CHAPTER 4
DISCUSSION AND CONCLUSIONS

Glycyl endopeptidase was explored in both papaya latex and peel proteases. The latex proteases were prepared by drying whole fresh latex in a tray dryer and obtained as enzyme powder. To prepare papaya peel proteases, the dried peels were ground and soaked with water before crushing through gauze to attain crude extract. The peel extract was clarified and subsequently subjected to precipitate out proteases. Both papaya proteases were compared in their proteolytic properties, protein components and portion of glycyl endopeptidase. Results indicated that glycyl endopeptidase was found only in the latex, but not in the peels. Therefore, fresh papaya latex was a good source for purifying of glycyl endopeptidase which was further used for catalysis of solid-to-solid peptide syntheses. Various parameters influencing on glycyl endopeptidase catalysed peptide synthesis via solid-to-solid system were also investigated.

4.1 Preparation of proteases from papaya peels

The papaya peels were obtained from peeling the green fruit maturation between 70 and 100 days. In order to store for a long period, the peels were dried in a built tray-dryer [Theppakorn, 2003] until the weight had been reduced to 10% w/w of the original (12 h of drying). Drying temperature was used at 55°C which has been reported as the optimum for drying papaya peel [Espin and Islam, 1998] and latex [Ortiz *et al.*, 1980; Theppakorn, 2003].

Three different methods were compared in extraction of proteases from the peels (**Table 3.1**). It was found that the arid blending of dried peels before soaking with water provided the best protease activity, highest concentration of proteases and shortest time consumed. Hence, this method was chosen for protease extraction. The third method which was previously described by Kanasawud *et al.* [2001] provided the least proteolytic content with larger volume of the extract. The crude extract of the first method obtained was a green-brown liquid containing solid particles of the peels. Therefore, clarification was necessary before precipitating of proteases. Coagulation by heating up to 40-50°C for 10-30 min has been used to clarify the green juice from leaves,

stems and trunks of young papaya trees [Burdick, 1964]. However, this might cause destruction of enzyme activity. In order to obtain more protease, repression of the extracted pulpous peels have been used. Even so, the purification of the enzyme later appeared to be more difficult because other constituents were coexistent at higher level [Burdick, 1964]. Here, clarification by centrifugation was chosen for eliminating of the peel particles. The extract pulp of papaya peels can be further utilised as an animal feedstuff [Burdick, 1964], pectin and hemicellulose preparation [Tongdeesuntorn, 2005].

Cysteine and cysteine-EDTA solution were not superior to water for extraction of the peel proteases. Moreover, enzyme stability in the crude extract from all three solution extractions was comparable. It was stable within 4 days and then extremely decreased until 14 days of storage. That means cysteine at 40 mM with or withour 20 mM EDTA used had no substantial effect on separation of proteases from papaya peels, as well as their stability. The results were consistent with previous report that proteolytic enzymes in juice from papaya leaves and stalks were stable within the first 2 days even storage at 5°C [Ball and Thompson, 1940]. The dramatically decreased activities of papaya peel proteases possibly explained by autolysis of enzymes in much diluted aqueous crude extract.

The clarified water-extracted crude solution of papaya peels was chosen for precipitation the proteases. Four precipitants used were methanol, ethanol, 2-propanol and ammonium sulfate. It was found that precipitation with 70% v/v ethanol provided the highest proteolytic yield and specific activity, while 67% v/v 2-propanol and 75% v/v methanol had less efficacy. All solvent concentrations used have been reported that they proficiently separated the proteases from papaya latex. Nonetheless, 67% iso-propanol offered the proteolytic yield slightly greater than 70% ethanol and 75% methanol [Lesuk, 1961]. Variation of precipitants' ability to separate the papaya enzymes between the peel extract and latex can be explained by difference in protein compositions of both sources [Kanasawud *et al.*, 2001].

Table 3.2 shows that 60% saturated ammonium sulfate possessed much lower potency than all three alcohols for separation of proteases from the papaya peels. This salt concentration was used to precipitate the proteases from liquid extract of papaya peels [Kanasawud *et al.*, 2001] and juice of young papaya trees [Burdick, 1964]. The lower yield of papaya peel proteases obtained by ammonium sulfate precipitation possibly explains that papaya peels are composed of enzymes that are less hydrophobic

on their surface. In addition, the peel extract contained a number of non-proteolytic proteins rather than the clear latex solution. These might interfere the salting-out effect on the enzymes in the peel extract. The results were consistent with report of Ball and Thompson [1940] that precipitation of proteases from papaya leaves juice showed saturated ammonium sulfate provided smaller proteolytic yield than 82% v/v of alcohol did. Generally, enzyme precipitation by alcohol offers the advantage to provide antibacterial, as well as separate the enzymes from the major portion of alcohol soluble contaminants [Lesuk, 1961]. The alcohol also produces a purer enzyme product than salting out agents despite the salts seem to be more economical [Burdick, 1964].

Spray drying is obviously a useful alternative method and a less time consuming process for preparation of proteases from papaya latex. The highest solid yield about 30% was obtained by drying the clarified papaya latex at 100°C and 110°C [Theppakorn, 2003]. The temperature of 100°C was used to spray dry the papaya peel extraction. In this study, the peel extract was much dilute resulted as very low yield of spray dried powder (4.84%) was attained. Basically, spray drying of solution containing low solid content lead to difficulty to collect the solid powder. Higher solid content required short time at relatively low temperature [Poulter and Caygill, 1985]. It was found that solid contents between 10% and 30% w/v were suitable for preparation of spray dried papain [Theppakorn, 2003]. In this experiment, addition of ammonium sulfate to the papaya peel extract giving solid content of 20% w/v in the extract resulted as the increase of spray dried powder. As a consequence, the remaining protease activity was increased from 4.0 to 21.6%. The mass of the obtained solid powder from spray dryer was rather low because the powder was stuck on drying chamber. It should be however noted that if all of the spray dried powder can be collected, the proteolytic yield will be 91.2%.

