# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals for investigation of papaya peel proteases

| Chemical | MW | Company |
|---|---|---|
| Acetic acid (glacial, d=1.05 kg/l) | 60.05 | Merck |
| Acrylamide | 71.08 | Fluka |
| Agarose | - | Sigma |
| β-Alanine | 89.09 | Sigma |
| Ammonium persulfate | 228.2 | Sigma |
| Ammonium sulfate | 132.14 | Carlo Erba |
| L-Arginine | 174.2 | Sigma |
| Basic fuchsin (chloride) | 323.80 | Sigma |
| Boric acid | 61.83 | Sigma |
| Bovine serum albumin | 67000 | Fluka |
| Bromophenol blue | - | Fluka |
| Casein | - | Sigma |
| Citric acid | 61.83 | Sigma |
| Coomassie Brilliant Blue G-250 | 854.0 | Sigma |
| Coomassie Brilliant Blue R-250 | 826.0 | Sigma |
| L-Cysteine | 121.16 | Fluka |
| Disodium hydrogen phosphate anhydrous | 141.96 | Fluka |
| Ethyl alcohol (absolute) | 46.07 | Merck |
| Ethyl alcohol 95% | 46.07 | Merck |
| Ethylene diamine tetraacetic acid disodium salt ($Na_2$·EDTA) | 372.44 | Sigma |
| Glycerol | 92.09 | Sigma |
| L-Glycine | 75.07 | Fluka |
| Hydrochloric acid (35.4%, d=1.19kg/l) | 36.46 | BDH |
| 2-propyl alcohol | 60.10 | Merck |
| L-Leucine | 131.18 | Fluka |
| Methyl alcohol | 32.04 | Merck |

| Chemical | MW | Company |
|---|---|---|
| *N,N'*-methylene-bis-acrylamide | 154.17 | Fluka |
| L-Phenylalanine | 165.19 | Sigma |
| Phosphoric acid 85% | 98.0 | Carlo Erba |
| Potassium hydroxide | 56.11 | Merck |
| Purified chymopapain | - | Sigma |
| Purified papain | - | Sigma |
| Silica gel 60 | - | Merck |
| Sodium dihydrogen phosphate dihydrate | 156.01 | Fluka |
| Sodium hydroxide | 39.99 | Merck |
| Sucrose | 342.30 | Carlo Erba |
| L-Tyrosine | 181.19 | Aldrich |
| *N,N,N',N'*-Tetramethylethylenediamine (TEMED) | 116.20 | Sigma |
| Trichloroacetic acid (TCA) | 163.39 | Carlo Erba |
| Tris (hydroxymethyl) methylamine | 121.14 | Merck |

### 2.1.2 Chemicals for purification of glycyl endopeptidase from papaya latex

| Chemical | MW | Company |
|---|---|---|
| Acetic acid (glacial, d=1.05 kg/l) | 60.05 | Merck |
| Acrylamide | 71.08 | Fluka |
| β-Alanine | 89.09 | Sigma |
| Ammonium persulfate | 228.2 | Sigma |
| Ammonium sulfate | 132.14 | Carlo Erba |
| Basic fuchsin (chloride) | 323.80 | Sigma |
| *N*-Benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA) | 434.88 | Bachem |
| *t*-Butyloxycarbonyl-L-alanyl-L-alanyl-L-glycine-p-nitroanilide (Boc-Ala-Ala-Gly-pNA) | 437.45 | Bachem |
| Bovine serum albumin | 67000 | Fluka |
| Coomassie Brilliant Blue G-250 | 854.0 | Sigma |
| Coomassie Brilliant Blue R-250 | 826.0 | Sigma |
| L-Cysteine | 121.16 | Fluka |

| Chemical | MW | Company |
|---|---|---|
| Dimethylsulfoxide (DMSO) | 78.13 | Sigma |
| Disodium hydrogen phosphate anhydrous | 141.96 | Fluka |
| Ethylene diamine tetraacetic acid disodium salt ($Na_2$·EDTA) | 372.44 | Sigma |
| Hydrochloric acid (35.4%, d=1.19kg/l) | 36.46 | BDH |
| Methyl alcohol | 32.04 | Merck |
| *N,N'*-methylene-bis-acrylamide | 154.17 | Fluka |
| Polyethylene glycol (PEG) | 8000 | Sigma |
| Purified papain | - | Sigma |
| Sodium acetate | 82.03 | Sigma |
| Sodium chloride | 58.44 | Carlo Erba |
| Sodium chloroacetate | 116.48 | Aldrich |
| Sodium dihydrogen phosphate dihydrate | 156.01 | Fluka |
| Sodium hydroxide | 39.99 | Merck |
| Sucrose | 342.30 | Carlo Erba |

