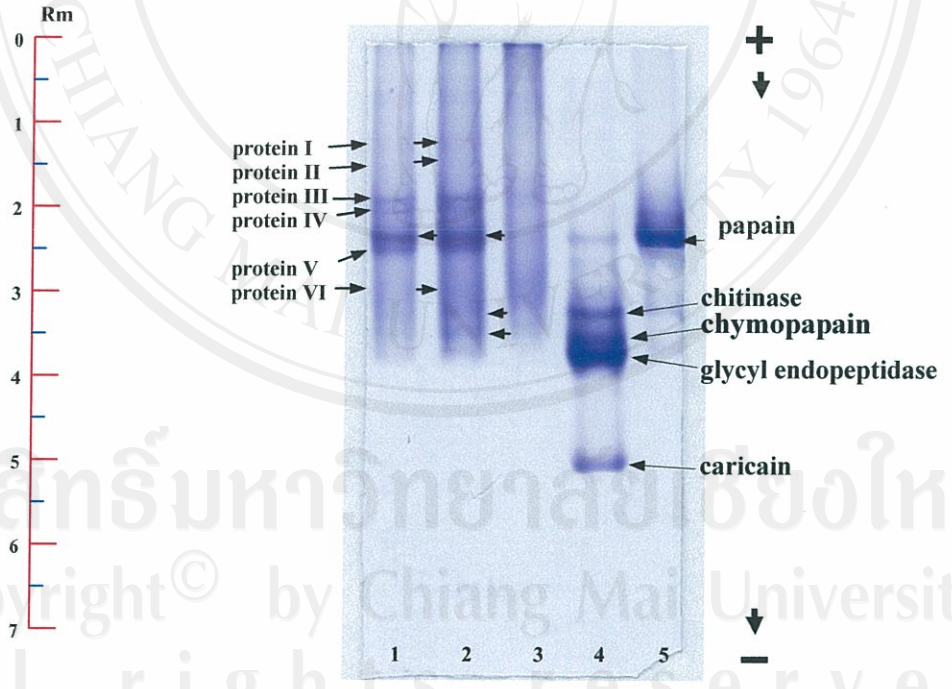


Figure 3.13 Separation of proteins by cathodic gel electrophoresis, stained with Coomassie Brilliant Blue. Lanes 1 and 2; papaya peel protease; (9.0 & 17.09 μg protein, respectively), lane 3; papaya peel crude extract (20.0 μg protein), lane 4; latex proteases (9.0 μg protein) and lane 5; standard papain (8.0 μg protein).

Protein bands of papaya peel proteases in lane 2, latex proteases in lane 4 and standard papain in lane 5 of the gel in **Figure 3.13** were investigated by densitometric analysis. The intensity of each band was interpreted by the peak area of densitogram. The relative mobility and percentage area of each protein on the gel was shown in **Table 3.4**. Standard papain from sigma contained one major component papain (peak 1) consisting at 97.470% and small amount of two impurities (**Figure 3.14 and Table 3.4**). The relative mobility indicated that these two purities were identical to chitinase and chymopapain. Protein bands of latex enzymes were also determined. **Figure 3.15** shows intensity of each five components which the major composition, glycyl endopeptidase, (peak 4) existing at 48.309%. Papain (peak 1) which was present in small amount in this latex sample appeared in rather low intensity reflecting to 1.323% of the total (**Table 3.4**). Chymopapain peak 3 appearing 25.003% was quite similar to glycyl endopeptidase on their electrophoretic properties. As a result, their intensive peaks were probably overlapped (**Figure 3.15 and Table 3.4**). Densitometric analysis of papaya peel proteases was shown in **Figure 3.16 and Table 3.4**. The proteases were composed of four major bands and five of minor proteins. In comparison to the relative mobility of standard papain and latex proteases, papaya peel enzymes were composed of papain (peak no. 5) as a major component at 29.749%. The band of protein V which very close to papain (peak 6) was existed at 18.384%. It should be noted that glycyl endopeptidase and caricain were not detected in the peels.

Results of triplicates of densitometric analyses revealed that glycyl endopeptidase was not present in papaya peels. Therefore, if this enzyme was desired, papaya latex containing the highest proportion of the glycyl endopeptidase should be chosen.

Table 3.4 Relative amount of papaya enzymes from fruit peels and latex. The enzymes were separated by cathodic gel electrophoresis and analysed by densitometry.

Papaya enzyme	No.	Relative mobility (Rm)	Area	Relative area (%)
1. Standard papain (**Figure 3.14**)	-	-	1431.071	100.00
papain	1	0.34	1394.827	97.470
chitinase	2	0.45	24.017	1.678
chymopapain	3	0.50	12.227	0.854
2. Papaya latex proteases (**Figure 3.15**)	-	-	2449.695	100.00
papain	1	0.34	32.421	1.323
chitinase	2	0.45	427.877	17.467
chymopapain	3	0.50	612.502	25.003
glycyl endopeptidase	4	0.54	1183.416	48.309
caricain	5	0.71	193.479	7.898
3. Papaya peel proteases (**Figure 3.16**)	-	-	1778.333	100.00
protein I	1	0.18	12.984	0.730
protein II	2	0.21	20.535	1.155
protein III	3	0.27	167.200	9.402
protein IV	4	0.30	163.554	9.197
papain	5	0.34	529.031	29.749
protein V	6	0.36	326.929	18.384
protein VI	7	0.43	264.405	14.868
chitinase	8	0.46	168.143	9.455
chymopapain	9	0.51	125.552	7.060

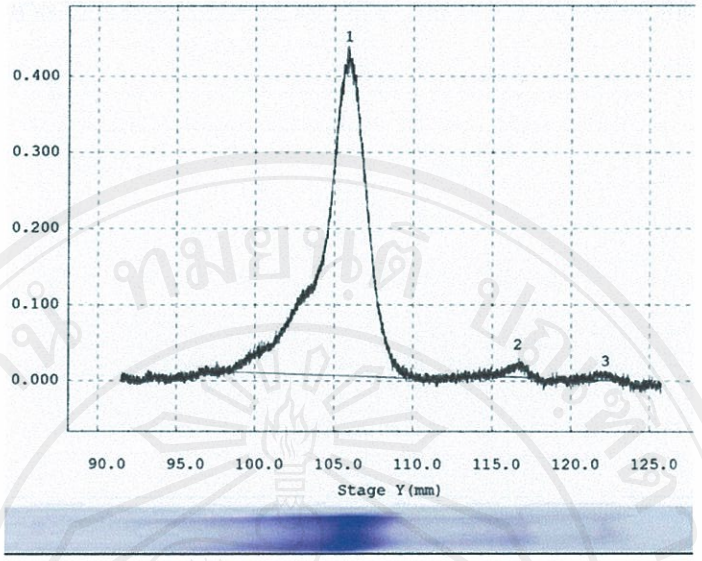


Figure 3.14 Protein band intensity of standard papain in lane 5 of **Figure 3.13**. Band No. 1; papain, No. 2 and 3; chitinase and chymopapain, respectively.

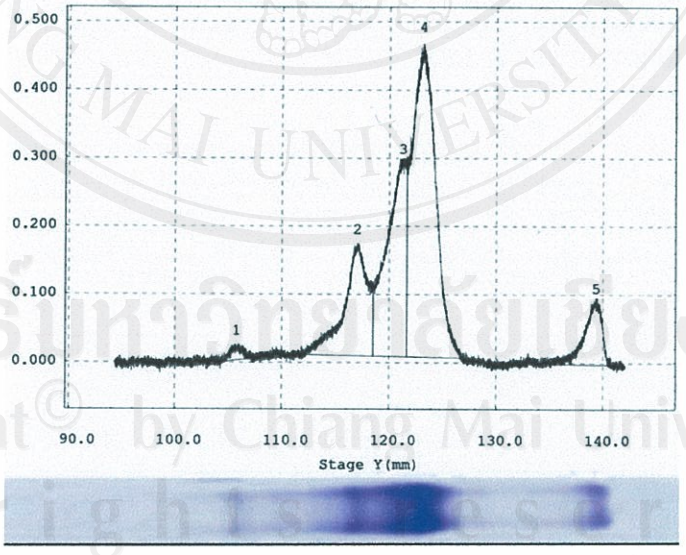


Figure 3.15 Protein band intensity of latex proteases in lane 4 of **Figure 3.13**. Band No. 1; papain, No. 2; chitinase, No.3; chymopapain, No. 4; glycyl endopeptidase and No. 5; caricain.

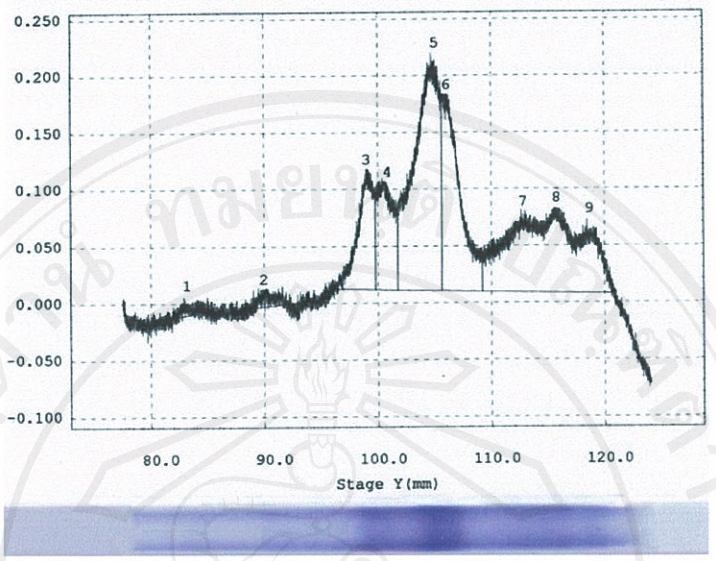


Figure 3.16 Protein band intensity of papaya peel proteases in lane 2 of **Figure 3.13**. Band No. 1-4; protein I-IV, respectively, No. 5; papain, No. 6; protein V, No. 7; protein VI, No. 8; chitinase and No. 9; chymopapain.

3.5 Purification of glycyl endopeptidase from fresh papaya latex

Because papaya latex contains a large quantity of glycyl endopeptidase, it was chosen for the source of enzyme purification. Glycyl endopeptidase was purified from fresh papaya latex by a two-step process. The first step was carried out by 6% PEG-15% $(NH_4)_2SO_4$ aqueous two-phase system. Papain was totally extracted into a PEG rich top phase, whereas other papaya enzymes were consisted in the salt rich bottom phase. The PEG and the salt phase were easily separated by high speed centrifugation. As shown in **Table 3.5**, the aqueous phase after removed papain was the salt enrichment containing most of the papaya enzymes. The further process for enzyme purification was the two-step salts precipitation with $(NH_4)_2SO_4$ and NaCl. After separated out chymopapain and caricain by precipitation with 11.3% $(NH_4)_2SO_4$, the clarified solution contained glycyl endopeptidase and chitinase possessing 76.0% of proteolytic yield. The precipitation out of chitinase by 20% NaCl separated the glycyl endopeptidase in the supernatant which was later precipitated by 33% NaCl. After re-dissolving, the enzyme contained 56.2% proteolytic yield. After dialysis overnight and lyophilization for 8 h, the enzyme powder

recovered 48.2% of proteolytic yield with 2.57 folds of purification. It was observed that the amount of active form of enzyme was quite similar during the process of purification (**Table 3.5**).

Table 3.5 Purification of glycyl endopeptidase from 100 g fresh papaya latex by aqueous two-phase following with salt precipitation. Enzyme activity was assayed by using Boc-Ala-Ala-Gly-pNA as substrate.

Purificaion step	Activity			Protein (mg)	Specific activity (u/mg)	Purification fold
	Total activity ($x10^6$ u)	% active form	Proteolytic yield			
1. Latex solution	9.94	57	100	4568	2176	1.00
2. Aqueous phase after removed papain	8.49	51	85.5	3516	2415	1.11
3. Solution after removed chymopapain and caricain	7.55	49	76.0	2749	2746	1.26
4. Glycyl endopeptidase before dialysis	5.58	52	56.2	1007	5541	2.55
5. Glycyl endopeptidase after lyophilization	4.79	59	48.2	856	5596	2.57

Electrophoresis patterns in **Figure 3.17** indicated that glycyl endopeptidase was successfully purified from papaya latex by this procedure. It can be totally separated from papain, chymopapain and caricain. Its purity of 93.47% was accounted by densitometric analysis of lanes 2 and 3 in **Figure 3.17**. However, densitogram revealed that the purified enzyme also contained chitinase as an impurity of 6.53%. One hundred grams fresh latex provided approximately 0.25 g of purified glycyl endopeptidase.

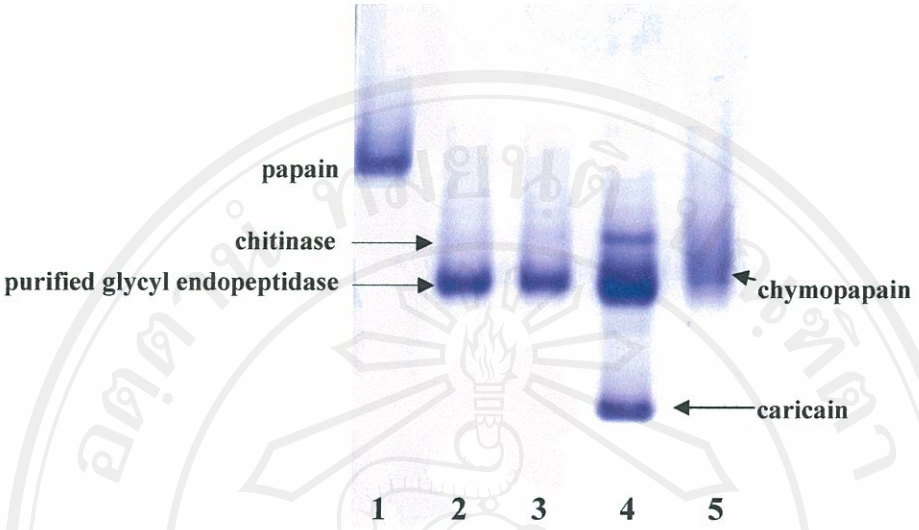


Figure 3.17 Cathodic gel electrophoresis of papaya cysteine proteases which lane 1; standard papain from Sigma (5.5 µg protein), lanes 2 and 3; purified glycyl endopeptidase (5.5 and 4.0 µg protein, respectively), lane 4; latex solution (12 µg protein) and lane 5; standard chymopapain from Sigma (5.5 µg protein).