4.2 Proteolytic contents in papaya proteases from peels and latex

Papaya peel and latex proteases showed the difference in maximum casein hydrolysis in Tris-HCl buffer pH 8.0 and 7.0, respectively. It has been reported that papain, chymopapain and caricain hydrolysed casein at the optimal pH of 8, 7 and 8, respectively [Kang and Warner, 1974]. The difference in the pH optimum between proteases from papaya peels and latex is the most likely result from their unequivalent proportion of enzyme contents. The result found that both papaya proteases were more

active in basic than acidic region. Their activities were nearly zero at pH 2.0. This was consistent with previous report that caseinolytic activities of caricain, papain and chymopapain were almost 0% of the optimum in sodium carbonate buffer pH 2. On the other hand, their activities were retained more than 50% in buffer pH 10 [Kang and Warner, 1974].

Proteases from the peels seem likely to be more stable in a wide range of pH, especially in basic side, than those of the latex (**Figure 3.7**). Moreover, the peel proteases displayed good resistance to high temperature (over 70°C) greater than the latex proteases (**Figure 3.8**). It has been shown that chymopapain was more stable at 70-80°C than caricain and papain, respectively [Kang and Warner, 1974]. The papaya cysteine proteases could be ranked in order of relative stability as chymopapain > caricain > papain > glycyl endopeptidase throughout the pH range 1.9-3.9 [Sumner *et al.*, 1993]. Therefore, the difference in stability profiles of papaya proteases from the peels and latex suggests once again that their proportions of protease compositions are unequilalent.

Papaya enzymes separated quite well by cathodic PAGE (**Figure 3.10**). However, mobility of chymopapain and glycyl endopeptidase were quite similar, but the second enzyme moved a bit further [Polgar, 1981; Dubois *et al.*, 1988; Buttle *et al.*, 1989; Dekeyser *et al.*, 1994]. This was because these two enzymes have very similar charge in cathodic PAGE system. Thus, 6 residues at N-terminal was analysed to verify each of protein band and the results corresponded to previously reported [Michel *et al.*, 1970; Dubios *et al.*, 1988; Ritonja *et al.*, 1989; Jacquet *et al.*, 1989; Watson *et al.*, 1990]. The N-terminal of each protein resolves the migration pattern with decreasing mobility as caricain, glycyl endopeptidase, chymopapain, chitinase and papain, respectively. Latex proteases in lane 2 of **Figure 3.10** contained a very small quantity of papain because this latex sample was obtained from the first time tapped fruits that probably reflected small proportion of papain as recently described [Azarkan *et al.*, 2004; 2006].

Cathodic PAGE pattern (**Figure 3.10A**) demonstrated that papaya peel proteases were composed of proteins that were different from the latex enzymes. It seems like only papain and chymopapain in the peels were analogous to the latex compositions. Difference in enzyme proportions between the two sources, one from extruded latex and another from pulp peels of the fruits, was clearly shown. There have been reported the difference amount of enzymes in fruit and non-fruit latex.

Chymopapain A was a major constituent in the non-fruit latex, while chymopapain B was found in the fruit latex only [Brocklehurst and Salih, 1985]. Another study indicated that the non-fruit latex contained chymopapain as the main component, whereas glycyl endopeptidase and caricain were present in larger amount of the fruit latex [Mckee and Smith, 1986]. This difference can also be found even in the latex from the same papaya fruits. A series of low molecular weight non-proteolytic proteins were found only in the first time tapping fruit latex [Akarzan *et al.*, 2004], whereas repeatedly wound fruits extruded the latex accumulated with several enzymes [Akanzan *et al.*, 2006].

There were at least three major protein bands of peels proteases which were different from the latex enzymes. One of these might be a protease due to caseinolytic activity on the gel (**Figure 3.10B**). Because papain has a fairly broad specificity and is quite stable under pH 4.3 [Barrett *et al.*, 1998] of the pH of electrophoresis, it potently digested casein into small peptides resulting a clear zone. Other papaya cysteine proteases possess lower efficiency or higher specificity for casein hydrolysis reflected the darker area. It was found that chymopapain, caricain and glycyl endopeptidase hydrolysed casein to be 37, 37 and 17%, respectively, compared to proficiency of papain [Nitsawang, personal communication]. This work explained the appearance of the darker zone from casein precipitation on the gel by papaya cysteine proteases (**Figure 3.10B**). 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 outside hydrophilic part away. Further cleavage of casein by sufficient protease produces the complete hydrolysis [Walsh, 2002]. This suggests that lower caseinolytic activities of other papaya proteases, except papain, could result in cloudy precipitation which appears as dark band rather than the clear zone.

Results of anodic PAGE and Mono Q anion-exchange FPLC firmly validated the distinction of protein components between papaya proteases from the peels and latex. All of latex enzymes possess pI values higher than the 8.3 of the pH buffer used in the anodic PAGE, while the peel proteases contained numerous proteins possessing pI lower and higher than 8.3. In case of those lower than 8.3, they would acquire positive net charge and thus had mobility toward cathode pole of anodic gel. It was interesting that at least one of these proteins exhibited caseinolytic activity (**Figure 3.10B**). Anion-exchange corroborated the difference in protein compositions between these two sources of enzymes. The pH of buffer used was 10.6 that provided the protein pI lower than this

value obtained the negative net charge and bound to the exchanger. The protein which had a pI value much lower than 10.6 would possess highly negative molecule and was eluted later by high ionic strength. As mentioned above, papaya enzymes had a greater pI value than 8.3 which obtained low negative charge. As a result, they were completely excluded within fraction number 20th by ionic strength about 0.3 (**Figure 3.12B**).