### 2.1.3 Chemicals for glycyl endopeptidase catalysed solid-to-solid peptide synthesis

| Chemical | MW | Company |
|---|---|---|
| Acetonitrile | 41.05 | Aldrich |
| 30% Acrylamide/bis-acrylamide | - | Sigma |
| Ammonium persulfate | 228.19 | Sigma |
| L-Aspartic acid benzylester (H-Asp-OBzl) | 223.23 | Bachem |
| Benzyloxycarbonyl-L-glycine (Z-Gly-OH) | 209.2 | Bachem |
| Benzyloxycarbonyl-L-glycine (Z-Gly-OH) | 209.2 | Novabiochem |
| Benzyloxycarbonyl-L-glycine-L-phenylalanine amide (Z-Gly-Phe-$NH_2$) | 355.39 | Bachem |
| *t*-Butyloxycarbonyl-L-alanyl-L-alanyl-L-glycine-p-nitroanilide (Boc-Ala-Ala-Gly-pNA) | 437.45 | Bachem |
| Bromophenol blue | - | Merck |
| L-Cysteine | 121.16 | Fluka |
| Dimethylsulfoxide (DMSO) | 78.13 | Sigma |
| Disodium hydrogen phosphate anhydrous | 141.96 | Fluka |

| Chemical | MW | Company |
|---|---|---|
| Ethylene diamine tetraacetic acid disodium salt ($Na_2$·EDTA) | 372.44 | Sigma |
| Glycerol | 92.09 | Sigma |
| Glycine | 75.07 | Fluka |
| Hydrochloric acid (35.4%, d=1.19kg/l) | 36.46 | BDH |
| L-Leucine amide (H-Leu-$NH_2$) | 130.19 | Bachem |
| Low molecular protein standard marker | - | Sigma |
| β-Mercaptoethanol | 78.13 | Sigma |
| Methyl alcohol | 32.04 | Merck |
| *N,N'*-methylene-bis-acrylamide | 154.17 | Fluka |
| L-Phenylalanine amide (H-Phe-$NH_2$) | 164.21 | Bachem |
| L-Proline amide (H-Pro-$NH_2$) | 114.15 | Bachem |
| Sodium acetate | 82.03 | Sigma |
| Sodium chloroacetate | 116.48 | Aldrich |
| Sodium dihydrogen phosphate dihydrate | 156.01 | Fluka |
| Sodium dodecyl sulfate (SDS) | - | Fluka |
| *N,N,N',N'*-Tetramethylethylenediamine (TEMED) | 116.20 | Sigma |
| Trifluoro acetic acid (TFA) | | Aldrich |
| L-Tyrosine amide (H-Tyr-$NH_2$) | 180.21 | Bachem |
| L-Tyrosine ethylester (H-Tyr-OEt) | 209.2 | Sigma |

### 2.1.4 Instruments

| Instrument | Model | Company |
|---|---|---|
| Adjustable micropipette | - | Gilson, Biohit |
| Balance | - | Sartorius |
| Blender | HR1701 | Philips |
| Centrifuge, refrigerated | 6800 | Kubota |
| Densitometer | DS-9301PC | Shimadzu |
| Dialysis membrane tubing (MW cut-off 10,000) medical | - | Spectrum Indus. Inc. |
| Digital camera | DSC-W5 | Sony |
| Electrophoresis set | Mini-PROTEAN | Bio-rad |

| Instrument | Model | Company |
|---|---|---|
| Electrophoresis set | Mini VE | Hoefer |
| FPLC system | - | Pharmacia |
| Hot plate and stirrer | - | Heiddoph |
| HPLC system | 2695 | Waters Alliance |
| Incubator | - | Memmert |
| Lyophiliser | FE-3-55D-MP | FTS System Inc. |
| Oven | 400 | Memmert |
| pH Meter | 713 | Metrohm |
| Platform shaker | STR6 | Stuart Scientific |
| Power supply | 1000/500 | Bio-rad |
| Shaking water bath | 1083 | GFL |
| Sonicator | 8891 | Cole-Parmer |
| Spectrophotometer UV/VIS | PU 8625 | Philips |
| Spectrophotometer UV/VIS | DU 800 | Beckman |
| Coulter | - | - |
| Spray dryer | - | - |
| Top loading balance | PG802-S | Mettler Toledo |
| Water bath | WBM 15 | Falc. |
| Vortex mixer | - | IKA |

## 2.2 Methods

### 2.2.1 Preparation of papaya peel proteases

#### 2.2.1.1 Extraction of proteases from papaya peels

*Carica papaya* L. fruits were harvested from 70-100 days of maturation of papaya trees planted in Chiang Mai, Thailand. The fruits were washed with water and then allowed the outside to be dried before peeling the fruits. Papaya peels were cut into small pieces and dried at 55°C in a tray-dryer [Theppakorn, 2003] until the weight has been reduced to 10% of the original.

Three different methods were used to extract enzyme from 20 g portions of dried papaya peels (200 g fresh). In the first, dried papaya peels were finely ground before soaking with 180 mL water until whole water was absorbed into the dried peels.