3.6 Properties of purified glycyl endopeptidase

Glycyl endopeptidase purified from fresh papaya latex was studied its catalytic properties. Because preference of peptide bond cleavage of this enzyme is contrasting with other papaya enzymes, substrate specificity of glycyl endopeptidase was investigated. Other principal properties, including optimal pH and temperature, effect of activation time and activator and stability of enzyme were also determined. Results from the study would be beneficial for further applications of the enzyme.

3.6.1 Substrate specificity

The substrate specificity of glycyl endopeptidase was studied using two synthetic amide substrates, DL-BAPNA (Bz-Arg-pNA) and Boc-Ala-Ala-Gly-pNA. The first is a usual substrate for most of papaya proteases, but not glycyl endopeptidase [Buttle *et al.*, 1990b]. The second substrate contains glycine residue for discrimination in favour cleavage of glycyl endopeptidase. **Table 3.6** shows that glycyl endopeptidase

appears to hydrolyse only glycyl bonds efficiently which rapidly cleaved Boc-Ala-Ala-Gly-pNA. This substrate was less sensitive to papain and very low sensitivity to chymopapain. The initial rate of glycyl endopeptidase (93.47% purity) releasing *p*-nitroaniline from DL-BAPNA appeared to be 7.55 and 10.53% of the rate of papain and chymopapain, respectively. This is consistent with those previously reported that substrate specificity of glycyl endopeptidase is very different from that of other members of papaya cysteine proteases [Buttle *et al.*, 1990b].

Table 3.6 Selective hydrolysis of glycyl endopeptidase on the two amide synthetic substrates comparing with standard proteases and latex solution.

Protease	Initial rate (μmol min^{-1} mg protein^{-1})* toward	
	Boc-Ala-Ala-Gly-pNA	DL-BAPNA
1. Papaya latex	2718	89
2. Purified glycyl endopeptidase	7728	8
3. Papain (Sigma)	759	106
4. Chymopapain (Sigma)	12	76

* initial rate of releasing *p*-nitroaniline from both substrates.

3.6.2 Optimal pH

The effect of pH on the proteolytic activity of glycyl endopeptidase was investigated by using Boc-Ala-Ala-Gly-pNA as the substrate. As can be seen in **Figure 3.18**, the enzyme had an optimal pH of 7.5, but its activity still retained higher than 90% of the optimum at the pH around 6.5-7.0. It should be noted that glycyl endopeptidase was more active in higher pH than lower pH region. Its activity was mildly decreased in basic region and retained about 78% of the optimum at pH 12.0. On the other hand, its activity was extremely decreased at pHs lower than 6.5 and retained only 10% at pH 2.0. Even though glycyl endopeptidase maximally hydrolysed the substrate at the neutral, the enzyme was obviously active in basic media greater than the acidic one.

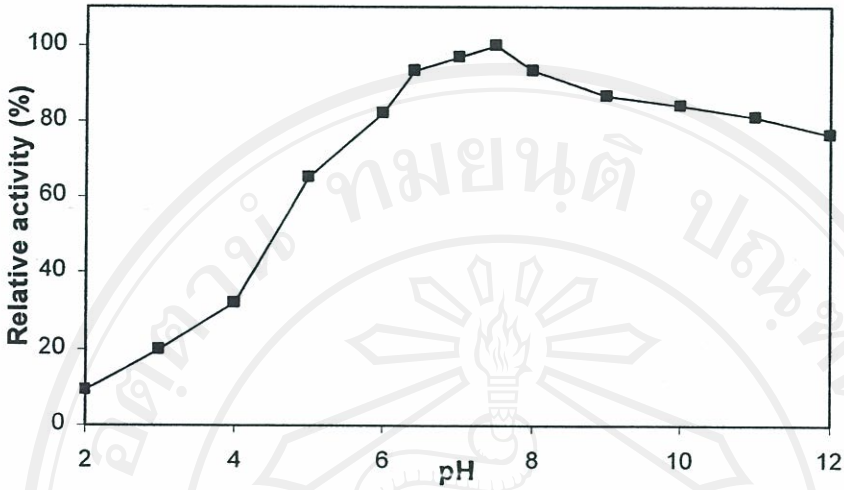


Figure 3.18 The pH-activity profile of glycyl endopeptidase. Buffer solutions were 50 mM sodium phosphate/phosphoric acid (pH 2, 7, 7.5 and 12); sodium citrate/ citric acid (pH 3 and 6); acetate/acetic acid (pH 4 and 5); citrate phosphate (pH 6.4); Tris-HCl (pH 8); sodium borate/ boric acid (pH 9 and 10) and glycine/NaOH (pH 11).

3.6.3 Optimal temperature

Effect of temperature on the proteolytic catalysis of glycyl endopeptidase was also examined. The result of enzyme activity toward Boc-Ala-Ala-Gly-pNA at various temperatures is shown in **Figure 3.19**. The enzyme was most active at the temperature 60°C, while 55°C its activity retained 96% of the optimum. At 20°C, the enzyme activity was 30% of the optimum and it was increased with respect to temperature extended to the optimal point. Over 60°C, activity of glycyl endopeptidase was rapidly decreased and subsequently retained about 8% at 80°C.

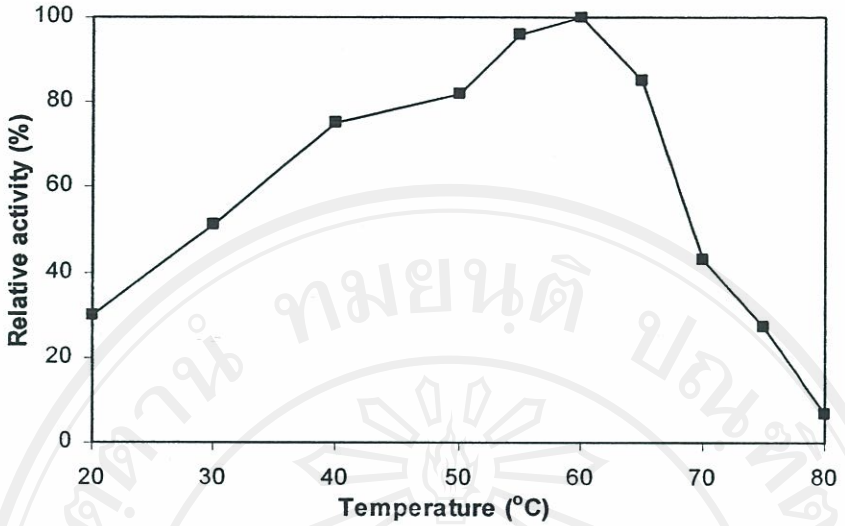


Figure 3.19 The temperature-activity profile of glycyl endopeptidase in sodium phosphate buffer pH 7.5 at temperatures ranging between 20 and 80°C.

3.6.4 Optimal activation before catalysis of enzyme

Effect of activation time and activator on enzyme catalysis was investigated. The enzyme, dissolved in buffer pH 7.5 or in normal activating agent, was incubated at 40°C at various time intervals before assay its activity. **Figure 3.20** shows that presence of activator (cysteine and EDTA) in the buffer increased about 120% of the enzyme activity compared to the absence. This signifies that activation of this enzyme before catalysis is important. It was found that incubating enzyme for 5 min was the most suitable for giving its activity. Higher activation time resulted as decline of glycyl endopeptidase activity.

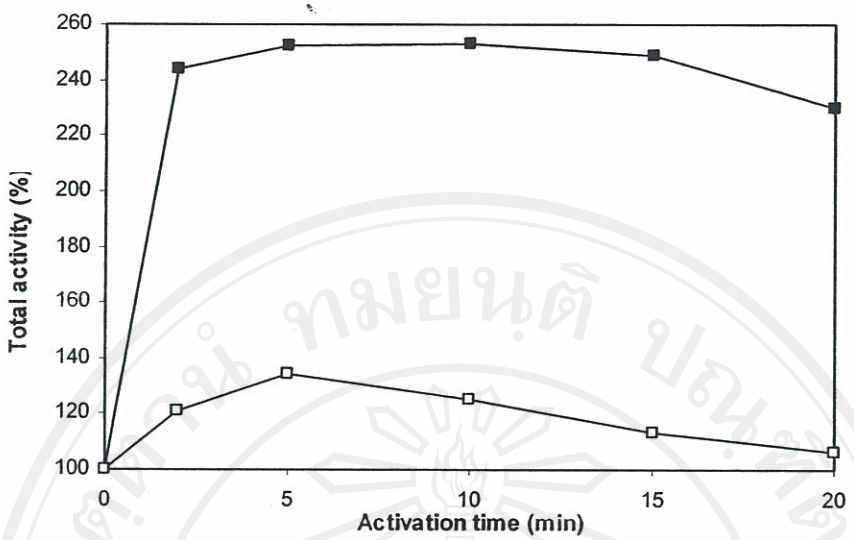


Figure 3.20 Effects of activator and incubation time on glycyl endopeptidase activity. The enzyme was dissolved in buffer pH 7.5 with presence (■) and absence (□) of cysteine and EDTA and then activated at 40°C at various times before determination of its activity.

3.6.5 Lack of inhibition by cystatin

As other members of the papaya cysteine proteases, glycyl endopeptidase would be expected to be inhibited by cystatin. With regard to substrate specificity, Boc-Ala-Ala-Gly-pNA was used as substrate for glycyl endopeptidase compared to other two enzymes which would hydrolyse DL-BAPNA. **Table 3.7** shows that initial rate of substrate hydrolysis of glycyl endopeptidase was not inhibited by chicken egg-white cystatin. It should be noted that differences in amidase activity between the reaction mixtures presence (7432 u/mg protein) and absence (7458 u/mg protein) of cystatin were not significant. In contrast, initial activities of papain and chymopapain were apparently disappeared when there was existence of chicken cystatin in the reaction mixture. This indicated that chicken cystatin was able to inhibit the activity of papain and chymopapain, like other member of papain family, but not glycyl endopeptidase.

Table 3.7 Amidase activity of papaya cysteine proteases with presence and absence of chicken cystatin.

Protease	Activity (u/mg protein)*	
	Absence of cystatin	**Presence of cystatin**
Glycyl endopeptidase	7458	7432
Papain (Sigma)	114	0
Chymopapain (Sigma)	109	0

* From hydrolysis of Boc-Ala-Ala-Gly-pNA for glycyl endopeptidase, and DL-BAPNA for papain and chympapain

3.6.6 Stability of glycyl endopeptidase

Purified glycyl endopeptidase was determined for its stability in both solid and soluble forms at room temperature and 40°C. It should be noted that 40°C was the temperature employed for further catalytic reaction by this enzyme. **Figure 3.21** shows stability of the enzyme storage at room temperature (approximately 30°C). Without any addition of activator, solid form of glycyl endopeptidase rapidly lost its acitivty to 76% over the first 3 days. After that, the enzyme seems likely to retain its activity during storage of 15 days. On the other hand, activity of soluble glycyl endopeptidase in buffer pH 7.5 was extremely decreased until 15% through the 15 days of storage.

Stability of glycyl endopeptidase at 40°C is shown in **Figure 3.22**. The solid form of enzyme activity was rapidly lost during first 2 days and then much more slowly over the next 13 days of storage. It was obvious that stability of glycyl endopeptidase at room temperature was greater than 40°C, even as solid form. Glycyl endopeptidase in buffer pH 7.5 at concentration 5% w/v exhibited truely low stability at high temperature. Its activity was extremely decreased over the first 5 days and then constantly declined to roughly 1% at the last day.

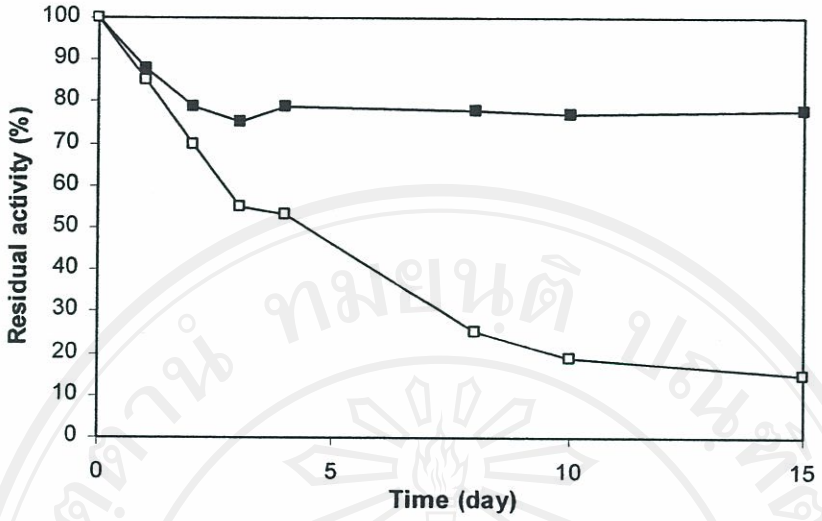


Figure 3.21 Stability of glycyl endopeptidase at room temperature (~30°C), the enzyme was incubated as solid form (■) and liquid form in buffer pH 7.5 at concentration of 5% w/v (□).