The peel enzymes consisted of more proteins, especially those eluted by the highest ionic strength at fractions number 36th to 39th. Apart from the absorption at 280 nm, additional results revealed that these molecules formed Ruhemann's purple with ninhydrin but failed to react with Bradford reagent, and showed no proteolytic activity toward casein. The enzymatic casein or ovalbumin hydrolysis could decrease the absorption of blue complex at 595 nm. Further hydrolysis resulted as the peptide products which could not react with coomassie brilliant blue [Guo and Jiang, 1998]. For these reasons, the latest eluted molecules in **Figure 3.10A** were probably peptides or amino acids containing aromatic structure that were produced from hydrolysis of proteins or enzymes in the peel proteases.

The first peak containing protein which did not bind to the Mono Q anion-exchanger in **Figure 3.12** has been reported as a protein which runs on cathodic PAGE slower than any protein in papaya latex except papain [Goodenough and Owen, 1987]. This location was approved by N-terminal analysis as described above to be the band of chitinase. Also, this unbound protein was possibly a caricain for which its pI value is greater than 10.6 of pH buffer used. Nevertheless, it lacks the caseinolytic activity as proteases did. Moreover, it has been reported that the caricain was bound to Mono Q anion-exchanger and eluted by 0.1-0.3 M NaCl in buffer pH 10.8 [Goodenough and Owen, 1987]. A Caseinolytic activity of eluted fractions in pools a, b and c were the same between papaya proteases from the peels and latex. Pool c was substantiated as papain by comparing to elution fraction of standard papain from Sigma. Fraction number 21st and 32nd of the peel enzymes displaying proteolytic activities in pools d and e were excluded from column later than papain. This indicated that they possessed pI value lower than 8.75. Anion-exchange analysis substantiated the result of anodic PAGE that there were at least two more proteases appeared in the peels further from the latex. Nevertheless, identification of these new enzymes requires further investigation. More recently, a new highly hydrophobic cysteine protease from papaya latex was found in the latex from newly wounded fruits [Azarkan *et al.*, 2006]. It might correspond to one of

the new proteases discovered in papaya peels. However, further work is required to elucidate how identical they are.

4.3 Screening of glycyl endopeptidase from papaya peel proteases

Glycyl endopeptidase was absent in papaya peels in which papain appeared to be abundant at 29.7% of the relative total amount. This is remarkable since glycyl endopeptidase exhibits as a major component in the latex. From analysis result, it displays 48.3% of the relative total amount while papain appeared only 1.3% in the latex. This is consistent with a number of reports that glycyl endopeptidase is a great majority of papaya latex proteases [Buttle *et al.*, 1989; Buttle, 1994; Barrett *et al.*, 1998]. However, there were different amounts of enzyme depending on source of the latex and the quantification method. Glycyl endopeptidase was accounted by single radial immunodiffusion or dot-blot ELISA as 23-28.2% of the total protein [Buttle *et al.*, 1989; 1990a; Buttle 1994], while densitometric analysis showed that enzyme constitutes of 40% [Nitsawang *et al.*, 2006a]. Therefore, difference in glycyl endopeptidase amount in between this thesis and the previous reports is probably due to variation of latex source and analysis method.

Papaya peels were expected to be great alternative source for isolation of glycyl endopeptidase since there is a lot of waste from consumption and they are also easy to discover. Nevertheless, they did not contain this enzyme. It is consequently not feasible to utilise the papaya peels for preparing glycyl endopeptidase which would be further used in catalysis of peptide synthesis. For this reason, papaya latex which is a rich source of glycyl endopeptidase was chosen for purification of the enzyme.

4.4 Purification and properties of glycyl endopeptidase from fresh papaya latex

The fractionation of the papaya latex soluble protein fraction by aqueous two-phase system followed with salt precipitation showed separation of individual papaya enzyme. The method was greatly successful for purifying glycyl endopeptidase from the latex solution, even though the papaya enzymes have similar physicochemical property that can be difficult to separate. The result in **Table 3.5** showed quite low purification value at 2.57 folds, but the enzyme obtained was of reasonably high purity 93.47%. Concentration of the two-phase components used at 6% (w/w) PEG and 15% (w/w)

ammonium sulfate was consistent with the method of Nitsawang *et al.* [2006a] that the method for partitioning of papain from other papaya proteases was evaluated. This condition was optimum for extraction of papaya enzymes, though the maximal concentrations providing the separated phase were 12% (w/w) PEG-15% (w/w) ammonium sulfate [Salabat, 2001]. Highly viscous mixture will be obtained when using PEG higher than 12%, while the ammonium sulfate greater than 15% provokes the enzyme precipitation.

The purification method in this study provided gram scale (0.25 g) of glycyl endopeptidase from 100 g of the whole fresh papaya latex. It also offered high recovering enzyme of 48% proteolytic yield or 18.74% protein yield. This was the first time using aqueous two-phase and salt precipitation for purifying glycyl endopeptidase. It has been reported that all of previous purification methods used the chromatographic techniques. The 80 mg protein portion of 0.5 g latex [Buttle *et al.*, 1989] or 25 mg spray dried [Thomas *et al.*, 1994] were applied to a site-directed affinity ligand, Sepharose-Ahx-Gly-Phe-$NHCH_2CN$, incorporated with Mono S HR 5/5 cation-exchange column. This method provided only 4 mg glycyl endopeptidase from 80 mg of latex protein (5% protein yield). Buttle [1994] reported that other latex enzymes were treated with iodoacetate and glycyl endopeptidase was later separated by Sepharose-Ahx-Gly-Phe-$NHCH_2CN$ column. This yielded 10 mg of purified enzyme from 200 mg latex protein [Buttle, 1994]. Glycyl endopeptidase was also purified by using a column series of S-Sepharose HP, Shodex HEC PH-814 and Mono S 5/5 [Dekeyser *et al.*, 1994]. The other chromatographic methods required 15 g papaya latex for application to S-Sepharose Fast Flow column accompanying with covalent chromatography of Sepharose-glutathione-2-pyridyl disulfide gel [Thomas *et al.*, 1994] or Sepharose-2-hydroxypropyl-2'-pyridyl disulfide gel [Thomas *et al.*, 1995]. However, for these last three methods the yield of purified enzyme was not reported.