In the second method, the dried peels were soaked with the same amount of water before grinding in a blender. The last procedure was performed by soaking the peels with 180 ml water before blending with 400 mL water (giving the ratio of water:fresh peels = 2:1). After filtration through gauze, the filtrates were clarified by centrifugation at 9000xg and 4°C to obtain a clear solution of papaya peel crude extracts. These 3 extracts were assayed for proteolytic activity.

**2.2.1.2 Study on the effect of cysteine and EDTA on protease extraction**

Twenty grams of dried papaya peels were finely ground, followed by soaking with 180 mL water for 10 min. The extract was filtered and centrifuged at 9000xg and 4°C to obtain a clear solution. After determination of protease activity and protein concentration, the papaya peel crude extract was stored at either -20°C, 4°C or room temperature (30°C approximately).

The same method was also used but water was replaced by 40 mM cysteine solution or 40 mM cysteine-20 mM $Na_2$·EDTA solution.

**2.2.1.3 Precipitation of proteases from papaya peel crude extract**

Proteases from papaya peel crude extract were precipitated with organic solvents following the method for protease precipitation from papaya latex [Lesuk, 1961]. Four portions of 38 mL crude extract were pre-chilled to 4°C. The precipitants, methanol, ethanol, and 2-propanol were slowly added to obtain final concentrations of 75% (v/v), 70% (v/v) and 67% (v/v), respectively. To the final portion, ammonium sulfate was added giving 60% of saturation with the salt (26.2 g/100 mL solution). The solutions were stirred at 4°C for 30 min. The precipitates of proteases were separated by centrifugation at 9000xg and 4°C for 10 min, and then dialysed 6 times against deionised water. The papaya peel proteases were lyophilised to obtain enzyme powders which were assayed for proteolytic activity and protein concentration.



**2.2.1.4 Spray drying of papaya peel crude extract**

Papaya peel proteases were also prepared by spray drying. Ammonium sulfate was added to 38 mL of papaya peel crude extract, giving a final concentration of 20% w/v. This helped increase the solids content which resulted in the highest yield of spray dried solid particles. The crude extract containing ammonium sulfate was dried by spray

dryer, built at Department of Chemistry, Chiang Mai University [Theppakorn, 2003]. Inlet temperature was 100°C and the solution was fed to a nebuliser at 2 mL $min^{-1}$.

### 2.2.2 Preparation of papaya latex proteases

#### 2.2.2.1 Latex collection

Fresh latex was collected from 70-100 days maturation of locally grown *Carica papaya* fruits (Hang Dong District, Chiang Mai, Thailand). A stainless steel knife was used to make 4-6 longitudinal incisions on the surface of unripe fruits in the morning. The liquid latex dripped onto the collecting tray attached to the trunk with wire supports (Theppakorn, 2003) and some latex coagulated on the fruits. The latex was filtered with a screen (50 mesh) to remove impurities before storing at -20°C in a sealed plastic container until used.

#### 2.2.2.2 Drying of papaya latex proteases

Fresh papaya latex was loaded on trays and placed into the drying chamber of a tray-dryer [Theppakorn, 2003]. The latex was dried at 55°C for 1 h. The dried product was scraped and then ground by hand with a mortar to obtain crude papain. It was stored at -20°C until used.

### 2.2.3 Proteolytic activity of papaya peel and latex proteases

#### 2.2.3.1 Assay for proteolytic activity

Proteolytic activity was measured using casein as a substrate according to the procedure of Arnon [Arnon, 1970] with slight modification. The reaction mixture containing 0.10 mL of enzyme solution, 0.30 mL of 50 mM Tris-HCl buffer pH 8.0 and 0.10 mL of activating agent (40 mM cysteine/20 mM $Na_2$·EDTA pH 8.0) was incubated in a water bath at 37°C for 5 min. The reaction was initiated by adding 0.50 mL of 1% (w/v) casein solution. After 10 min, 1.50 mL of 5% (w/v) cold trichloroacetic acid was added to terminate the reaction. The supernatant of the mixture was separated by centrifugation at 9000xg for 20 min. The absorbance of the supernatant was measured at 275 nm.

One unit of proteolytic activity was defined as the amount of enzyme releasing product equivalent to 1 μmole tyrosine $min^{-1}$ (see Appendix A for standard curve of tyrosine).

**2.2.3.2 Determination of optimal pH**

To determine the pH optimum of papaya peel and latex proteases, the reaction were performed at 37°C using a number of buffers at a concentration of 50 mM in the range of pH 2-11: sodium-phosphate buffer pH 2, 7 and 11, sodium-citrate buffer pHs 3 and 6, sodium-acetate buffer pHs 4 and 5, Tris-HCl buffer pH 8 and sodium borate buffer pHs 9 and 10.

**2.2.3.3 Determination of optimal temperature**

Catalytic activities of proteases from papaya peels and latex were determined at constant temperatures of 30, 40, 50, 60, 65, 70, 75, 80 and 90°C in Tris-HCl buffer pH 8.0.