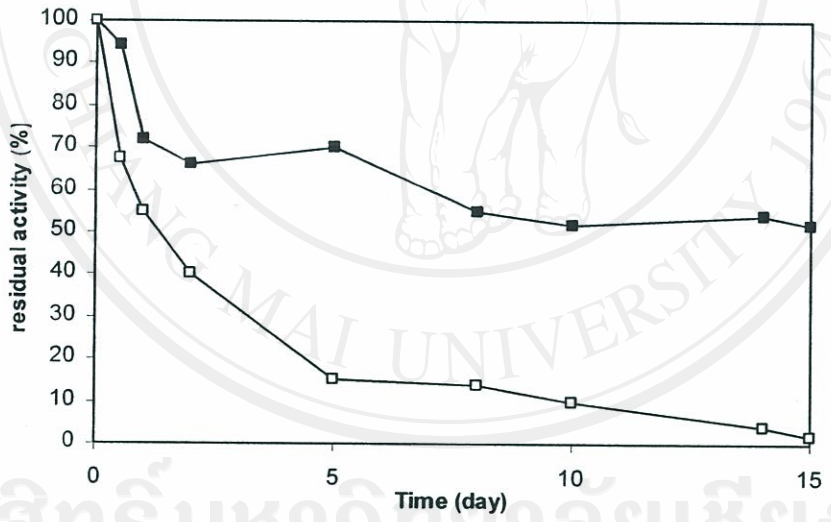


Figure 3.22 Stability of glycyl endopeptidase at 40°C, the enzyme was incubated as solid form (■) and liquid form in buffer pH 7.5 at concentration of 5% w/v (□).

3.6.7 Alteration of enzyme's form

Spray dried papain at solid content higher than 5% w/w retained its activity roughly 70% over storage period by changing its directly active to reversibly inactive form [Theppakorn, 2003]. This was taken place by maintenance stabilisation of enzyme. Therefore, it was interesting to investigate this change in glycyl endopeptidase as well.

Glycyl endopeptidase at a concentration of 50% w/w in phosphate buffer pH 7.5 was incubated at 40°C without any activating agent for analysis its alteration. The mixtures appeared as wet solid form at starting time and became visible as concentrated liquid after 5 h of incubation. As can be seen in **Figure 3.23**, the enzyme seems likely not altered it form apparently. At 0 h, glycyl endopeptidase exhibited in the form of directly active at 38% and reversibly inactive at 62%. The reversibly inactive form can be transformed to the active enzyme by treating with cysteine. Both direct and reversible inactive activities were gathered to be 100% of the total activity. At 27 h of incubation, the total activity of enzyme retained 75% of the starting. This activity was accounted from 2% of directly active and 73% of reversibly inactive enzyme. It can be seen that directly active enzyme was continuously decreased during incubation. On the other hand, the reversibly inactive enzyme was rather stable and it should be noted that this form was represent almost of the total activity of enzyme at 27 h. It is thus suggested that the reversibly inactive form prevented loss of stability of glycyl endopeptidase.

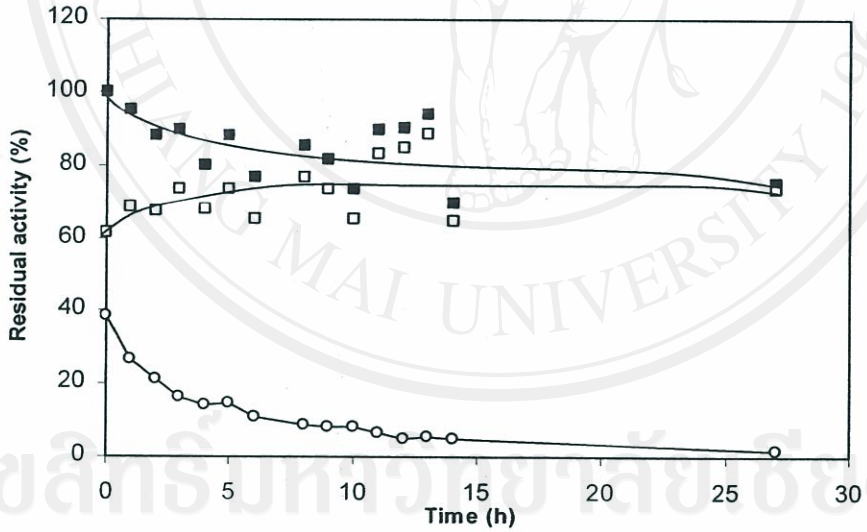


Figure 3.23 Change in total recoverable (■), directly active (○) and reversibly inactive (□) activities of 50% solid glycyl endopeptidase in phosphate buffer pH 7.5, incubated at 40°C. Lines just illustrate the trend.

Spray dried papain and clarified latex solution were investigated to compare with glycyl endopeptidase. **Figure 3.24** shows activity profile of 50% w/w spray dried papain in buffer solution pH 7.5. Total recoverable activity was decreased over the first 5

h and then increased in the next 6 days. At this time, the protease could not be activated with activating agent. This period is the transformation of enzyme from directly active to reversibly inactive form for protecting its activity loss [Theppakorn, 2003]. Directly activity of spray dried latex was still constant at the lower proportion over the testing. More clearly, **Figure 3.25** shows the alteration forms of the 16% solid clarified papaya latex. While directly activity was decreased since starting, reversibly inactivity was increased continuously. This indicated that directly active enzyme transformed to reversibly inactive enzyme.

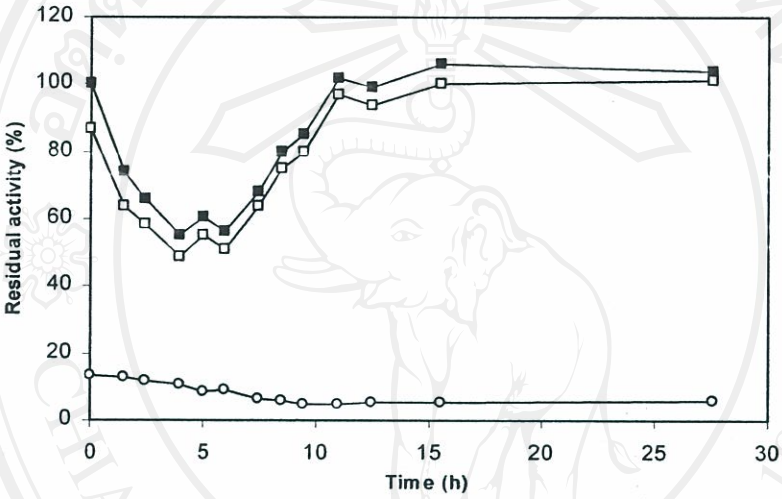


Figure 3.24 Change in total recoverable (■), directly active (○) and reversibly inactive (□) activities of 50% solid spray dried papain in phosphate buffer pH 7.5, incubated at 40°C.

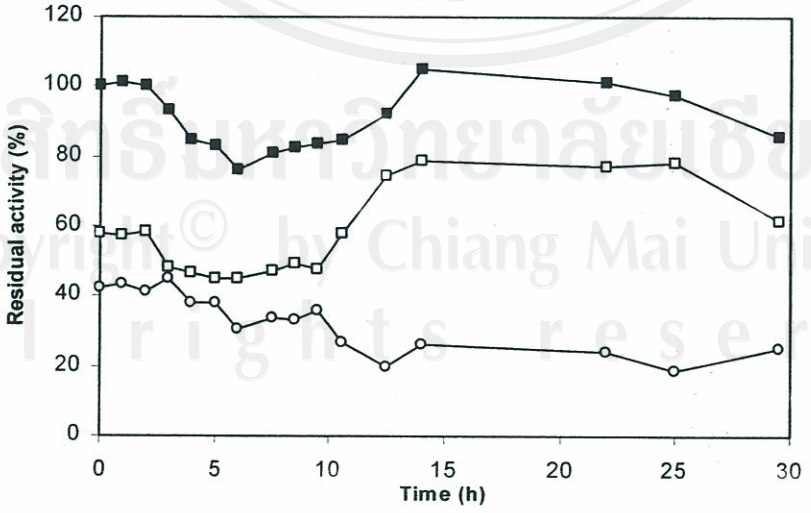


Figure 3.25 Change in total recoverable (■), directly active (○) and reversibly inactive (□) activities of 16% solid clarified papaya latex solution, incubated at 40°C.

3.7 Glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2

Purified glycyl endopeptidase was used to catalyse coupling of Z-Gly-OH and H-Phe-NH_2 via solid-to-solid system. Because this enzyme is greatly specific for N-terminal of glycine, Z-Gly-OH was consequently selected as an acyl donor. Nucleophile, H-Phe-NH_2 (free base), was used in this study. A number of principle parameters in the reaction were investigated including, substrate molar ratio, optimal enzyme amount, sources of acyl donor and effect of enzyme activators.

3.7.1 Effect of substrate molar ratios

Influent of various substrate molar ratios of Z-Gly-OH:H-Phe-NH_2 on the synthesis reaction was determined. **Figure 3.26** shows high dependence of reaction rate and yield of Z-Gly-Phe-NH_2 synthesis on the substrate molar ratios. Even a very slight excess of Z-Gly-OH, the rate of synthesis and final conversion was lead to a substantial increase. At higher excess of Z-Gly-OH (substrate ratios of 1.3:1, 1.5:1 and 2:1), a further increase in the final conversion was achieved, although the initial rate is slightly lower. The ratio of 2:1 seems to provide the highest conversion and it was consequently used in further experiments. It is noted that the scatter in the experimental points of solid reaction is greater than that of a typical reaction in solution, which is normal for these heterogeneous mainly solid systems. The experiments of substrate molar ratios at 1:2 and 1:3 were also carried out. However, no peptide product was found. This was probably resulted from inappropriate condition from the excess of H-Phe-NH_2.

Figure 3.27 shows, as a visual example, the different components of a typical reaction of glycyl endopeptidase catalysed solid-to-solid peptide synthesis. These are the two substrates in large excess (1 and 2), the enzyme (3) and the activating agent (liquid phase) used in the reaction (4). When all substances are mixed together, the reaction mixture can appear like a largely dry powder (**Figure 3.27B**) or like a wet paste [Ulijn *et al.*, 2003] depending on the solubilities of the individual substrates.

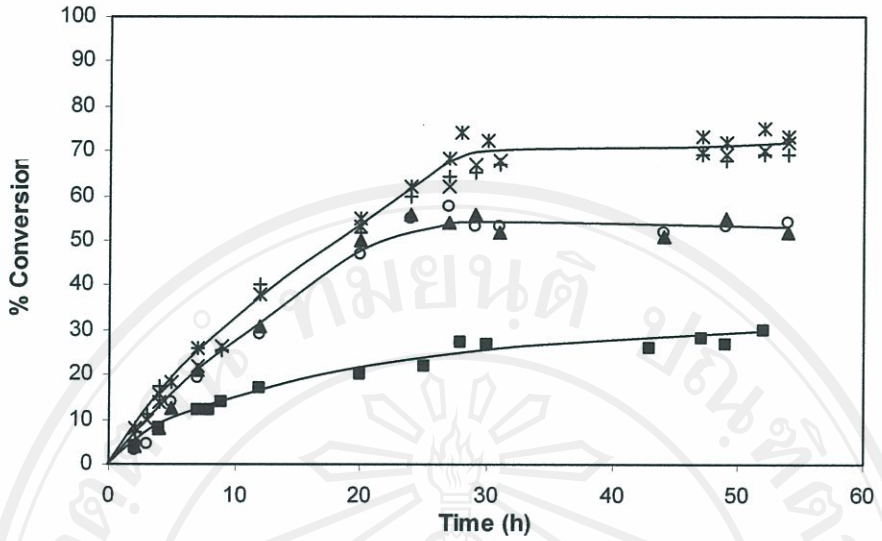


Figure 3.26 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2, with the substrate molar ratios (Z-Gly-OH : H-Phe-NH_2) at 1:1 (■), 1.05:1 (○), 1.1:1 (▲), 1.3:1 (+), 1.5:1 (×) and 2:1 (∗).

Figure 3.27 A typical glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2 before (A) and after (B) mixing of reactants with the substrate molar ratio 2:1. The four containers in (A) were 1.28 mmol of Z-Gly-OH (1), 0.64 mmol of H-Phe-NH_2 (2), 20 mg of both solid cysteine and enzyme powder (3) and 46 µl of activating agent (4).

3.7.2 Composition of liquid phase in substrate mixture

The liquid phases equilibrated with the two substrates as reaction of various molar ratios were analysed. **Table 3.8** shows the volume of saturated substrate mixture obtained was increased with increasing of Z-Gly-OH excess; even equal amount of water was used (1050 μL). At equimolar of the two substrates used, their concentrations in the equilibrated liquid phase showed nearly equal. With excess solid Z-Gly-OH either 1.5 and 2 times to H-Phe-NH_2, its total concentration in the equilibrated liquid phase was found to be around 0.24 M, which was higher than the total concentration of H-Phe-NH_2 (approximately 0.18 M). Due to electroneutrality balance [Halling, personal communication], the ionised forms of these two substrates must be present in equal concentrations. Therefore, about 0.06 M of Z-Gly-OH was present in the acidic form. This would give rise to relatively acidic conditions in the liquid phase (around pH 5 in the equivalent dilute system). This relates to the result in **Figure 3.26** which it seems clear that mildly acidic conditions are optimal for solid-to-solid peptide synthesis catalysed by glycyl endopeptidase.

Table 3.8 Liquid phase compositions at 27 h of substrate mixture equilibrated with 1050 μL of water at 40°C.

Substrate molar ratios	Volume of liquid phase	Concentration (M)	
(Z-Gly-OH:H-Phe-NH_2)	(μl)	Z-Gly-OH	H-Phe-NH_2
1:1	70	0.157	0.149
1.5:1	160	0.234	0.177
2:1	260	0.240	0.175

3.7.3 Analysis result of reaction mixture

Samples from solid-to-solid synthesis of Z-Gly-Phe-NH_2 were analysed by HPLC system with C-18 reverse phase column. Elution was performed with both gradient and isocratic systems. The gradient elution acquired longer time (30 min) than the latter one (13 min). It also eluted the substances with much lower efficiency when comparing to the flatted mobile phase ratio. Hence, the isocratic elution was used for the HPLC analysis of peptide product.