Glycyl endopeptidase was identified by cathodic PAGE and 6 residues of N-terminal analysis that showed the protein band of glycyl endopeptidase possessed 6 amino acids as LPESVD [Nitsawang, personal communication]. This corresponds with previous report of amino acid sequence of glycyl endopeptidase [Ritonja *et al.*, 1989]. Some properties were also unique to glycyl endopeptidase, at least among the papaya cysteine proteases, including successful hydrolysis of P_1 glycine substrate but not P_1 arginine substrate and the lack of inhibition by chicken cystatin [Buttle *et al.*, 1989].

Although, the low rate of inactivation by iodoacetate and iodoacetamide is unusual, it is not a unique property of this enzyme [Buttle, 1994]. In the present work, glycyl endopeptidase was investigated its specific peptide bond cleavage by using two synthetic substrates. Boc-Ala-Ala-Gly-pNA was represented the P_1 glycine substrate, while DL-BAPNA did P_1 arginine one. The result corresponded to previous reports that glycyl endopeptidase was greatly preferable cleavage the first one, while the second substrate is most suitable for papain and chymopapain. This feature readily distinguishes glycyl endopeptidase from the other enzymes in fractions from chromatographic purification [Buttle *et al.*, 1989; Thomas *et al.*, 1994; 1995; O'Hara *et al.*, 1995; Azarkan *et al.*, 2006]. For other P_1 glycine substrates, Boc-Ala-Ala-Gly-NHMec has also been used for the enzyme activity assay [Buttle *et al.*, 1990b; 1990c; Buttle, 1994], while Z-Gly-$OPhNO_2$ and Boc-Gly-$OPhNO_2$ were rapidly spontaneously hydrolysed and consequently not recommended [Polgar, 1981; Buttle, 1994].

In case of protein substrate, due to more restriction of glycyl endopeptidase, the enzyme appeared to hydrolyse azocasein at only about 10% of the activity of papain [Buttle *et al.*, 1989]. By 60 min of casein digestion, glycyl endopeptidase produced a number of discrete peptide products, whereas papain completely degraded [Butlle *et al.*, 1989]. The initial study of casein hydrolysis corresponded to this reports that glycyl endopeptidase exhibited to have only 17.6% of the papain activity. The lower efficiency for hydrolysis of both casein and azocasein of glycyl endopeptidase was not from losing its activity, but from its strictly specific cleavage [Buttle *et al.*, 1989]. The great specificity to Gly of glycyl endopeptidase was shown by 42 peptide bonds cleaved from protein substrates, 3 are glycyl bonds, while the 4 remains suggested from contaminating endopeptidases [Buttle *et al.*, 1990b; Buttle, 1994; Barrett *et al.*, 1998]. Lack of inhibition by cystatin has also usually been recognised for discrimination of glycyl endopeptidase from the other. While the cystatin inhibits most of the cysteine proteases, it acts as a substrate for glycyl endopeptidase. The enzyme cleaved at position Gly9-Ala10 of chicken cystatin or Gly11-Gly12 of cystatin C [Buttle *et al.*, 1990c].

Glycyl endopeptidase was highly active at pH around 6.5-7.5 and was the most active at pH 7.5. It was more active in basic side than the acidic one. This is consistent with the report of Buttle [1994] showing that the enzyme has pH optimum of 7.0-7.5, but still retains about 75% of the optimal activity at pH 9.5, while remaining 15% at pH 3.0. Earlier view [Thomas *et al.*, 1994] also found that optimum k_{cat}/K_m of glycyl

endopeptidase appeared at pH 6.5-7.5 compared to pH 6.0-6.5 of papain. In the present study, glycyl endopeptidase maximally hydrolysed the substrate at 60°C and extremely lost capability at temperature higher than this. As well, it was not likely to be stable at room temperature or 40°C, particularly the enzyme in solution form which showed dramatic decline of stability since the first day (**Figure 3.21-3.22**). There has been a report that glycyl endopeptidase had the lowest stability among the four papaya cysteine proteases [Sumner *et al.*, 1993]. It was revealed that at pH1.9-2.9, glycyl endopeptidase was denatured at temperature around 40°C. Sumner *et al.* [1993] suggested that Pro/Gly ratio in the protein molecule is the most important factor for stability of the three papaya proteases. While papain and caricain had Pro/Gly ratio of 0.36 and 0.42, respectively, glycyl endopeptidase possessed the least of 0.27 reflecting its lowest stability. In case of chymopapain, even though its Pro/Gly appeared at high ratio (0.40), its highest stability was highly related to additional free cysteine residue [Sumner *et al.*, 1993].

Papaya cysteine proteases possesses three forms of molecules; active, reversibly inactive and irreversibly inactive forms. The spray dried latex in citrate-phosphate buffer pH 6.4 at concentration higher than 5% w/w displayed alteration of enzyme's form during incubation at 40°C. This occurred by directly active transformed to reversibly inactive form via apparently inactivated structure [Theppakorn, 2003]. In this study, the alteration of their forms was found in spray dried and clarified solution latex, but not glycyl endopeptidase. This can be explained by the transformation might required the complex of enzymes and/or other promoting compounds as present in the spray dried and solution of the latex.