**2.2.3.4 Determination of enzyme stability at various pHs**

Stability of papaya proteases was analysed by incubating the enzyme solution in the buffer pHs 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 at a constant temperature of 37°C for 10 min. The incubated enzymes were then assayed for their proteolytic activity in pH 8.0 at 37°C.

**2.2.3.5 Determination of enzyme stability at various temperatures**

The proteases were dissolved in Tris-HCl buffer pH 8.0 and incubated at temperatures of 20, 30, 40, 50, 60, 70 or 80°C for 10 min. Then, the enzymes were taken for determination of their proteolytic activity in pH 8.0 at 37°C.

**2.2.3.6 Effect of cysteine**

The effect of cysteine on enzyme catalysis was investigated by performing reaction at 37°C in buffer pH 8.0. The reaction was carried out as in Section 2.2.3.1 except that the activating agent was substituted by 20 mM $Na_2$·EDTA with various concentration of cysteine (0, 2.5, 5, 10, 20, 30 and 40 mM).

**2.2.4 Protein composition of proteases from papaya peel and latex**

**2.2.4.1 Protein determination**

The protein content in enzyme samples was measured using protein-dye binding method [Bradford, 1976]. The sample (0.10 mL) at appropriate concentration was mixed

with 0.40 mL distilled water and 5.0 mL of Bradford reagent. The mixture was kept at room temperature for 15 min and then the absorbance was measured at 595 nm. The amount of protein in the enzyme sample was calculated from a BSA standard curve (see Appendix B).

**2.2.4.2 Cathodic gel electrophoresis and *in situ* proteolysis assay**

Cathodic polyacrylamide gel electrophoresis was carried out on a slab gel using Hoefer miniVE electrophoresis system. The experiment was modified from the method of Reisfeld et al. [Reisfeld *et al.*, 1962]. The slab gel comprised resolving gel of 15% acrylamide, pH 4.3 and stacking gel of 4% acrylamide at pH 6.7. The upper and lower chamber electrode buffer (pH 4.5) consisted of 0.36 M β-alanine–0.14 M acetic acid. Prior to loading onto the gel the protein sample was diluted 1:1 v/v with stacking buffer containing 10% sucrose and 0.002% tracking dye basic fuchsin. Electrophoresis was pre-run at a constant current 20 mA, 300 V for 30 min, and after loading protein samples it was then run at constant 40 mA, 300 V for 3 h. The protein sample migrated toward the cathode during operation.

After electrophoresis, the gel was cut into two equal parts. The first half was stained with Coomassie Brilliant Blue R-250 in a solution of acetic acid/methanol/water (1:5:4 by vol.) and then destained with acetic acid/methanol/water (2:3:1 by vol). The second half was assayed for *in situ* proteolytic activity by a modification of the method of Moutim *et al.* [1999]. The gel was rinsed twice with Tris-HCl buffer pH 8.0. The same buffer containing 0.5% (w/v) agarose and 1.8% (w/v) casein was then applied to the gel surface. After incubation at 37°C for 24 h, complete and partial casein digestion resulted as clear zones and precipitation regions on the gel, respectively.

**2.2.4.3 Anodic gel electrophoresis and *in situ* proteolysis assay**

Anodic polyacrylamide gel electrophoresis was performed according to the method of Hames [1981] using Hoefer miniVE electrophoresis system. The gel consisted of 7% acrylamide separating gel and 5% acrylamide stacking gel. The buffer in both electrode chambers was 0.025 M Tris–0.192 M Glycine, pH 8.3 and bromophenol blue was used as a dye marker. Electrophoresis was pre-run at a constant current 20 mA, 300 V for 30 min. After loading samples, it was run at constant 40 mA, 300 V for 1.5 h. The protein bands were stained with Coomassie Brilliant Blue R-250.

**2.2.4.4 Anion-exchange FPLC system**

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

**2.2.5 Analysis of glycyl endopeptidase in protease from papaya peels and latex**

Because glycyl endopeptidase is the major protease in papaya latex, it is consequently expected to be abundant in papaya peels. Therefore, glycyl endopeptidase content in papaya peel proteases was investigated by comparing to that in the latex. The method used in this section was cathodic electrophoresis and staining as in Section 2.2.4.2. The relative concentrations of glycyl endopeptidase and other papaya enzymes were quantified by measuring the absorbance of protein bands at 595 nm using CS-9301PC program attached in Shimadzu densitometer.

**2.2.6 Purification of glycyl endopeptidase from fresh papaya latex**

The results of above experiments (Section 2.2.5) indicated that glycyl endopeptidase is absent in papaya peels. Hence, papaya latex was subsequently used for purification of glycyl endopeptidase for further experiments in this thesis.