Figure 3.28 shows a typical HPLC chromatogram of the reaction progress at 27 h. At the mobile phase condition of 30% v/v acetonitrile mixed with 70% v/v aqueous containing 0.01% TFA, nucleophile, acyl donor and product peptide Z-Gly-Phe-NH_2 were eluted at retention times of 2.94, 4.78 and 7.95 min, respectively. Although the sample was taken to analyse at the time of reaction stopped at 27 h, it is important to note that both of reactants still remained in the mixture. Therefore, exhaustion of one or both reactants were not a cause of synthesis termination.

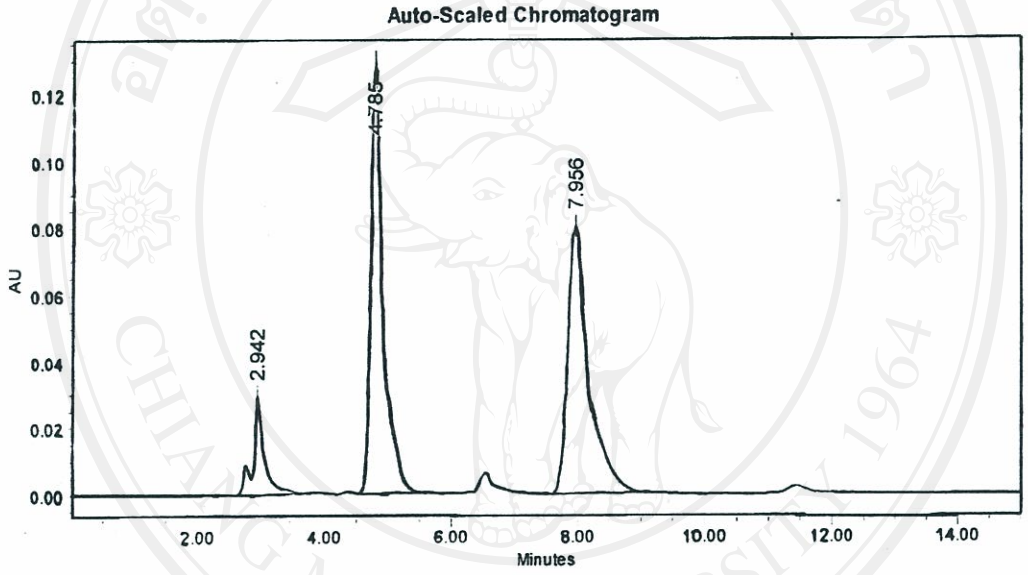


Figure 3.28 Chromatographic profile at reaction progress of solid-to-solid condensation between Z-Gly-OH and H-Phe-NH_2 at the substrate molar ratio 2:1, 20 mg of both glycyl endopeptidase and solid cysteine. H-Phe-NH_2, Z-Gly-OH, and Z-Gly-Phe-NH_2 were eluted at the retention times of 2.942, 4.785 and 7.956 min, respectively.

It is noted that in the solid-to-solid reaction with equimolar substrates (but not those with any excess of Z-Gly-OH), a by-product at retention time 11.42 min was observed as shown in **Figure 3.29**. It was not investigated for the progression curve during reaction taking place. However, the observation found that in the early stages of reaction the byproduct was a very small proportion of the products, but it eventually rose to about 26% of the total. It is presumably formed by hydrolysis of Z-Gly-Phe-NH_2

synthesised earlier. To ensure this presumption, the fractions of this unknown and product peptide (at retention times of 11.42 and 7.87 min, respectively from **Figure 3.29**) were collected and identified by high-resolution mass spectrometry. Accurate mass spectra are shown in **Figure 3.30** that peptide product ($M+H^+$ = 356.2) and by-product ($M+H^+$ = 357.3) were different just one mass. Both molecules were fragmented to a number of similar sizes indicating that their structures were quite similar. Their accurate mass analysis showed the product (Z-Gly-Phe-NH_2) and the unknown possessed formula of $C_{19}H_{21}N_5O_2$ and $C_{19}H_{20}N_4O_3$, respectively. This corroborated that the unknown compound was the hydrolysed product from the synthesised peptide and its structure was Z-Gly-Phe-OH .

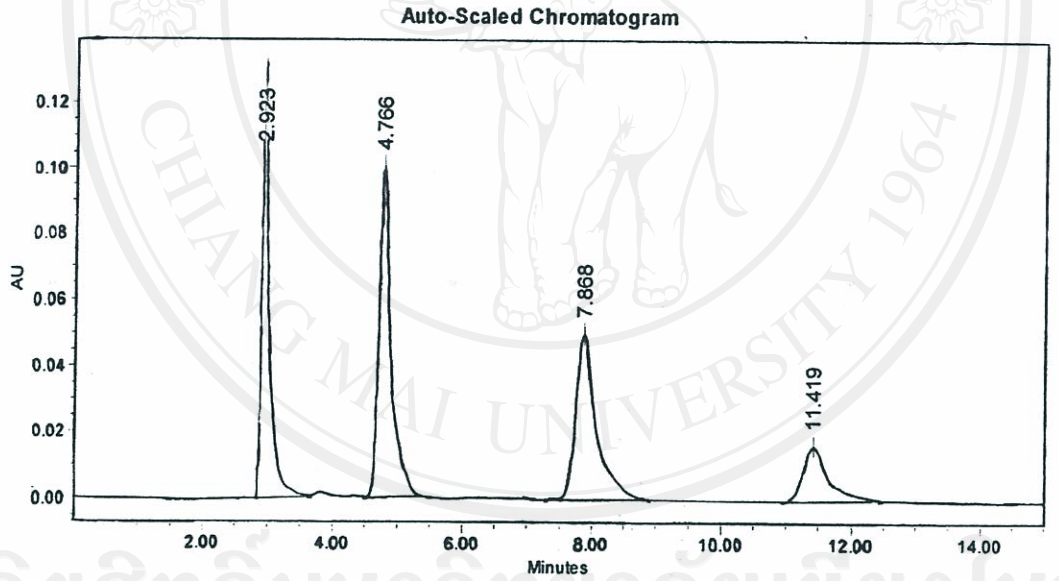


Figure 3.29 Chromatographic profile at reaction progress of solid-to-solid condensation between Z-Gly-OH and H-Phe-NH_2 at the substrate molar ratio 1:1, 20 mg of both glycyl endopeptidase and solid cysteine. H-Phe-NH_2, Z-Gly-OH, Z-Gly-Phe-NH_2, and by-product Z-Gly-Phe-OH were eluted at retention times of 2.923, 4.766 and 7.868 and 11.419 min, respectively.

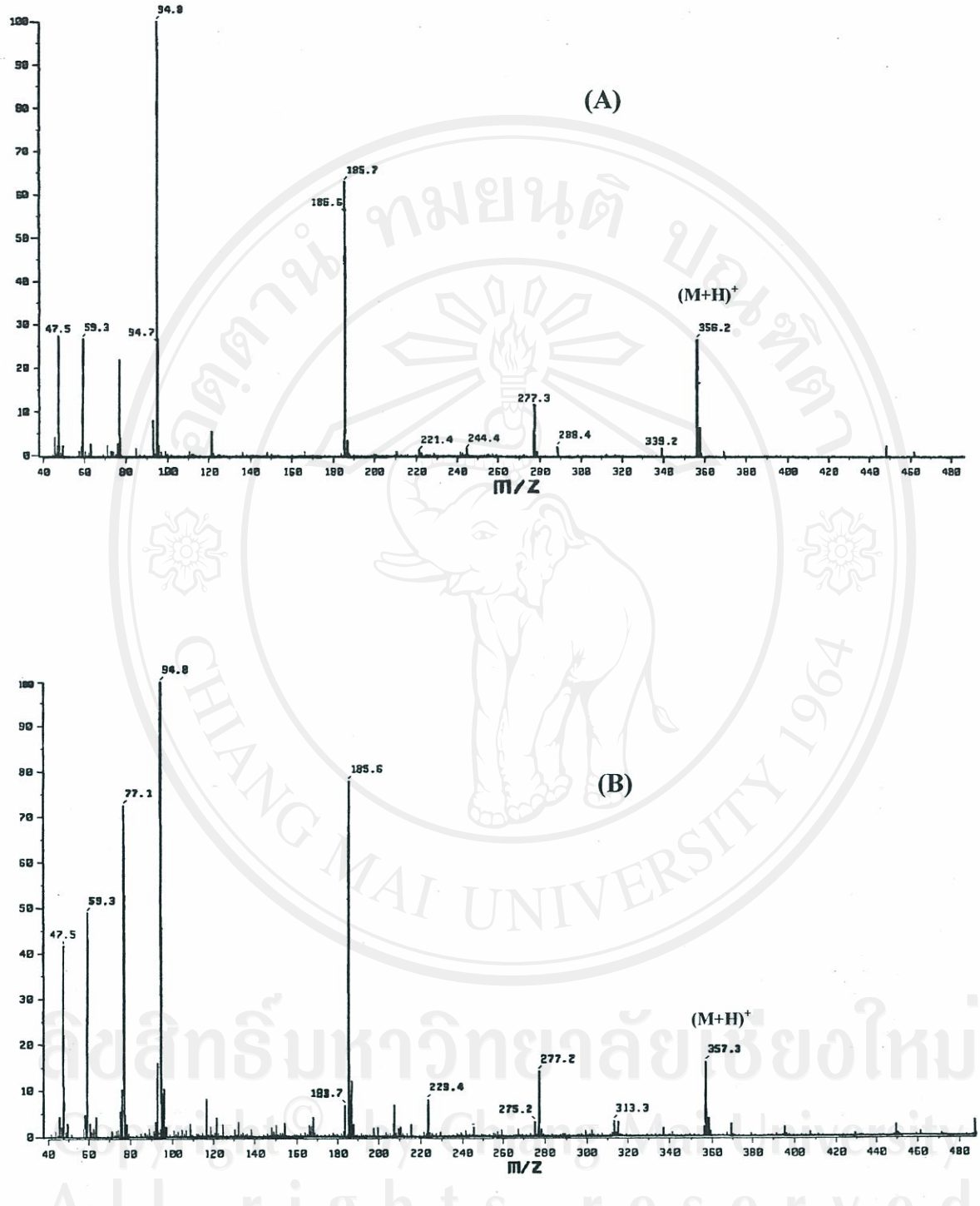


Figure 3.30 Accurate mass spectra of peptide product, Z-Gly-Phe-NH_2 (A) and by-product, Z-Gly-Phe-OH (B) from glycyl endopeptidase catalysed solid-to-solid peptide synthesis.

3.7.4 Optimal amount of glycyl endopeptidase

The effect of enzyme quantities on the solid-to-solid peptide synthesis was examined. **Figure 3.31** shows the initial rates of reaction progress as a function of enzyme amounts. At the lower quantities of glycyl endopeptidase used, increase in enzyme amount resulted as an increase in the reaction rate. The figure shows the reaction reached mass transfer limited region at 20 mg per reaction. The initial rate above this enzyme level was constant at around 50% nmol min^{-1}. The lack of further increase in rate at more enzyme using was probably due to mass transfer limitation. Therefore, 20 mg of enzyme per reaction was the most suitable for using in further experiments of Z-Gly-Phe-NH_2 synthesis.

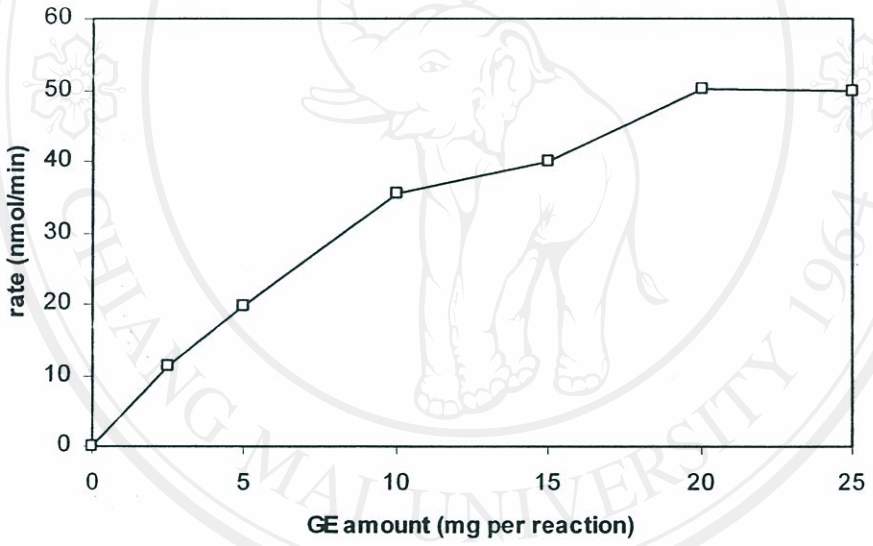


Figure 3.31 Effect of glycyl endopeptidase (GE) amount on the initial rate of solid-to-solid synthesis of Z-Gly-Phe-NH_2.

3.7.5 Effect of Z-Gly-OH sources

Because Z-Gly-OH was differently obtained from the two companies, Bachem and Novabiochem, they consequently were compared as acyl donor in the synthesis reaction of Z-Gly-Phe-NH_2. As shown in **Figure 3.32**, the initial rate and product yield were quite similar in both sources. However, Z-Gly-OH from Bachem provided a substantial higher synthesis progress than that from the other. Its reaction proceeded faster until stopped after 30 h, whereas using acyl donor from Novabiochem gave the

slower process and reached the highest conversion at about 50 h. Consequently, the substrate from Bachem was chosen for all of the syntheses. The appearances of solid powders of the two Z-Gly-OH are shown in **Figure 3.33**. Even though their powder size and shape are in the same, the colour was quite different which the one from Bachem was more whitish than the other.