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

Glycyl endopeptidase was used to catalyse the solid-to-solid peptide synthesis from two solid substrates, Z-Gly-OH as acyl donor and H-Phe-NH_2 as nucleophile. The aqueous phase used in the reaction was 10% w/w of the whole mixture. This water concentration has been reported as the optimum for catalysis in this system [Erbeldinger *et al.*, 1998a; 1998b]. Increasing amount of water at >20% (w/w) led to decline the final yield, while the reaction could no longer be described as solid-to-solid at water concentration > 30% (w/w) [Erbeldinger *et al.*, 1999]. Using aqueous buffer or just water in thermolysin catalytic solid-to-solid synthesis had no influence on the reaction performance [Erbeldinger *et al.*, 1998b]. In the current thesis, the activating agent

(phosphate buffer pH 7.5 consisting of cysteine and EDTA) was consequently used to dissolve glycyl endopeptidase for its full activation before catalysis the coupling of peptides.

As can be seen from **Figure 3.26** that excess of Z-Gly-OH improved the synthetic progress, but excess of H-Phe-NH_2 completely prevented the reaction. This contrasts to the reaction of thermolysin catalysed Z-Gln-Leu-NH_2 synthesis [Erbeldinger *et al.*, 1999]. A very large increase in the initial rate and final yield were achieved when using an excess of H-Leu-NH_2, but the opposite effect was found with excess of Z-Gln-OH. It was suggested that slightly unequal substrate molar ratio can have a large effect on reaction rate and conversion, because the composition of the liquid phase in which the reaction takes place may be greatly dependent on this ratio [Erbeldinger *et al.*, 1999]. They also proposed that acid-base conditions were critical, and supported this theory by showing greatly increased reaction rate after adding basic salt $KHCO_3$ to the reaction mixture of 1:1 substrate molar ratio [Erbeldinger *et al.*, 2001c]. The acid-base effect has also been investigated for solid-to-solid synthesis of Z-Asp-Phe-OMe in which 1:1 ratio of Z-Asp-OH to H-Phe-OMe·HCl making the pH of saturated liquid phase must be very low. Neutralisation by addition of basic salts, $KHCO_3$ or K_2CO_3 or $NaHCO_3$, obviously improved the reaction rate, the last one was however less effective [Erbeldinger *et al.*, 2001c]. In the synthesis of Z-Gly-Phe-NH_2 catalysed by glycyl endopeptidase, analysis of saturated liquid phase from the two substrates mixture could provide an insight into this small volume where reaction would occur. If the solid phases present are Z-Gly-OH and H-Phe-NH_2, the liquid phase concentrations were the same, mutually saturated, whatever the relative amounts of solid were (**Table 3.8**). At calculated condition pH 7.2, non-ionised COOH and NH_2 pK_a of these two substrates were reported at 3.7 and 7.3, respectively [Ulijn *et al.*, 2001]. Therefore, the saturated liquid phase at excess of Z-Gly-OH over H-Phe-NH_2 was relatively acidic. It is presumably that mildly acidic in Z-Gly-Phe-NH_2 condensation had important effect on enzyme behaviour to catalyse the reaction proceed faster. However, attempt to add conc. HCl (100% by mol of substrates) to the reaction mixture of 1:1 ratio fully prevented the synthesis reaction. This is probably too strongly acidic and might require weak acidic salt instead.

It should be noted that scatter in the experimental points, as found in most of the results of solid-to-solid peptide synthesis, is greater than for a typical reaction in solution. This is normal for this heterogenous mainly solid system. Erbeldinger [1999]

has stated that unequal ratio of substrates was predominant over unequal water distribution for the cause of this scatter. If each Eppendorf tubes contain slightly different molar ratios of substrates, the initial rate of reaction in each tube can extremely change. Hence, mixing well of the reaction mixture at starting before incubation is the best choice to carry out.

It can be seen that the reaction reached mass-transfer limited region at about 20 mg enzyme per reaction (**Figure 3.31**). Before this level, the enzyme concentration was important since the reaction was limited by the reaction rate itself and consequently the rate was increased according to higher enzyme used. After 20 mg/reaction, this enzymatic process was predominant by mass-transfer limited region and adding more enzyme did not make any effect. This has also been observed by previous article [Erbeldinger *et al.*, 1998b] that mass-transfer limitation was attained at around 3 g enzyme per mol substrate in the thermolysin catalysed solid-to-solid synthesis of Z-Gln-Leu-NH_2. They also suggested that mass transfer can be reduced by increasing amount of water used in the reaction [Erbeldinger *et al.*, 1998b], however, the peptide conversion is possibly declined. It is very likely that mixing well during the reaction can shift the enzymatic process from mainly mass-transfer limited region to the reaction-rate limited region [Erbeldinger *et al.*, 1998b]. It was also found that continuous sonication or magnetic stirring increased the rate and productivity of the reaction, and furthermore, the initial rate was a function of sonication power Kuhl *et al.* [1992]. Nevertheless, Erbeldinger *et al.* [1999] reported later that the ultrasonication did not improve any proceed of solid-to-solid Z-Gln-Leu-NH_2 synthetic reaction. They suggested that this contrast was probably from very different reaction systems.

In aqueous media, cysteine proteases need to be activated by a reducing agent like cysteine and a chelating agent such as EDTA before catalysis. The cysteine functions to convert the reversibly inactive form toward active form. In addition, it also maintains the enzyme activity during catalysis by prevent oxidation at active site thiol group of Cys25. It is most likely that cysteine in solid-to-solid synthesis of Z-Gly-Phe-NH_2 played important role in either changing form of enzyme or protection the activity during catalysis, because it improved both initial rate and final product (**Table 3.9**). It has also been reported that other reducing agents such as 2-mercaptoethanol was added to the reaction of papain or chymopapain catalysed mainly solid (eutectic mixture) peptide synthesis of Z-Tyr-Gly-NH_2 [López-Fandiño *et al.*, 1994a] or delicious peptide

fragments [Jorba *et al.*, 1995]. In the different low water media, recent article found that addition of cysteine increased the conversion of Z-Gly-Phe-NH_2 synthesis catalysed by immobilised papain that was prepared by the propanol-rinsed method [Theppakorn *et al.*, 2004]. In case of EDTA, it functions to eliminate free metal ions which inhibit the enzyme activity. No substantial effect from the inclusion of Na_2·EDTA in the reaction mixture indicated that the reaction mixture did not contain any ion and/or EDTA consisting in activating agent for dissolving enzyme is sufficient.