Glycyl endopeptidase was purified from fresh latex of *C. papaya* L. by an aqueous-two-phase system followed by salt precipitation. One hundred grams of fresh papaya latex were agitated with 33 mL of water at 4°C and then filtered through the Whatman No.1 paper to separate the insoluble materials. The clear solution was adjusted to pH 5.0 and then papain was removed by an aqueous-two-phase system of 6% w/w PEG (7.60 g) and 15% ammonium sulfate (19.00 g). Chymopapain and caricain were separated from the salt-rich bottom phase by precipitation with 11.3% w/v ammonium sulfate. After centrifugation at 9000xg, 4°C, the supernatant protein concentration was adjusted to 8 mg/mL and then NaCl (20% w/v) was added to the solution to precipitate

out chitinase. Further NaCl was added to the supernatant giving 33% w/v to precipitate glycyl endopeptidase. The enzyme was re-dissolved in deionised water and dialysed 6 times against deionised water. After lyophilisation, enzyme powder was stored at -20°C. The purity of the enzyme was verified by cathodic polyacrylamide gel electrophoresis and its ability to resist cystatin inhibitor and hydrolyse the specific substrate Boc-Ala-Ala-Gly-pNA but not DL-BAPNA.

**2.2.7 Assay for glycyl endopeptidase activity on DL-BAPNA**

This assay was performed following the previous method [Dekeyser *et al.*, 1994] with some modification. The substrate solution was prepared by dissolving 50 mg of DL-BAPNA with 2 mL of DMSO. This solution was then diluted 1:50 v/v in 50 mM sodium phosphate buffer pH 7.5. Two hundred milligrams of enzyme powder was dissolved in 1 mL of water. One hundred microlitres of enzyme solution was mixed with 200 µL of the sodium phosphate buffer pH 7.5 and 200 µL of activating agent (40 mM cysteine/ 20 mM $Na_2$·EDTA in the same buffer). The mixture was incubated at 40°C for 5 min to activate the enzyme. The reaction was initiated by adding 500 µL of substrate solution. After exactly 10 min, 500 µL of 30% (v/v) acetic acid was added to terminate the reaction. Released *p*-nitroaniline was determined by measurement of absorbance at 410 nm. One unit of activity was defined as the release of 1 nmol of *p*-nitroaniline $min^{-1}$ from DL-BAPNA at 40°C, pH 7.5 ($\varepsilon_{410}$ = 8800 $M^{-1}$ $cm^{-1}$).

**2.2.8 Assay for glycyl endopeptidase activity on Boc-Ala-Ala-Gly-pNA**

The procedure was modified from the method of Buttle [1994]. One milligram of glycyl endopeptidase was dissolved in 1 mL of water. A sample of this enzyme solution (200 µL) was mixed with 500 µL of activating agent (40 mM cysteine/20 mM $Na_2$·EDTA in 50 mM sodium phosphate buffer pH 7.5). The volume was made up to 975 µL with the same buffer and the mixture was then incubated at 40°C for 5 min to give fully active enzyme. The reaction was started by adding 25 µL of substrate stock solution (50 mM Boc-Ala-Ala-Gly-pNA in DMSO). After exactly 8 min, 1 mL of stopping reagent (100 mM sodium chloroacetate/30 mM sodium acetate/70 mM acetic acid, pH 4.3) was added and $A_{410}$ was measured. One unit activity of enzyme was defined as the release of 1 nmol of *p*-nitroaniline $min^{-1}$ from Boc-Ala-Ala-Gly-pNA at 40°C, pH 7.5 ($\varepsilon_{410}$ = 8800 $M^{-1}cm^{-1}$).

### 2.2.9 Determination of properties of glycyl endopeptidase

Because the results showed that glycyl endopeptidase hydrolysed Boc-Ala-Ala-Gly-pNA preferably over DL-BAPNA, all studies on properties of glycyl endopeptidase were investigated using the first substrate.

#### 2.2.9.1 Optimal pH

This assay was investigated using a range of 50 mM aqueous buffer solutions: sodium phosphate buffer pH 2.0, 7.0, 7.5 and 11.0, sodium citrate buffer pH 3.0 and 6.0, sodium acetate buffer pH 4.0 and 5.0, citrate phosphate buffer pH 6.4, Tris-HCl buffer pH 8.0 and sodium borate buffer pH 9.0 and 10.0. One mg of glycyl endopeptidase powder was dissolved in 1 mL of water. The enzyme solution was used for amidase activity assay in buffers of various pHs at 40°C.

#### 2.2.9.2 Optimal temperature

One mg of enzyme powder was dissolved in 1 mL of sodium phosphate buffer pH 7.5. This enzyme solution was used for amidase activity assay in the same buffer at temperatures of 20, 30, 40, 50, 55, 60, 65, 70, 75 and 80°C.

#### 2.2.9.3 Activation time of enzyme and effect of activator

Influence of activation before catalysis by glycyl endopeptidase was investigated. One milligram of enzyme powder was dissolved in sodium phosphate buffer pH 7.5 and diluted with either the same buffer or activating agent (40 mM cysteine/20 mM $Na_2$·EDTA in the same buffer). Both enzyme solutions were incubated in a water bath at 40°C for 0, 2, 5, 10, 15 and 20 min before determination of the enzyme activity at 40°C, pH 7.5.