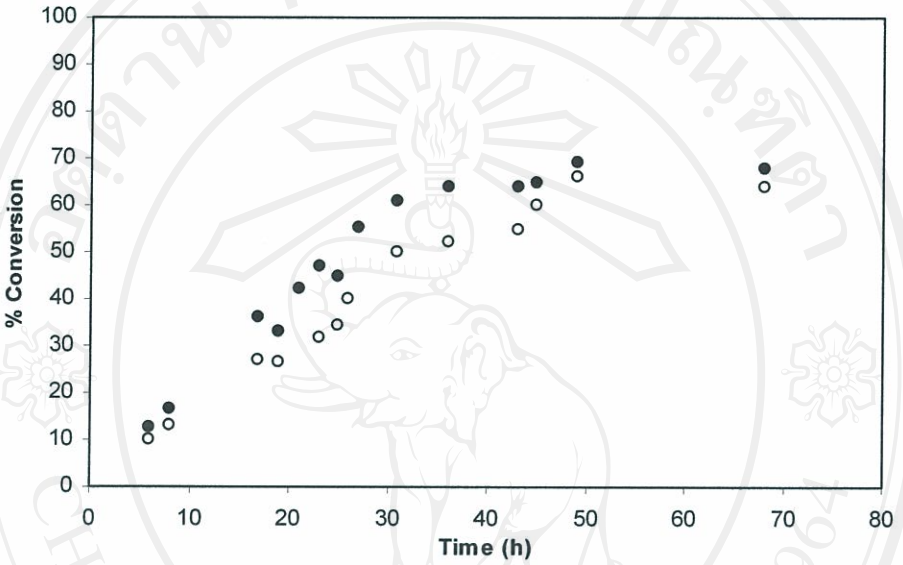


Figure 3.32 Comparison in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2 by using Z-Gly-OH from Bachem (●) and Novabiochem (○) and H-Phe-NH_2 at molar ratio of **2:1**, 20 mg of both enzyme and solid cysteine.

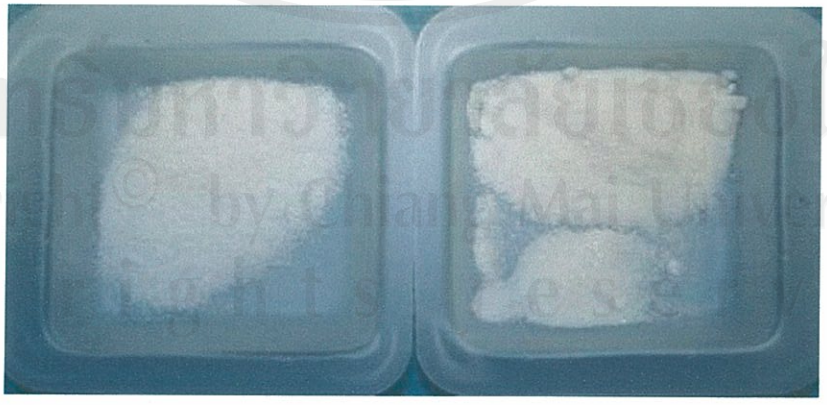


Figure 3.33 A typical appearance of Z-Gly-OH powders from Bachem (left) and Novabiochem (right). The left one is more whitening powder, while the right one is quite creamy colour. Their particle sizes are equal.

3.7.6 Effect of cysteine on glycyl endopeptidase catalysis

As mentioned previously in this thesis, papaya cysteine proteases, including glycyl endopeptidase, need to be activated by a reducing agent such as cysteine for maximum activity in the aqueous media. As a consequence, the effect of cysteine on glycyl endopeptidase catalysed peptide synthesis in the solid-to-solid system was investigated. It was present both in the initial enzyme solution and as solid form in the reaction mixture. **Table 3.9** shows that the initial rate was doubled when dissolving glycyl endopeptidase with 20 mM cysteine in a phosphate buffer pH 7.5 containing 20 mM EDTA (activating solution). The presence of solid cysteine in the reaction mixture had a substantial effect as the reaction proceeds, with the initial rate increased from 11 nmol min^{-1} mg $enzyme^{-1}$ to around 12-16 nmol min^{-1} mg $enzyme^{-1}$ upon the cysteine quantity. In addition, solid cysteine in the mixture also increased the highest conversion approximately 10% from that absence. As the **Table 3.9** shown, the optimal addition of solid cysteine was 100% w/w relative to the enzyme amount.

Table 3.9 Initial rate and % conversion from glycyl endopeptidase catalysed solid-to-solid Z-Gly-Phe-NH_2 synthesis in the presence and absence of cysteine. Reactions were performed at substrate molar ratio 2:1 and using 20 mg enzyme per reaction.

Presence of cysteine in		**Initial rate** (nmol min^{-1} mg $enzyme^{-1}$)	**Highest conversion** (%)
activating solution (mM)	reaction mixture (% w/w of enzyme)		
0	0	6	59
0	100	7	67
20	0	11	63
20	50	12	74
20	100	16	76
20	200	16	76

3.7.7 Effect of EDTA on glycyl endopeptidase catalysis

In the aqueous catalysis of cysteine proteases, EDTA was used accompanying with cysteine for complement of enzymes activation. In this study, 20 mM EDTA was already present in the activating agent which was used to dissolve enzyme before mixing with the solid substrates. However, influence of solid EDTA on the further catalysis of enzyme in peptide conversion was still examined. **Figure 3.34** shows that the addition of 1 mg EDTA to the reaction mixture had no significant effect on the synthetic progress. This amount was approximately 3 times higher than that already in the activating agent (0.34 mg). EDTA which functions to eliminate free metal ions, and its presence in the activating solution is presumably sufficient for this study. Therefore, the further experiment of dipeptide synthesis will not require the addition of solid EDTA.

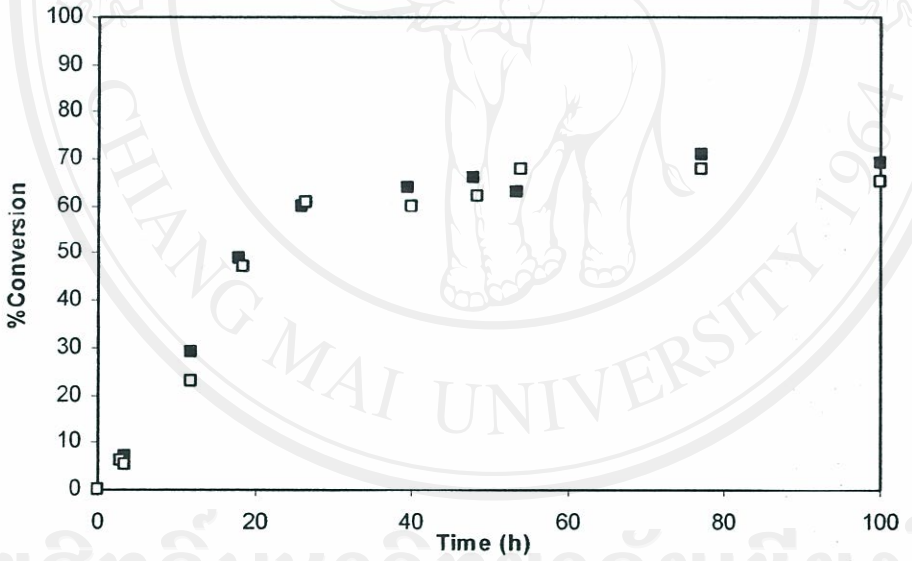


Figure 3.34 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2. Two reactions were compared with the presence (■) and absence (□) of 1 mg EDTA in the mixture.

3.8 Various nucleophiles coupling with Z-GlyOH in glycyl endopeptidase catalysed solid-to-solid synthesis

Glycyl endopeptidase is specific for carboxyl end of glycine, so Z-Gly-OH was used as a fixed substrate for dipeptide synthesis. Although its catalysis of solid-to-solid

synthesis of Z-Gly-Phe-NH_2 occurred very well, preference of the enzyme to various nucleophiles condensed with Z-Gly-OH in this system was interesting. Therefore, five different nucleophiles including, H-Leu-NH_2, H-Tyr-NH_2, H-Tyr-OEt, H-Asp-OBzl and H-Pro-NH_2 were chosen to the reactions. Because 20 mg of enzyme and 20 mg of solid cysteine provided the highest efficiency, they were consequently used in the experiment.

3.8.1 Solid-to-solid synthesis of Z-Gly-Leu-NH_2

Various substrate molar ratios, 1:1, 1.1:1 and 1.5:1 of Z-Gly-OH to H-Leu-NH_2 were used in the reaction. **Figure 3.35** shows about 35-54% conversion were obtained from the synthesis of Z-Gly-Leu-NH_2 by glycyl endopeptidase. At the substrate ratios 1.1:1 and 1.5:1, the initial rates and final conversions of peptide were similar but higher than those of the ratio 1:1. The initial rates of all experiments were very fast, nevertheless, the progresses were stopped at around 10 h. The highest conversion at 48% was obtained when using the substrate ratio 1.1:1 and 1.5:1.

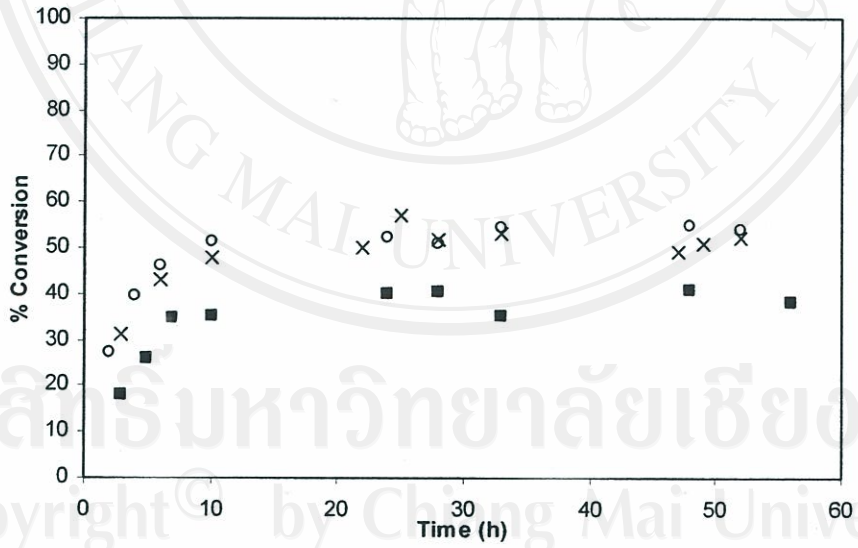


Figure 3.35 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Leu-NH_2, with 20 mg of both glycyl endopeptidase and solid cysteine per reaction. The substrate molar ratios (Z-Gly-OH:H-Leu-NH_2) were varied at 1:1 (■), 1.1:1 (×) and 1.5:1 (○).

3.8.2 Solid-to-solid synthesis of Z-Gly-Tyr-NH_2

The solid-to-solid condensation of Z-Gly-OH and H-Tyr-NH_2 was also studied at the substrate molar ratios of 1:1 and 1.1:1. The nuclophile could be compared with H-Leu-NH_2 and H-Phe-NH_2 in case of specific of enzyme to different coupled amino acids with the same amino blocker. The result was quite similar to the coupling of Z-Gly-Phe-NH_2 and Z-Gly-Leu-NH_2 in term of reaction pattern. As shown in **Figure 3.36**, the small excess of acyl donor Z-Gly-OH resulted as the higher initial rate and final conversion than the equimolar ratio which possessed the highest conversion at around 30%. However, their reaction progresses were quite slow and then stopped after 30 h of reactions. The highest peptide product was achieved at 52% when using substrate molar ratio 1.1:1.

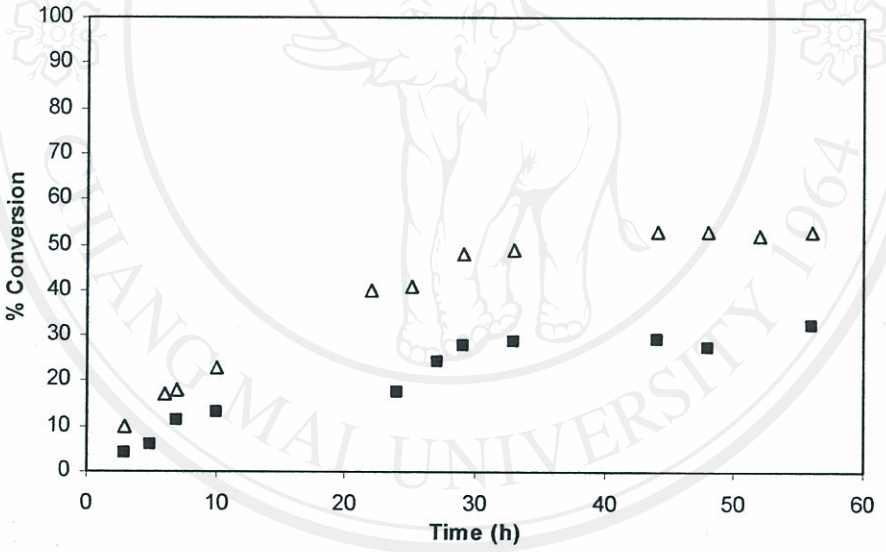


Figure 3.36 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-NH_2, with 20 mg of both glycyl endopeptidase and solid cysteine per reaction. The substrate molar ratios (Z-Gly-OH:H-Tyr-NH_2) were varied at 1:1 (■) and 1.1:1 (Δ).

3.8.3 Solid-to-solid synthesis of Z-Gly-Tyr-OEt

Another nucleophile, H-Tyr-OEt, was also used in the study. This tyrosine ester was able to compare with H-Tyr-NH_2 in case of enzyme specificity to dissimilar amino blocking groups. As **Figure 3.37** demonstrates, the higher final yield was obtained when

using the excess of Z-Gly-OH, whether 1.1:1 or 1.5:1. Equimolar of substrate ratio slightly decreased the final conversion while its initial rate was similar with those of unequal ratios. However, though the reaction progress well up righted but it has terminated at around 7 h which the highest conversion of 70% was attained. After this level, the peptide conversion was still constant.