4.6 Nucleophile selectivity of glycyl endopeptidase in solid-to-solid synthesis

Glycyl endopeptidase catalysed solid-to-solid synthesis by coupling Z-Gly-OH with a number of each nucleophile, H-Phe-NH_2, H-Leu-NH_2, H-Tyr-NH_2, H-Tyr-OEt, H-Asp-OBzl and H-Pro-NH_2. It was found that the peptides could only be attained by using just four amino acid amides, whereas H-Asp-OBzl and H-Pro-NH_2 could not proceed the reaction. The reaction rate and final conversion by using these nucleophiles are summarised in **Table 4.1**. In comparison among different amino acid amides, at initial stage, glycyl endopeptidase seemed to accept the amino acid with uncharged aliphatic side chain like Leu greater than those with uncharged aromatic of either Phe or Tyr. The highest peptide product Z-Gly-Leu-NH_2 was thus obtained by 10 h of reaction, whereas Z-Gly-Phe-NH_2 and Z-Gly-Tyr-NH_2 were accomplished within longer time (30 h). Yet, the final conversions of these three peptides were almost similar (50-54%). The difference in the initial rates was probably from preference of the enzyme to each amino acid, while their amide protecting group at C-terminal might influence on limitation of the further synthesis. It has been reviewed that 59% of 41 peptide bonds cleaved by glycyl endopeptidase occupied uncharged aliphatic residues at P_2 site. It should be noted that glycyl endopeptidase strictly hydrolyses the peptide bond at between P_1 and P_1' site in which Gly residue denoted at P_1. The uncharged aliphatic amino acids were also found 24 times in P_3 site [Buttle, 1994]. Therefore, it can be suggested that glycyl endopeptidase marked more preference for uncharged aliphatic than aromatic residue.

The result showed that amino acids with polar and nonpolar aromatic side chains (Tyr and Phe, respectively) were practically the same regarding the initial rate, the final conversion and time to attain the final conversion. This indicates that steric effect of amino acids is more crucial than polar effect one on the enzyme's active site during catalysis of solid-to-solid synthesis. When comparing the same amino acid but different

protecting group at C-terminal (ethyl ester and amide) glycyl endopeptidase catalysed the coupling of Z-Gly-OH with H-Tyr-OEt much greater than with H-Tyr-NH_2. This demonstrates that the protecting group also has substantial effect on the glycyl endopeptidase catalysis in predominantly solid system. The result agreed well with previous report of influence of *N*-protecting groups on mainly solid synthesis of Z-Tyr-Gly-NH_2, Ac-Tyr-Gly-NH_2 and TFAc-Tyr-Gly-NH_2 by subtilisin [López-Fandiño *et al.*, 1994a]. The peptide yields at 61, 33 and 2% were obtained when protecting the N-terminal of Tyr with acetyl (Ac), benzyloxycarbonyl (Z) and trifluoroacetyl (TFAc), respectively [López-Fandiño *et al.*, 1994]. Thermolysin has also been studied its favourability of acyl donor and nucleophile in solid-to-solid peptide synthesis [Eichhorn *et al.*, 1997]. While the enzyme accepted all type of amino acid as carboxy components, only hydrophobic residues could be nucleophiles [Eichhorn *et al.*, 1997].

Table 4.1 Glycyl endopeptidase catalysed solid-to-solid synthesis of various peptides. Acyl donor was used at 1.1 per mol nucleophiles, with 20 mg enzyme and 20 mg solid cysteine.

Nucleophile	Initial rate (nmol min^{-1} mg $enzyme^{-1}$)	Highest conversion (%)	Time to highest conversion (h)
H-Phe-NH_2	23	54	30
H-Leu-NH_2	100	50	10
H-Tyr-NH_2	21	52	30
H-Tyr-OEt	125	70	7
H-Asp-OBzl	-	-	-
H-Pro-NH_2	-	-	-

In case of H-Asp-OBzl, it can be explained by two possibilities why product did not occur. Firstly, this nucleophile is zwitterionic compound that might be less effective to form the peptide. Secondly, glycyl endopeptidase might not accept this amino component to couple with Z-Gly-OH. It is now clear that reactant ionisation can play important role in solid-to-solid synthesis. The product precipitation is observed when

neutral peptides are synthesised from opposite single charged substrates. Reaction that involved zwitterionic substrates did not always lead to precipitated product [Ulijn, 2001; Ulijn *et al.*, 2003]. It has been reported that *N*-protected basic amino acids (Z-Lys and Z-Arg) were condensed to L-Leu-NH_2 [Eichhorn *et al.*, 1997]. These acyl donors are zwitterions which yielded the single charged peptide product of Z-Lys-Leu-NH_2 and Z-Arg-Leu-NH_2. As a result, product precipitation was not observed [Eichhorn *et al.*, 1997]. When the amino group in the side chain of Z-Lys was protected to create an acid acyl donor instead of zwitterions, precipitation driven synthesis of Z-Lys-Leu-NH_2 became possible [Ulijn *et al.*, 2003]. It is also possible that glycyl endopeptidase did not accept H-Asp-OBzl probably due to the enzyme selectivity. From previous reports, Asp was not seen in any of the 41 bonds hydrolysed by glycyl endopeptidase. Moreover, most of these cleavages denoted as amino acids with uncharged side chain [Buttle *et al.*, 1990b; Buttle, 1994]. For the last nucleophile tested, H-Pro-NH_2 did not couple with the acyl donor to form the peptide as well. This is most likely that glycyl endopeptidase strictly not accepted the Pro residue. The presence of Pro at P_1' or P_2 site efficiently inhibited the enzyme to cleave Gly at P_1 [Buttle *et al.*, 1990b]. However, glycyl endopeptidase could hydrolyse Gly-Pro bond from protein substrate. This cleave bond was always adjacent to Cys residue at P_2 site which belongs to disulfide bridge [Bernard *et al.*, 1995]. It is almost certain that the enzyme appears to act efficiently in the vicinity of disulfide bonds, being at P_2, although Pro residue was present at P_1' site [Buttle, 1994; Bernard *et al.*, 1995]. In other word, even though glycyl endopeptidase does not cleave Gly-Pro, it will cut this bond if P_2 site is the cystine residue. The peptide yields from solid-to-solid syntheses of Z-Gly-Xaa (Xaa = amino acid amides and esters) were variable depending on types of nucleophile. This is well consistent with previous review that the product yield of this reaction was greatly influenced by the chemical nature of each amino acid [Erbeldinger, 1999]. From discussion above, however, this is just an initial study of glycyl endopeptidase. More different types of nucleophiles are truly worth for more precision of the enzyme behaviour.