#### 2.2.9.4 Inhibition of enzyme by cystatin

The inhibition of glycyl endopeptidase by chicken cystatin was investigated using Boc-Ala-Ala-Gly-pNA as substrate. Two hundred microlitres of enzyme solution in phosphate buffer pH 7.5 (1 μg/μL) was mixed with 200 μL of the same buffer with or without 5 μg/μL of chicken egg-white cystatin and 575 μL of activating agent pH 7.5. The mixture was then incubated at 40°C for 20 min. Then 25 μL of substrate solution

was added and the substrate hydrolysis was allowed for 8 min. The reaction was terminated and analysed as described in Section 2.2.8.

Papain and chymopapain from Sigma were also determined using DL-BAPNA as substrate. One hundred microlitres of each enzyme solution (5 μg/μL) was mixed with 200 μL of the buffer with or without 5 μg/μL chicken egg-white cystatin and 200 μL of activating agent pH 7.5. The mixture was incubated at 40°C for 20 min and then carried out as described in Section 2.2.7.

**2.2.9.5 Stability of glycyl endopeptidase**

Stability of glycyl endopeptidase in both solid and solution form was determined at room temperature and 40°C. The enzyme powder (50 mg) or liquid (50 mg/mL in 50 mM phosphate buffer pH 7.5) was filled in Eppendorf tubes. Each tube was kept at room temperature or incubated at 40°C for 15 days. At interval time, the enzyme was taken for assay its amidase activity by using Boc-Ala-Ala-Gly-pNA as substrate.

**2.2.9.6 Alteration of enzyme's form**

Transformation of glycyl endopeptidase was investigated by weighing 1 mg of enzyme in an Eppendorf tube and then adding 1 μL 50 mM sodium phosphate buffer pH 7.5 to obtain solid content 50% w/w. The tubes were sealed with parafilm and then placed in an incubator at constant temperature 40°C. After a specified period of time, the samples were dissolved in 1 mL the same buffer above. The enzyme solution was used for assay of amidase activity with and without the activation by activating agent. The activity assay was carried out by using Boc-Ala-Ala-Gly-pNA as substrates.

The transformation of spray-dried and clarified solution of papaya latex were also investigated. Fresh papaya latex was agitated and then filtered through the Whatman No.1 paper to remove the insoluble materials and obtain clear latex supernatant. This solution was directly used to test alteration of enzyme or dried by the built spray-dryer at inlet temperature 100°C to obtain spray-dried latex (Theppakorn, 2003).



**2.2.10 Glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-$NH_2$**

**2.2.10.1 Effect of substrate molar ratios**

Various substrate molar ratios were obtained by mixing (1+x) mmol of Z-Gly-OH and (1-x) mmol of H-Phe-$NH_2$ with 20 mg solid cysteine in 10 mL vial. Glycyl

endopeptidase was used at 20 mg per reaction. The enzyme was first dissolved in activating agent (50 mM sodium phosphate buffer pH 7.5 containing 40 mM cysteine/20 mM $Na_2$·EDTA) and incubated at 40°C for 5 min. The enzyme solution was then added to the solid substrates giving a final water content of 10% w/w. The reaction mixture was then mixed well by hand with a spatula and separated into individual Eppendorf tubes, each with around 7% of the total. These sealed tubes were immersed in a water bath at 40°C.

**2.2.10.2 HPLC analysis**

At the required time intervals, individual portions of reaction mixtures were dissolved in 3.5 mL of acetronitrile/water/TFA 30:66.5:3.5 v/v/v to terminate the reaction. After removing solid particles by filtration (through 0.2 μm nylon membrane, Whatman), the samples were analysed by HPLC on a Waters Alliance system with C-18 reverse phase column and detected at 254 nm. The mobile phase consisted of 30% acetonitrile in 0.01% aqueous TFA at a flow rate of 1.0 mL $min^{-1}$ and 25°C. The retention times of Phe-$NH_2$, Z-Gly and Z-Gly-Phe-$NH_2$ were 2.9, 4.7 and 7.9 min respectively.

Because the weight of each sample was known, the ratio of the two HPLC areas (H-Phe-$NH_2$ and product) gave the conversion directly. This was complemented by calculation of the quantity of product using the area and external standards of Z-Gly-Phe-$NH_2$ (Appendix C).

**2.2.10.3 Analysis of liquid phase equilibrated with solid substrates**

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

**2.2.10.4 Optimal amount of glycyl endopeptidase**

Enzyme amount was investigated for optimal catalysis in solid-to-solid synthesis of Z-Gly-Phe-$NH_2$. Glycyl endopeptidase in the reaction mixtures were present

at 0, 2.5, 5, 10, 15, 20 or 25 mg per reaction. The enzyme powder was firstly dissolved with activating agent pH 7.5 and then activated for 5 min before mixing with 1.28 mmol of Z-Gly-OH (0.2689 g), 0.64 mmol of H-Phe-$NH_2$ (0.1045 g) and 20 mg of solid cysteine.