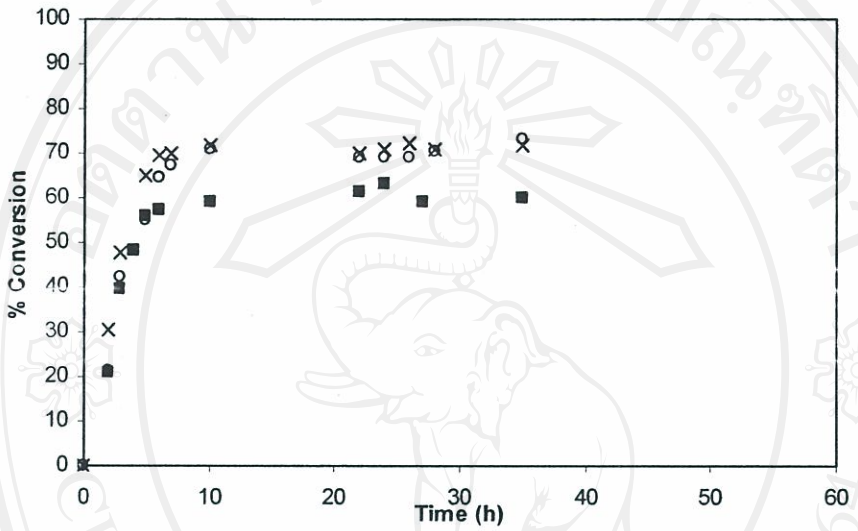


Figure 3.37 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-OEt, with 20 mg of glycyl endopeptidase and solid cysteine per reaction. The substrate molar ratios (Z-Gly:Tyr-OEt) were varied at 1:1 (■), 1.1:1 (×) and 1.5:1 (○).

3.8.4 Solid-to-solid synthesis of Z-Gly-Asp-OBzl and Z-Gly-Pro-NH_2

The last two nucleophiles, H-Asp-OBzl and H-Pro-NH_2 were used to condense with Z-Gly-OH via solid-to-solid system as well. The result obtained was opposite the all previous experiments. There was no peptide product occurred by using these two nucleophiles. This revealed that glycyl endopeptidase could not catalyse the coupling of Z-Gly-OH with H-Asp-OBzl and H-Pro-NH_2.

3.9 Improvement of peptide conversion of solid-to-solid synthesis catalysed by glycyl endopeptidase

As the result in Sections 3.7 and 3.8 show, the reaction progress in the solid-to-solid peptide synthesis catalysed by glycyl endopeptidase terminates before achieving

100% peptide conversion. Various factors have been investigated in order to improve the peptide conversion. This will be able to develop the enzyme catalysed peptide synthesis via mainly undissolved system.

3.9.1 Water evaporation from reaction mixture

During the solid-to-solid synthesis of Z-Gly-Phe-NH_2, some apparent separation of the reaction mixture (liquid droplets) were observed at longer incubation times as shown in **Figure 3.38**, although the reaction tubes were completely immersed in a water bath to prevent it. This separation might cause the incomplete of the reaction. It would be also expected that reducing the size of the Eppendorf tube from 1.5 to 0.50 mL could prevent this water evaporation. The results (**Figure 3.39**) showed that initial rates were still the same going in both the smaller and bigger tubes. However, the reaction progress when using smaller size was slightly going further and consequently the final conversion was increased from 75 to around 83%. The reduced size tubes were also periodically centrifuged during incubation. This would help the condensed liquid droplets completely fell back to the reaction mixture at bottom. The result obtained was shown in **Figure 3.39** that there was no change from non-centrifugation experiment when 0.5 mL tubes were used. Therefore, it was assumed that just reducing the tube size was enough to prevent the separation of reaction mixture. Nevertheless, the synthetic reaction was still not achieved 100%. Further experiments with other possible factors were required to be investigated.

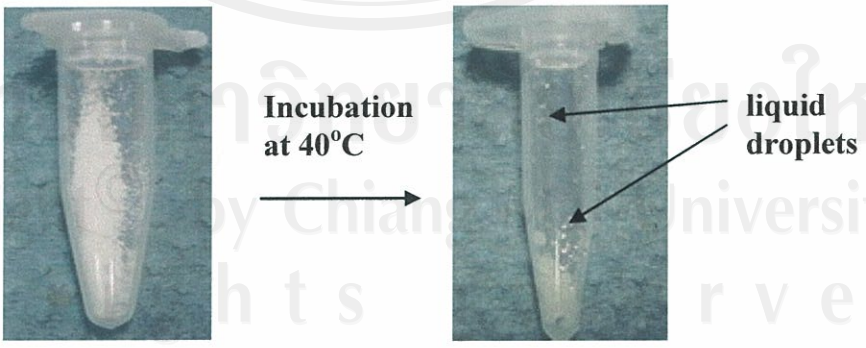


Figure 3.38 The separation of reaction mixture in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2; the reaction mixture before (A) and after (B) incubation in a water bath at 40°C for 25 h.

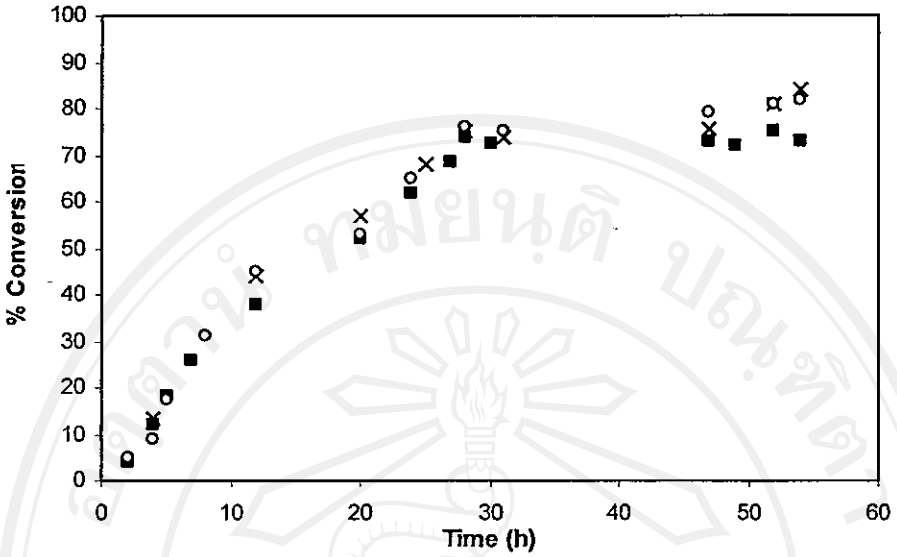


Figure 3.39 Effects of tube size and centrifugation on glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2. Substrate molar ratio (Z-Gly-OH : H-Phe-NH_2) was 2:1. The reaction was carried out in the tubes of 1.5 mL (■) and 0.5 mL with (o) or without (×) centrifugation.

3.9.2 Particle size of reactants

The particle size of solid substrates may become a key factor if mass transfer to all parts of the liquid is limited. The effect of grinding the solid substances on the reaction progress of glycyl endopeptidase catalysed solid-to-solid peptide synthesis was investigated. In this study, reactions from 4 synthetic peptides, Z-Gly-Phe-NH_2, Z-Gly-Leu-NH_2, Z-Gly-Tyr-NH_2 and Z-Gly-Tyr-OEt were examined at the substrate ratio 1.1:1 and the results were shown in **Figures 3.40-3.43**, respectively. Neither initial rates nor final conversions were significantly different, with any of the 4 nucleophiles used. This was suggested that all solid substrates were already in powder forms, which were small enough to avoid mass transfer limitation.

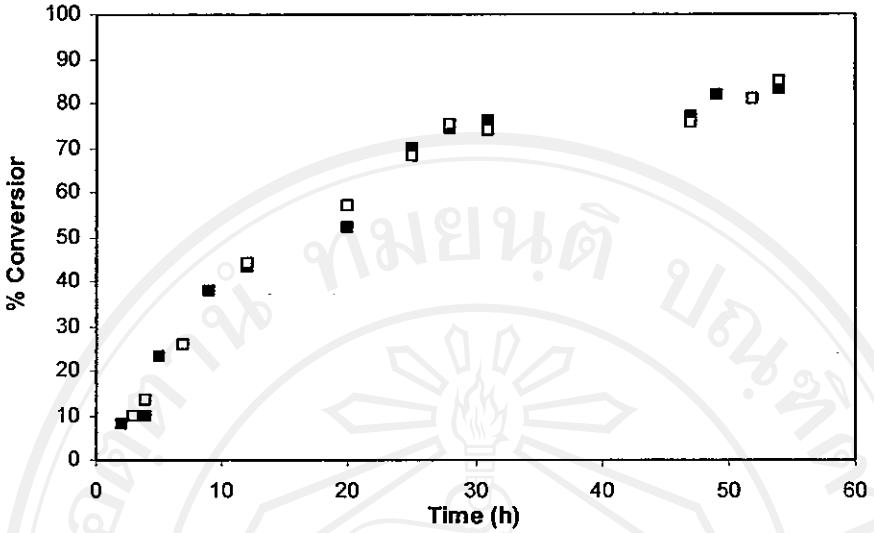


Figure 3.40 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2 with substrate molar ratio at 2:1, 20 mg of enzyme and solid cysteine per reaction. The reactions were compared between ground (□) and unground (■) of the two solid substrates.

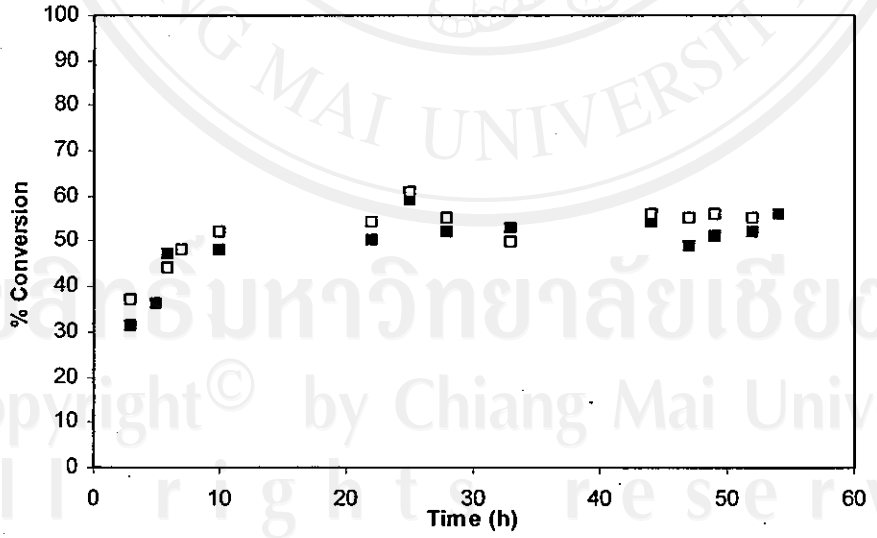


Figure 3.41 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Leu-NH_2 with substrate molar ratio at 1.1:1, 20 mg of enzyme and solid cysteine per reaction. The reactions were compared between ground (□) and unground (■) of the two solid substrates.

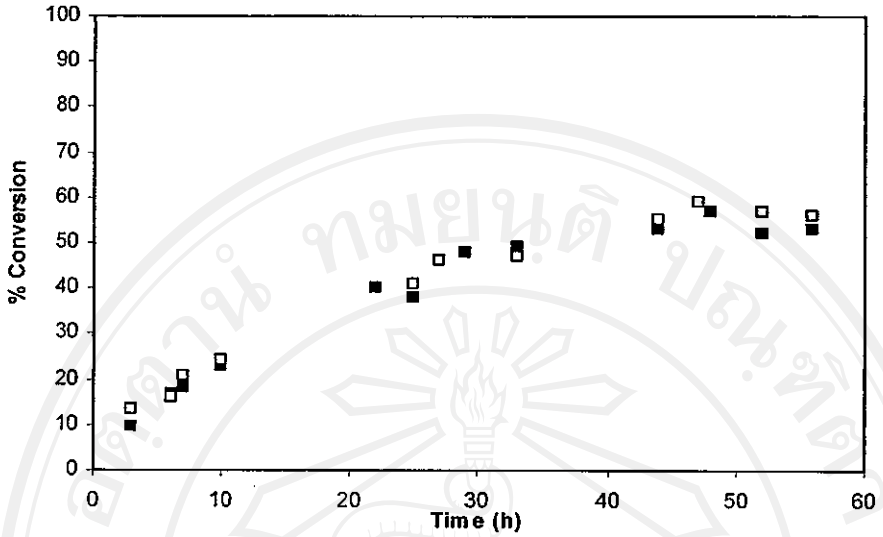


Figure 3.42 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-NH_2 with substrate molar ratio at 1.1:1, 20 mg of enzyme and solid cysteine per reaction. The reactions were compared between ground (□) and unground (■) of the two solid substrates.

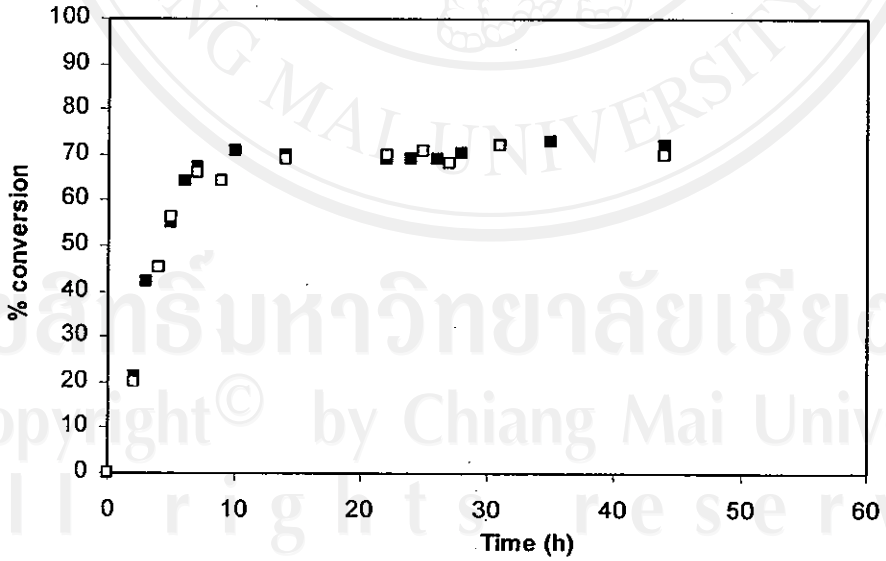


Figure 3.43 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-OEt with substrate molar ratio at 1.1:1, 20 mg of enzyme and solid cysteine per reaction. The reactions were compared between ground (□) and unground (■) of the two solid substrates.