4.7 Study on parameters improving solid-to-solid peptide conversion

Even though glycyl endopeptidase successfully catalysed the solid-to-solid synthesis of various glycyl peptides, all of these reactions did not achieve 100% conversion. Therefore, a number of factors affecting the reaction progress were listed

and investigated including, evaporation of water from reaction mixture, particle sizes of reactants, solid substrates were entrapped, reaction reached it equilibrium position, and loss of enzyme activity.

As generally well known even the reaction mixture is predominantly solid mixture, a key feature of this enzymatic solid-to-solid synthesis is the fact that the reaction takes place in small volume of the liquid phase saturated with both substrates [Erbeldinger *et al.*, 2001a; 2001b]. Therefore, the separation of liquid component from the reaction mixture during incubation was considered, even immersion the tubes in water bath firstly protected water vaporisation. It was found that reduction the size of Eppendorf tubes from 1.5 to 0.5 mL volume increased just 10% conversion (**Figure 3.39**). This reveals that water evaporation significantly affected to enzyme catalysis but not the main factor for stopping the reaction. Substrates particle size was also regarded. In solid-to-solid system, dissolution of solid particles into the small portion of liquid phase highly depends on those sizes. Smaller particle size also reduces mass transfer limitation [Kaschet and Galunsky, 1995]. It can be seen that grinding of the two substrates had no substantial effect on the reaction (**Figure 3.40-3.43**), contrasting with that of solid cysteine (**Figure 3.44**). This suggests that both solid substrates appear as small enough powder for dissolution, whereas solid cysteine shown up as larger crystal that is hard to dissolve. Thus, the solid cystine requires grinding beforehand. It was observed that substrate entrapment by precipitated product occurred in the synthesis reaction of Z-Gly-Tyr-OEt (**Figure 3.47**), but not for Z-Gly-Phe-NH_2 and Z-Gly-Leu-NH_2. This is explained by the first reaction was more rapid progress than the latter two. Consequently, the substrates were then surrounded by this quickly precipitated product. In case of the trapped substrates, mixing apparatus has to be considered built in a reactor during catalysis reaction. There have been considerable attempts to design the reactors containing a numerous types of mixer for solid-to-solid peptide synthesis in pilot-scale [Eichhorn *et al.*, 1997; Erbeldinger *et al.*, 2001; Gill and Valivety, 2002]. However in laboratory scale, just mixing well at starting reaction worked greater than ultrasonication, because the ultrasonic wave through the wall of Eppendorf tubes into the reaction mixture is difficult and inefficient [Erbeldinger *et al.*, 1999]. Reaching reaction equilibrium seemed unlikely to be a factor terminating the synthesis because addition or removal of the liquid phase did not alter the reaction direction (**Figure 3.48**). Morever, analysis of both solid and liquid portions of reaction mixture revealed that they were still

containing the two substrates. This indicated that the thermodynamical synthesis was not reached its equilibrium due to one (or two) substrates had been not exhausted [Ulijn *et al.*, 2001]. However, the equilibrium position in this thesis was not so clear. It was observed that addition of fresh enzyme to the normal stopped-reaction mixture resulted as shifting the reaction to hydrolysis side (**Figure 3.48-3.50**). This would support the idea that the reaction mixture is already near equilibrium for the 10% w/w of water level. Hence, when the mixture received more water from new added enzyme solution, the hydrolysis would occur and declined the product conversion. The query of reaction reaching its equilibrium in this solid-to-solid synthesis is still unexplained and requires further investigation to explore obvious answer for this.

Apart from reaching equilibrium, loss of glycyl endopeptidase activity is probably the reason to stop the synthetic progress. It should be noted that enzyme inactivation was the first thing taken care in this experiment. The obtained result showed the decline of peptide yield when re-adding new enzyme solution (as shown in **Figure 3.48-3.50**). This led to a thought that the enzyme still retained its activity and reversed the reaction toward hydrolysis side due to more water present. However, this reason had been re-considered due to confusing in **Figure 3.49** that the reaction proceed did not going further even new enzyme solution was added to the completely dried normal reaction mixture. This mixture was absolutely dried regarding to control the water amount as 10% after adding new enzyme, which appropriate to condense the peptide. As result showed that glycyl endopeptidase activity was rapidly decreased during catalysis of peptide synthesis via solid-to-solid system. Two possibilities were considered for loss of enzyme activity. The first is oxidation of the thiol catalytic site group to become a sulfinic acid or sulfonic acid. However, all reaction mixtures in the thesis contained sufficient reactive cysteine that should prevent the oxidation. Another possibility is the autolysis of glycyl endopeptidase itself during catalysis. This reason was validated by SDS-PAGE and C-4 column HPLC. The rapid autolysis of glycyl endopeptidase is probably explained by second order reaction. As shown in **Figure 4.1**, by using Raswin program, a numerous Gly residues are located at the surface of enzyme molecule. Due to this enzyme strictly cleaves after Gly, it is most likely that glycyl endopeptidase hydrolysed itself during catalysis of solid-to-solid peptide synthesis at 40°C. In conclusion, the rapid hydrolysis of glycyl endopeptidase itself in the mixture was an obvious factor in causing of incomplete conversion in this solid-to-solid synthesis.