**2.2.10.5 Effect of Z-Gly-OH sources**

One substrate, Z-Gly-OH, was obtained from both Bachem and Novabiochem companies. Therefore, these two different sources of Z-Gly-OH were compared. The two substrates at ratio of 2:1 were mixed with 20 mg solid cysteine. The activated glycyl endopeptidase solution pH 7.5 was added to this solid mixture. After thoroughly mixing, the reaction mixture was divided and incubated before analysis as usual.

**2.2.10.6 Effect of cysteine**

In this synthetic reaction, cysteine was present in both activating solution (to dissolve the enzyme) and as solid form in the reaction mixture. The role of cysteine in both these forms was thus investigated. Before mixing with solid substrate mixture, glycyl endopeptidase was dissolved in the activating solution (20 mM cysteine/20 mM $Na_2$·EDTA solution pH 7.5) or in the same solution but without cysteine. The effect of solid cysteine in the reaction mixture was also determined by varying it at 0, 50, 100 and 200% w/w relative of enzyme. All reaction mixtures were incubated and analysed as usual.

**2.2.10.7 Effect of EDTA**

The effect of EDTA on the glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-$NH_2$ was investigated. The reaction, consisting of 1.28 mmol of Z-Gly-OH, 0.64 mmol H-Phe-$NH_2$, 20 mg of enzyme and 20 mg of solid cysteine, was performed with and without 1 mg of solid $Na_2$·EDTA. The catalytic rate and peptide conversion were analysed as usual.

**2.2.11 Investigation of specificity of glycyl endopeptidase on nucleophiles in solid-to-solid peptide synthesis**

Other reactions of glycyl endopeptidase catalysed solid-to-solid synthesis were also studied. Z-Gly-OH was still used as acyl donor because the enzyme is specific for

glycine at $P_1$ of coupling site. All reactions were performed at the substrate molar ratios 1:1, 1.1:1 and 1.5:1 with 20 mg of glycyl endopeptidase and 20 mg of solid cysteine.

**2.2.11.1 Synthesis of Z-Gly-Leu-$NH_2$**

Substrate molar ratios at 1:1, 1.1:1 and 1.5:1 were obtained by mixing (1+x) mmol of Z-Gly-OH with (1-x) mmol of H-Leu-$NH_2$ and solid cysteine. Twenty milligrams of enzyme powder were dissolved in activating agent and activated at 40°C for 5 min before adding to the solid mixture to initiate the reaction. After mixing well with a spatula, the mixture was then divided into separated Eppendorf tubes and incubated at 40°C. Samples were analysed by HPLC with C-18 reverse phase column. The mobile phase consisted of 30% v/v acetonitrile mixed with 70% v/v aqueous containing 0.01% TFA (flow rate 1.0 mL $min^{-1}$). Detection of the Z-Gly-OH, H-Leu-$NH_2$, and product Z-Gly-Leu-$NH_2$ was at 225 nm.

**2.2.11.2 Synthesis of Z-Gly-Tyr-$NH_2$**

Z-Gly-OH [(1+x) mmol] and H-Tyr-$NH_2$ [(1-x) mmol] were mixed with solid cysteine and carried out as described in Section 2.2.11.1. The reaction mixture was analysed by C-18 reverse phase column on HPLC system with the mobile phase consisting of 25% v/v acetonitrile in aqueous solution containing 0.01% aqueous TFA (1.0 mL $min^{-1}$). The peptide product and substrates were detected at 225 nm.

**2.2.11.3 Synthesis of Z-Gly-Tyr-OEt**

Substrate molar ratios were obtained by mixing (1+x) mmol of Z-Gly-OH with (1-x) mmol of H-Tyr-OEt and solid cysteine. The reaction was performed as in Section 2.2.11.1 and analysed by HPLC system with C-18 reverse phase column. The mobile phase contained 35% v/v acetonitrile mixed with 65% v/v aqueous solution containing TFA at flow rate 1 mL $min^{-1}$. The product and substrate were detected at 254 nm.

**2.2.11.4 Synthesis of Z-Gly-Asp-OBzl**

The reaction was made up by mixing (1+x) mmol of Z-Gly-OH, (1-x) mmol of H-Asp-OBzl and solid cysteine with the activated enzyme solution and carried out as in Section 2.2.11.1. The reaction mixture was analysed by HPLC system with C-18 reverse phase column. The mobile phase consisted of 45% v/v acetonitrile in aqueous solution

containing 0.01% TFA. The flow rate was 1 mL $min^{-1}$ and the substrates were detected at 254 nm.

**2.2.11.5 Synthesis of Z-Gly-Pro-$NH_2$**

H-Gly-OH [(1+x) mmol] and H-Pro-$NH_2$ [(1-x) mmol] were mixed with solid cysteine and carried out as described in Section 2.2.11.1. Portions of reaction mixture were analysed by HPLC system with C-18 reverse phase column and detection of the substrates at 225 nm. The mobile phase consisted of 30% v/v of acetonitrile and 70% v/v of 0.01% aqueous TFA at flow rate 1.0 mL $min^{-1}$.