Effect of particle size of solid cysteine on the catalytic reaction of Z-Gly-Phe-NH_2 synthesis was also determined at the substrate molar ratio 2:1. The unground solid cysteine was observed to be in the form of larger crystals and it was usually ground before using in the normal reactions. In order to compare its particle size effect, the unground cysteine was used in the reaction comparing with the ground one. As the result shown in **Figure 3.44**, grinding of solid cysteine before adding to the reaction mixture increased both initial rate and further conversion. Although the condensation stopped at the same time about 28 h, the reaction with ground cysteine possessed around 20% higher product yield than that with larger size of solid cysteine.

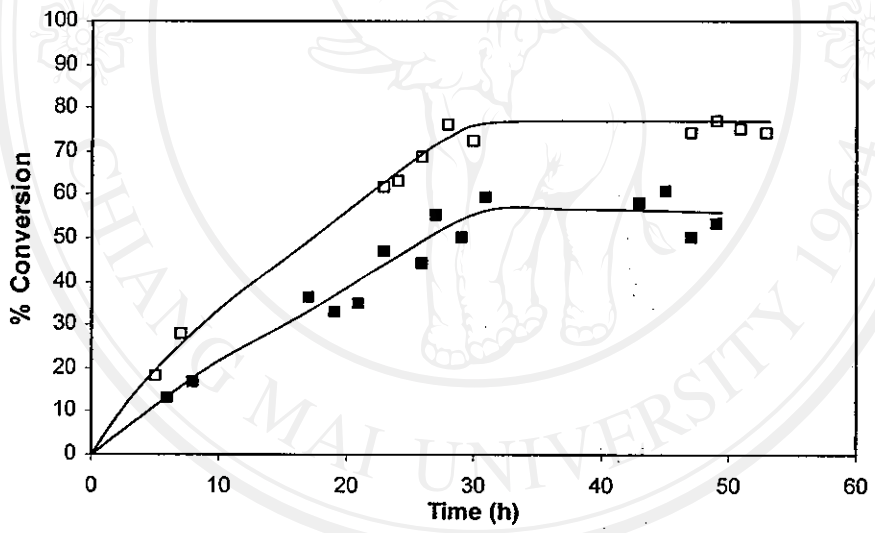


Figure 3.44 Effect of grinding solid cysteine on glycyl endopeptidase catalysed peptide synthesis. Substrate molar ratio (Z-Gly-OH : H-Phe-NH_2) of 2:1, added solid cysteine was ground (□) or unground (■).

3.9.3 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 substrates dissolving in the liquid phase, so their concentrations there

fall, and they are less available to the enzyme. Eventually their concentrations may become low enough so that further synthesis is not favourable. At this point, re-mixing of the reaction mixture might help the reaction continue further, by re-exposing the substrate particles. Three peptide synthesis reactions of Z-Gly-Phe-NH_2, Z-Gly-Leu-NH_2 and Z-Gly-Tyr-OEt were chosen to investigate such effect. At the time of each reaction stopped, the mixture was taken to be re-mixed and was then re-incubated for expectation of further reaction proceeds. For the synthesis using nucleophiles H-Phe-NH_2 and H-Leu-NH_2, there was no change in further peptide conversion when the mixture was re-mixed (**Figure 3.45-3.46**). This suggests that there was no entrapment of solid substrates by peptide product in these reactions and the reaction stopped was caused from other possible parameters. In contrast with the reaction using H-Tyr-OEt, the final conversion was increased from about 72 to 82% (**Figure 3.47**). The result indicated that the two solid substrates were around by the peptide product and needed to re-expose for more going reaction.

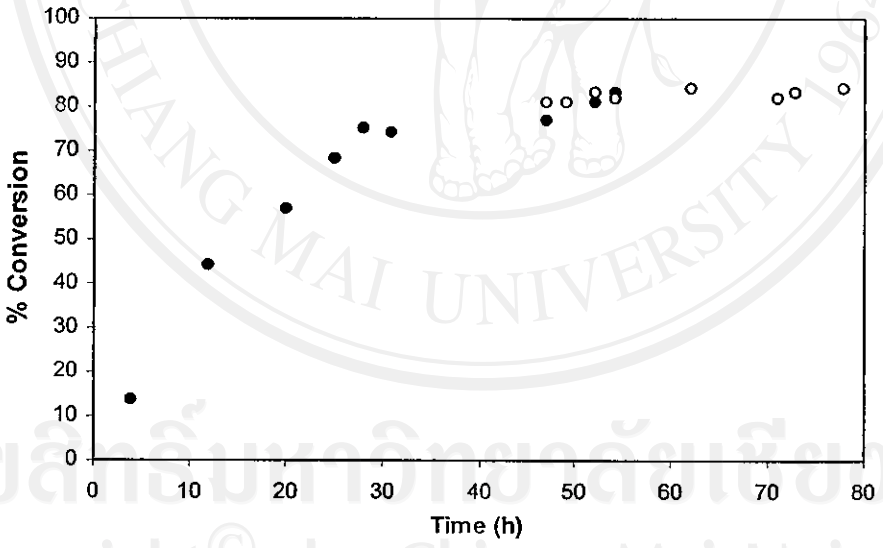


Figure 3.45 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2 with substrate molar ratio at 2:1, 20 mg of enzyme and solid cysteine per reaction. Reaction mixture was mixed once at starting time (●) and re-mixed after reaction stopped (○).

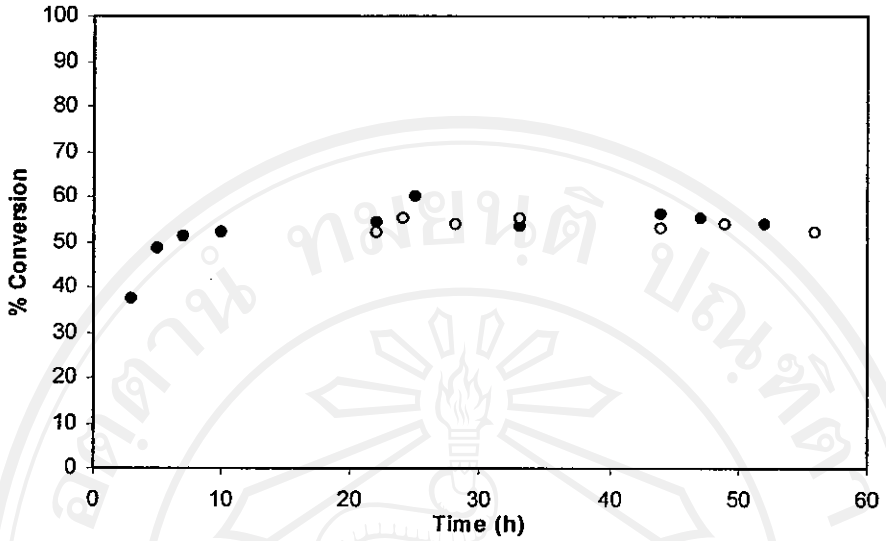


Figure 3.46 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Leu-NH_2 with substrate molar ratio at 1.1:1, 20 mg of enzyme and solid cysteine per reaction. Reaction mixture was mixed once at starting time (●) and re-mixed after reaction stopped (○).

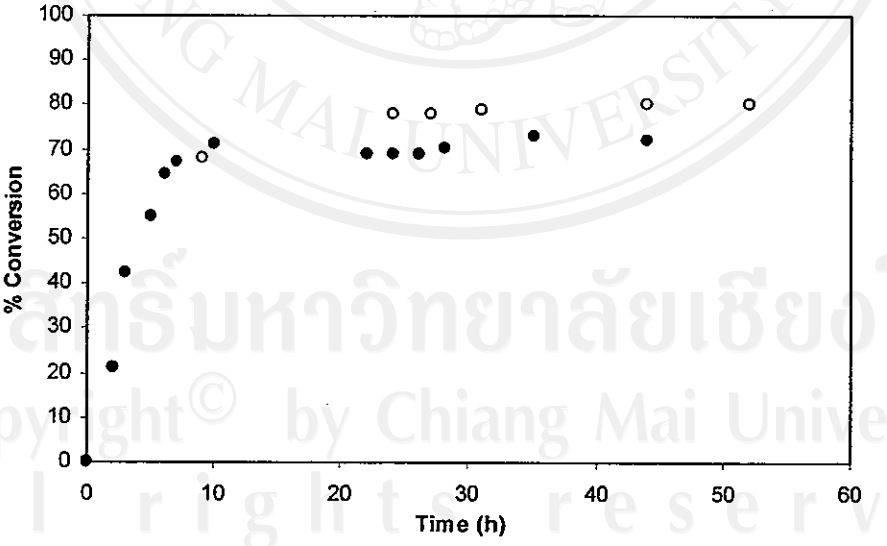


Figure 3.47 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-OEt with substrate molar ratio at 1.1:1, 20 mg of enzyme and solid cysteine per reaction. Reaction mixture was mixed once at starting time (●) and re-mixed after reaction stopped (○).

3.9.4 Equilibrium of reaction

In thermodynamically controlled peptide synthesis, another important factor is the reaction equilibrium. Clearly, conversion cannot continue as equilibrium is approached. Therefore, this solid-to-solid synthesis was stopped possibly because of reaching reaction equilibrium. To test this theory, the mixture at the time of reaction stopping was dried over molecular sieve until the moisture reached at a half point from starting. When the dried mixture was re-incubated, the reaction was expected to be going further due to equilibrium shift. Other experiments to verify this expectation was pure water, buffer pH 7.5, activating agent or enzyme solution were added to the mixture. This was expected to see the decline of peptide product due to hydrolysis more favoured. As **Figure 3.48** shown, there was no any substantial changing in peptide conversion, whether removed or added the liquid (without enzyme) to the mixture in order to change equilibrium position. From this point, it can be concluded that the equilibrium approaching was not the cause of reaction termination. However, results of addition liquid phase without enzyme were different from when the enzyme solution was added (**Figure 3.48**) which conversion was decreased due to more hydrolysis favoured than synthesis.

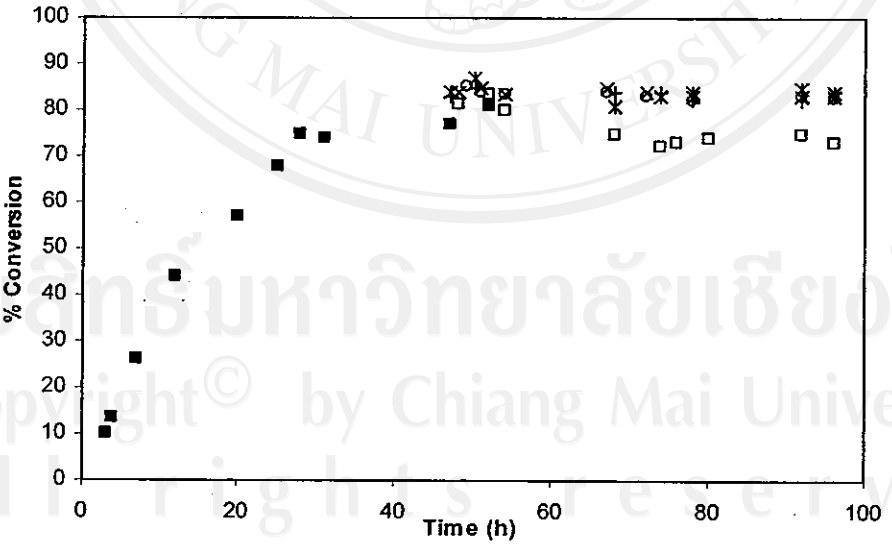


Figure 3.48 Glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2. Substrate molar ratio (Z-Gly-OH : H-Phe-NH_2) of 2:1. After normal reaction (■) stopped (48 hr), some liquid was removed (×) or added with enzyme solution (□), pure water (○), phosphate buffer pH 7.5 (∗) and activating agent (+).

In further investigation of reaction equilibrium, both liquid and solid phases comprised of the reaction mixture were analysed by HPLC. In theory, this solid-to-solid reaction can only reach equilibrium when one (or both) of solid substrates is exhausted from the reaction mixture [Ulijn *et al.*, 2001]. **Table 3.10** shows the two substrates were still present in the reaction mixture in both liquid and solid phases. This substantiated the result above that reaching equilibrium position was not the reason for termination of the synthesis.

Table 3.10 Quantity of liquid and solid phase components in reaction mixture of glycyl endopeptidase catalysed solid-to-solid Z-Gly-Phe-NH_2 synthesis.

Substance	Quantity ($x10^{-3}$ mmol)	
	Liquid phase	Solid phase
Z-Gly-OH	2.01	10.5
H-Phe-NH_2	0.558	4.40
Z-Gly-Phe-NH_2	0.350	21.9

3.9.5 Addition of new enzyme solution

Result from Section 3.9.4 showed that addition of new glycyl endopeptidase solution to the normal reaction mixture caused hydrolysis of peptide product. This might result from the enzyme nature favours which hydrolysis more than synthesis in the reaction containing higher amount of water. Therefore, the mixture was completely dried, for removing the whole water component, before adding new enzyme solution. This was expected that the conversion would continue by the new enzyme activity. Unfortunately, result in **Figure 3.49** shows the solid-to-solid conversion was not going further from the normal reaction. This indicated that loss of enzyme activity is probably a factor. However, as mentioned above, adding of new enzyme solution to that undried reaction mixture declined the peptide conversion, observed in both solid-to-solid synthesis of Z-Gly-Phe-NH_2 (**Figure 3.49**) and Z-Gly-Tyr-OEt (**Figure** 3.50). This demonstrated that reaction has reached its equilibrium and it would shift to hydrolysis rather than peptide synthesis when more liquid was added from new enzyme solution.