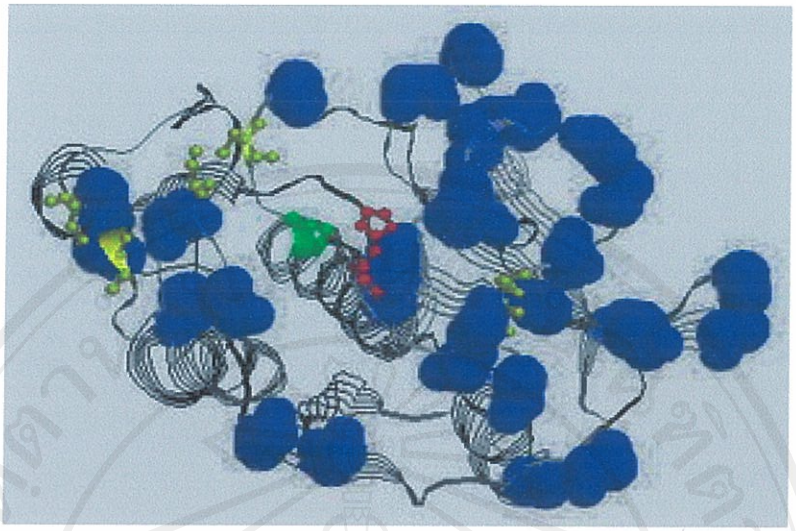

Figure 4.1 Three dimensional structure of glycyl endopeptidase molecule indicating the glycine residues at the surface (blue sphere shape). Balls & stick representing the catalytic Cys-25 (green), catalytic His-159 (red) and three disulfide bridges (yellow). Source: Brookhaven Protein Databank (Code 1GEC).

4.8 Conclusions

Papaya peels were investigated as an alternative source for preparing proteases, especially glycyl endopeptidase. The peel crude extract was precipitated out the proteases with alcohols C1-C3 or ammonium sulfate. The salt yielded lower proteases than the alcohols which 70% ethanol provided the highest yield. Cathodic and anodic gel electrophoresis and anion exchange FPLC indicated that papaya peel proteases were composed of protein components largely different from the latex proteases. This reflected the differences in optimal pH, activation property, and stability in various buffer solutions or at various temperatures. However, the optimal temperatures of the both were similar.

Screening of glycyl endopeptidase in papaya peel and latex proteases by cathodic gel electrophoresis and densitometry revealed that the peel proteases did not contain glycyl endopeptidase. Purification of glycyl endopeptidase by PEG-$(NH_4)_2SO_4$

aqueous two-phase following by two-step salts precipitation with $(NH_4)_2SO_4$ and NaCl gave the enzyme with 93.5% purity.

The studies on properties of glycyl endopeptidase showed the enzyme proficiently hydrolyses synthetic substrate Boc-Ala-Ala-Gly-pNA but not DL-BAPNA. It was also not inhibited the activity by egg-white chicken cystatin which is a protein inhibitor for most of the cysteine proteases. Glycyl endopeptidase had the optimal pH and temperature toward Boc-Ala-Ala-Gly-pNA at 7.0 and 60°C, respectively. The enzyme, especially solution form, showed rather low stability at room temperature or 40°C. Glycyl endopeptidase seemed likely not altered its form that might reflect to its low stability, while the clarified solution and spray dried of papaya latex showed transition stage of changing enzyme's form. This is probably related to the other compound present in the latter two enzymes.

Glycyl endopeptidase worked well as a catalyst for solid-to-solid synthesis of Z-Gly-Phe-NH_2. The reaction rate and conversion were very dependent on the substrate molar ratios and presence of cysteine. The highest conversion at around 83% was achieved with a 2:1 Z-Gly-OH to H-Phe-NH_2 ratio, and 20 mg enzyme and 20 mg solid cysteine per reaction. Glycyl endopeptidase had to be activated before catalysis. Analysis of saturated liquid phase of substrate mixtures showed liquid phase component was relative to proportion of each solid substrate. The acyl donor Z-Gly-OH obtained from Bachem provides better result than that obtained from Novabiochem.

The synthesis of other peptides, Z-Gly-Leu-NH_2, Z-Gly-Tyr-NH_2, and Z-Gly-Tyr-OEt could be also catalysed by glycyl endopeptidase. The enzyme seemed to accept aliphatic side chain amino acid rather than aromatic one. Therefore, the initial rate of Z-Gly-Leu-NH_2 synthesis was faster than those of Z-Gly-Phe-NH_2 and Z-Gly-Tyr-NH_2. Using the two nucleophiles, H-Phe-NH_2 and H-Tyr-NH_2, gave no significant difference in reaction progress, indicating steric effect play more important role than polar effect on the enzyme. Glycyl endopeptidase catalysed the synthesis of Z-Gly-Tyr-OEt more efficient than that of Z-Gly-Tyr-NH_2. This was suggested that C-protecting group was a great influence. It can be concluded that substrate binding sites in the active centres of proteases exhibit different specificity towards nucleophiles.

The studies on parameters improving conversion of peptide synthesis showed that grinding of the two substrates or re-mixing of the stopped reaction mixture or changing reaction equilibrium or adding new activated enzyme solution resulted in no

further peptide conversion. It was found that an important factor in synthesis termination at around 83% conversion was the rapid autolysis of glycyl endopeptidase. This requires additional investigation on ways to prevent enzyme autolysis and hence further improve the synthesis.