**2.2.12 Investigation of parameters improving peptide conversion**

These studies were all performed with the solid-to-solid synthesis of model peptide Z-Gly-Phe-$NH_2$ by using the substrate molar ratio 2:1, 20 mg of enzyme and 20 mg of solid cysteine. However, in some cases, other peptide syntheses were also investigated with 1.1:1 of substrate molar ratio.

**2.2.12.1 Reducing size and centrifugation of reaction tubes**

This was investigated in synthesis of the peptide Z-Gly-Phe-$NH_2$. As mentioned above, individual small portions of reaction mixture were incubated separately in order to monitor progress. In some cases liquid droplets were observed to form in the upper part of the tube, away from the solid mass at the bottom. In order to eliminate any effect of this separation on the reaction progress, smaller Eppendorf tubes (0.5 mL) were used instead, and these were centrifuged when liquid separation was observed, forcing it back down into the rest of the mixture. These treatments slightly increased the final conversion of the peptide synthesis reaction.

**2.2.12.2 Grinding of solid substances**

Substrate particles of smaller size might be more available for dissolving to proceed the reaction. All solid particles including the two substrates and solid cysteine were ground by hand in a mortar (previously dried at 100°C for 24 h and stored in a desiccator until used). The ground solid substances were subsequently used in the reaction as usual.

**2.2.12.3 Re-mixing of reaction mixture**

After normal reaction progress had stopped, the mixture was re-mixed thoroughly with a spatula. The mixture was separated into individual Eppendorf tubes and then re-incubated at 40°C and analysed by HPLC as usual.

**2.2.12.4 Alteration of liquid amount in reaction mixture**

Possible effects on reaction equilibrium were investigated with the solid-to-solid synthesis of peptide Z-Gly-Phe-$NH_2$. After peptide conversion had stopped, more water was added in the same quantity as initially (10% w/w), and mixed thoroughly. Other additions studied were buffer pH 7.5, activating agent and enzyme solution. Water was also removed by drying the reaction mixture over molecular sieve in a desiccator at 40°C. Removal of water was monitored by periodically weighing, until half that added originally had been removed (5% w/w). The reaction mixture was then re-incubated and analysed as usual.

**2.2.12.5 Analysis of phase composition of the reaction mixture**

The mixture of Z-Gly-Phe-$NH_2$ synthesis reaction was suspended in 1 mL deionised water, shaken for a few seconds and then the liquid phase was removed. The solid phase remaining in the tube was dissolved with 2 mL of acetonitrile/water/TFA 30:66.5:3.5 v/v/v. Both liquid portions were analysed by HPLC.

**2.2.12.6 Adding of new enzyme solution**

After the normal reaction of solid-to-solid synthesis of Z-Gly-Phe-$NH_2$ had terminated, new-activated enzyme solution was added to the mixture with and without completely drying beforehand. The water was removed by drying over molecular sieve in a desiccator at 40°C. Both reaction mixtures after adding enzyme solution were re-incubated and then analysed by HPLC as usual.

**2.2.13 Investigation of glycyl endopeptidase recovered from the reaction mixture of solid-to-solid Z-Gly-Phe-$NH_2$ synthesis**

**2.2.13.1 Enzyme assay for amidase activity**

The reaction mixture in Eppendorf tube was suspended briefly in 1 mL of deionised water. Insoluble material was removed by using filter paper (Whatman No.1)

and the enzyme activity in the clear solution was determined by using Boc-Ala-Ala-Gly-pNA as substrate as described in Section 2.2.8.

**2.2.13.2 SDS-PAGE**

Autolysis of glycyl endopeptidase in the reaction mixture was investigated by SDS-PAGE, using a slab gel in a Bio-Rad Mini-PROTEAN unit. The experiment was carried out according to the method of Laemmli [1970], with the separating gel 15% (w/v) acrylamide and the stacking gel 4%. The buffer in both electrode chambers was 0.124 M Tris–0.959 M glycine. Electrophoresis was run at a constant 200 V and bromophenol blue was used as a dye marker. The protein bands were obtained by staining with Coomassie Brilliant Blue R-250. Molecular weight standards were bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), rabbit muscle glyceraldehyde phosphate dehydrogenase (36 kDa), bovine erythrocytes carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), bovine milk α-lactalbumin (14.2 kDa) and bovine lung aprotinin (6.5 kDa).

**2.2.13.3 HPLC analysis on C-4 column**

The autolysis of glycyl endopeptidase in the reaction mixture was also analysed by $C_4$ column HPLC. The enzyme solution obtained as in Section 2.2.13.1 was injected to a Waters Alliance HPLC system with C-4 reverse phase column. The mobile phase consisted of 30% v/v acetonitrile in 70% v/v aqueous containing 0.01% TFA at a flow rate 1.0 mL $min^{-1}$. The enzyme molecule was detected at 280 nm.