Therefore, results of adding enzyme solution gave a doubt that whether loss enzyme activity or reaching equilibrium is a major factor to terminate the reaction.

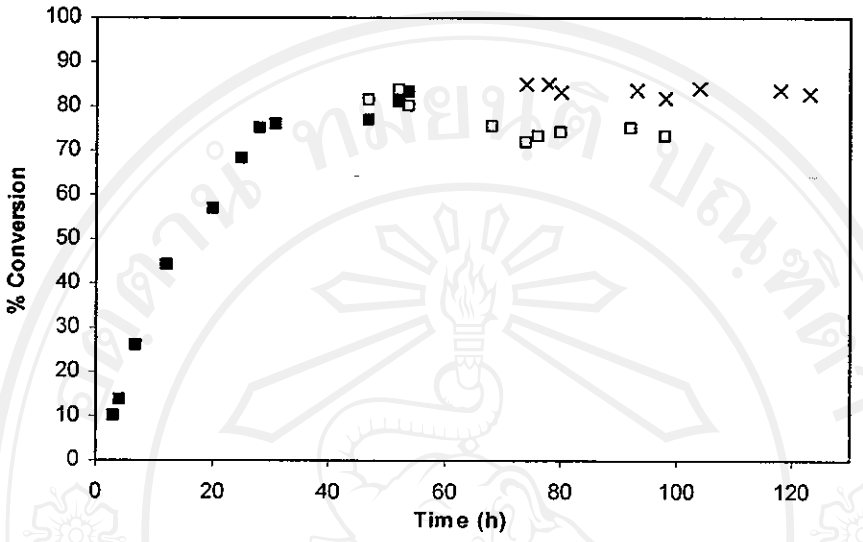


Figure 3.49 Effect of adding fresh enzyme after conversion had stopped in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH_2 at substrate molar ratio 2:1. After normal reaction (■) stopped (48 hr), more enzyme solution was added to the reaction mixture with (×) and without drying (□).

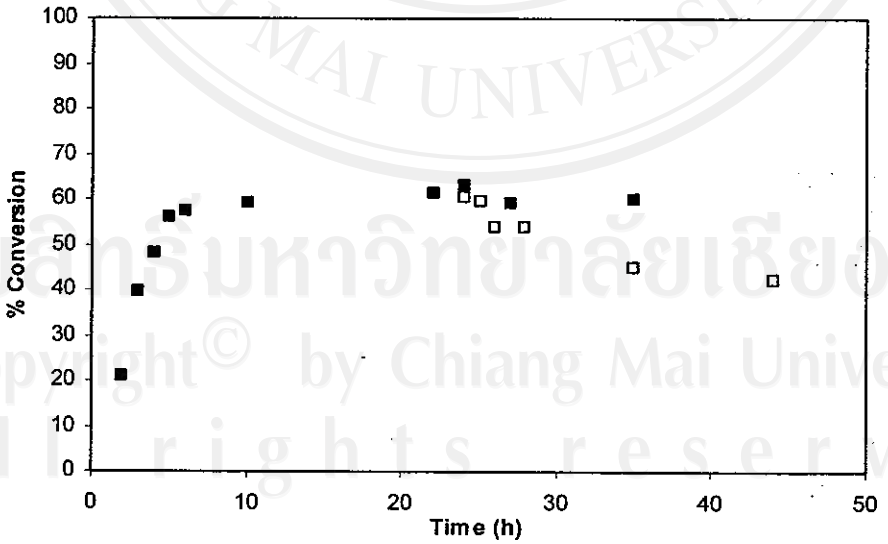


Figure 3.50 Effect of adding fresh enzyme after conversion had stopped in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-OEt at substrate molar ratio 1.1:1. After normal reaction (■) stopped (24 hr), more enzyme solution was added to the reaction mixture without drying (□).

3.10 Activity of glycyl endopeptidase recovered from solid-to-solid reaction mixture

3.10.1 glycyl endopeptidase activity

The results described above in Section 3.9.5 suggested that enzyme inactivation during the synthesis reaction is significant, so its residual activity was investigated. The glycyl endopeptidase assay revealed that its activity was dramatically decreased within 3 h of incubation and after that, it seems to decline further until 24 h (**Figure 3.51, Table 3.11**). It is noted worthy that the reaction is still proceeding well when only a few percents of enzyme activity remain. **Table 3.11** also shows the activity profile of glycyl endopeptidase after the 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 **Figure 3.49** that there was no further conversion going. Therefore, it seems likely that loss of enzyme activity is a significant factor causing the solid-to-solid peptide synthesis to stop at around 83% final conversion.

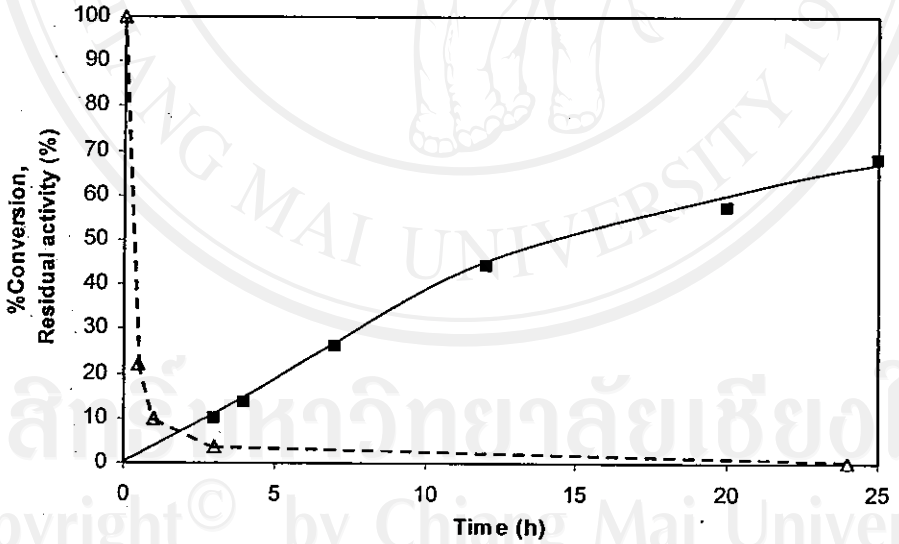


Figure 3.51 Inactivation of glycyl endopeptidase while catalysing solid-to-solid synthesis of Z-Gly-Phe-NH_2 with substrate molar of 2:1; progress of synthesis (—■—), and residual amidase activity of enzyme (---Δ---).

Table 3.11 Activity of glycyl endopeptidase in reaction mixture for Z-Gly-Phe-NH_2 synthesis. Assay substrate used was Boc-Ala-Ala-Gly-pNA and 1 unit is defined as 1 nmole product released within 1 min at pH 7.5 and 40°C.

Glycyl endopeptidase	Amidase activity (u/mg)	Residual activity (%)
Before mixing with solid substrates	2710	-
In standard reaction mixture at		
5 min	2530	100
20 min	1210	47.6
30 min	560	22.1
1 h	252	10.0
3 h	85.7	3.39
24 h	4.49	0.18
After second addition of enzyme*		
5 min	533	21.0
30 min	20.3	0.80
1 h	13.5	0.54

*After 48 h standard reaction, mixture was dried for 24 h, then fresh enzyme solution mixed in

3.10.2 SDS-PAGE

Besides determination of the protease activity, the presence of glycyl endopeptidase itself in the stopped reaction mixture was also analysed. The enzyme recovered from the reaction mixture was brought to investigate by running SDS-PAGE. As shown in **Figure 3.52**, by comparison to the molecular standard proteins, glycyl endopeptidase before catalysis appeared at approximately 23 kDa with the most intensity of protein band. After mixing enzyme with solid substrates, intensity of the band decreased rapidly when catalysis over 3 h. It was then disappeared within 24 h of reaction. This suggests that glycyl endopeptidase was autolysis during catalysis of peptide synthesis via solid-to-solid system. Hydrolysis of the catalyst itself is the main reason for the reaction stops.

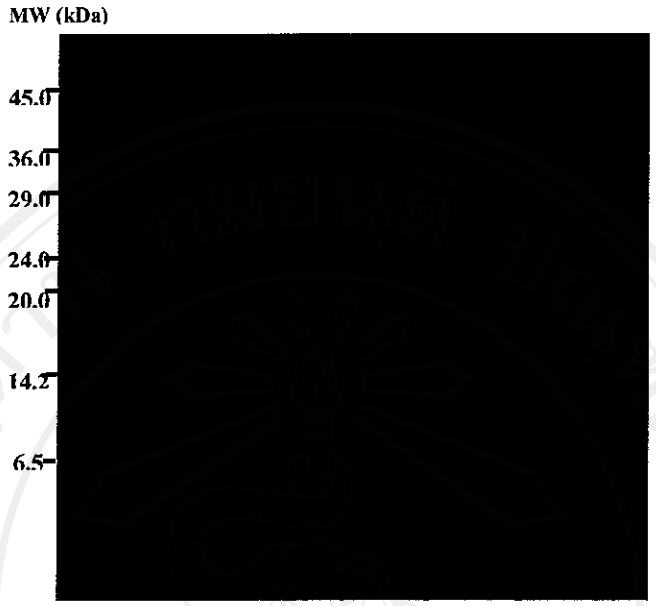


Figure 3.52 SDS-PAGE of glycyl endopeptidase recovered from the reaction mixture; after catalysis for 5 min (lane 3), 30 min (lane 4), 1 h (lane 5), 3 h (lane 6) and 24 h (lane 7), comparing to the enzyme before addition to the reaction mixture (lane 2).

3.10.3 HPLC analysis

Glycyl endopeptidase in the mixture of solid-to-solid reaction was also analysed by C-4 column HPLC. The result was similar to that of SDS-PAGE which the enzyme disappeared rapidly during catalysis. The enzyme was eluted from the column at elution time 18 min (**Figure 3.53**). The peak area of enzyme became less after incubation the reaction mixture at 1 and 3 h. However, a number of small peaks were simultaneously present at early elution time. Earlier elution of these peaks demonstrated that they were small peptides suggested as a product from autolysis of glycyl endopeptidase. This was consistent with the elution of enzyme recovered from incubation at 24 h. The peak of glycyl endopeptidase was almost disappeared, whereas those areas of the small peptides were increased. This is obvious to corroborate the result of enzyme activity assay and SDS-PAGE above that autolysis of glycyl endopeptidase during catalysis was the main cause for termination of solid-to-solid peptide synthesis.

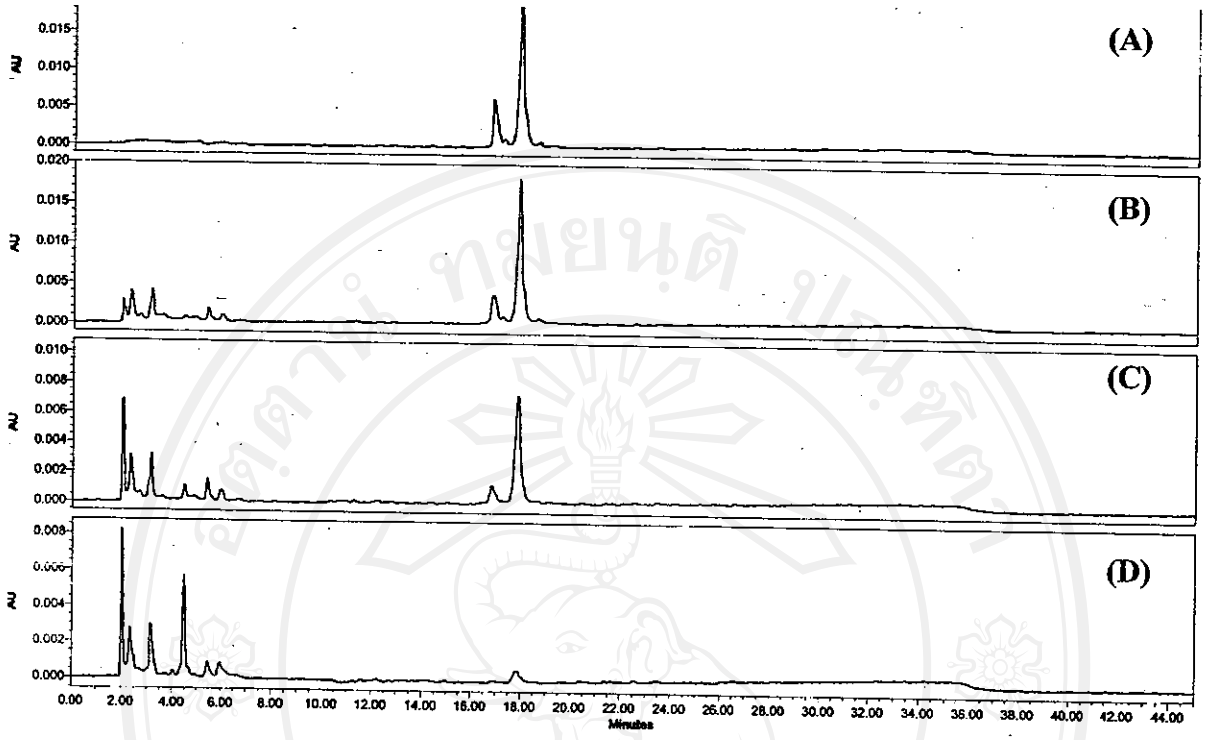


Figure 3.53 Absorption at 280 nm of glycyl endopeptidase recovered from the reaction mixture of solid-to-solid Z-Gly-Phe-NH_2 synthesis after mixing with the substrates and incubation for 1 h (B), 3 h (C) and 24 h (D), compared with the enzyme before catalysis (A).