

## TABLE OF CONTENTS

Title	Page
Acknowledgements	iii
Abstract (in English)	iv
Abstract (in Thai)	vi
Table of Contents	viii
List of Tables	xiv
List of Figures	xvi
Abbreviations and Symbols	xxiv
<b>Chapter 1 Introductions</b>	
1.1 Principle and rationale	1
1.2 Literature Review	2
1.2.1 Papaya cysteine proteases	2
1.2.1.1 Composition of cysteine proteases in fruit latex and other parts of the tree	2
1.2.1.2 Separation by ion-exchange/FPLC	6
1.2.1.3 Cathodic polyacrylamide gel electrophoresis pattern	9
1.2.1.4 Catalytic mechanism and form of enzyme	12
1.2.2 Glycyl endopeptidase	15
1.2.2.1 History	15
1.2.2.2 Physicochemical properties	16
1.2.2.3 Enzymatic properties	18
1.2.2.4 Preparation	20
1.2.3 Thermodynamically and kinetically controlled peptide synthesis	24
1.2.4 Solid-to-solid peptide synthesis	26
1.2.4.1 Historical background	26
1.2.4.2 General aspects	28
1.2.4.3 Switch like thermodynamics of reaction	31
1.2.4.4 Solvent selection	34
1.2.4.5 Form of enzyme	36

	Page
1.2.4.6 pH effect on catalytic reaction	37
1.2.5 Objective of study	39
<b>Chapter 2 Materials and Methods</b>	
2.1 Materials	40
2.1.1 Chemicals for investigation of papaya peel proteases	40
2.1.2 Chemicals for purification of glycyl endopeptidase from papaya latex	41
2.1.3 Chemicals for glycyl endopeptidase catalysed solid-to-solid peptide synthesis	42
2.1.4 Instruments	43
2.2 Methods	44
2.2.1 Preparation of papaya peel proteases	44
2.2.1.1 Extraction of proteases from papaya peels	44
2.2.1.2 Study on the effect of cysteine and EDTA on proteases extraction	45
2.2.1.3 Precipitation of proteases from papaya peel crude extract	45
2.2.1.4 Spray drying of papaya peel crude extract	45
2.2.2 Preparation of papaya latex proteases	46
2.2.2.1 Latex collection	46
2.2.2.2 Drying of papaya latex proteases	46
2.2.3 Proteolytic activity of papaya peel and latex proteases	46
2.2.3.1 Assay for proteolytic activity	46
2.2.3.2 Determination of optimal pH	47
2.2.3.3 Determination of optimal temperature	47
2.2.3.4 Determination of enzyme stability at various pHs	47
2.2.3.5 Determination of enzyme stability at various temperatures	47
2.2.3.6 Effect of cysteine	47
2.2.4 Protein composition of proteases from papaya peel and latex	47
2.2.4.1 Protein determination	47
2.2.4.2 Cathodic gel electrophoresis and <i>in situ</i> proteolysis assay	48
2.2.4.3 Anodic gel electrophoresis and <i>in situ</i> proteolysis assay	48
2.2.4.4 Anion-exchange FPLC system	49

	Page
2.2.5 Analysis of glycyl endopeptidase in proteases from papaya peels and latex	49
2.2.6 Purification of glycyl endopeptidase from fresh papaya latex	49
2.2.7 Assay for glycyl endopeptidase activity on DL-BAPNA	50
2.2.8 Assay for glycyl endopeptidase activity on Boc-Ala-Ala-Gly-pNa	50
2.2.9 Determination of properties of glycyl endopeptidase	51
2.2.9.1 Optimal pH	51
2.2.9.2 Optimal temperature	51
2.2.9.3 Activation time of enzyme and effect of activator	51
2.2.9.4 Inhibition of enzyme by cystatin	51
2.2.9.5 Stability of glycyl endopeptidase	52
2.2.9.6 Alteration of enzyme's form	52
2.2.10 Glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub>	52
2.2.10.1 Effect of substrate molar ratios	52
2.2.10.2 HPLC analysis	53
2.2.10.3 Analysis of liquid phase equilibrated with solid substrates	53
2.2.10.4 Optimal amount of glycyl endopeptidase	53
2.2.10.5 Effect of Z-Gly-OH sources	54
2.2.10.6 Effect of cysteine	54
2.2.10.7 Effect of EDTA	54
2.2.11 Investigation of specificity of glycyl endopeptidase on nucleophiles in solid-to-solid peptide synthesis	54
2.2.11.1 Synthesis of Z-Gly-Leu-NH <sub>2</sub>	55
2.2.11.2 Synthesis of Z-Gly-Tyr-NH <sub>2</sub>	55
2.2.11.3 Synthesis of Z-Gly-Tyr-OEt	55
2.2.11.4 Synthesis of Z-Gly-Asp-OBzl	55
2.2.11.5 Synthesis of Z-Gly-Pro-NH <sub>2</sub>	56
2.2.12 Investigation of parameters improving peptide conversion	56
2.2.12.1 Reducing size and centrifugation of reaction tubes	56
2.2.12.2 Grinding of solid substances	56
2.2.12.3 Re-mixing of reaction mixture	57

	Page
2.2.12.4 Alteration of liquid amount in reaction mixture	57
2.2.12.5 Analysis of phase composition of reaction mixture	57
2.2.12.6 Adding of new enzyme solution	57
2.2.13 Investigation of glycyl endopeptidase recovered from the reaction mixture of solid-to-solid Z-Gly-Phe-NH <sub>2</sub> synthesis	57
2.2.13.1 Enzyme assay for amidase activity	57
2.2.13.2 SDS-PAGE	58
2.2.13.3 HPLC analysis on C-4 column	58
<b>Chapter 3 Results</b>	
3.1 Preparation of proteases from papaya peels	59
3.1.1 Preparation of papaya peel crude extract	59
3.1.2 Effect of cysteine and EDTA on proteases extraction	60
3.1.3 Yield of papaya peel protease preparation	62
3.1.4 Spray dried papaya peel proteases	63
3.2 Comparison of enzyme catalysis between proteases from papaya peel and latex	65
3.2.1 Optimal pH	65
3.2.2 Optimal temperature	66
3.2.3 Stability at various pHs	66
3.2.4 Stability at various temperatures	67
3.2.5 Effect of cysteine	67
3.3 Comparison of protein composition between proteases from papaya peel and latex	69
3.3.1 Cathodic gel electrophoresis and <i>in situ</i> proteolysis	69
3.3.2 Anodic gel electrophoresis and <i>in situ</i> proteolysis	70
3.3.3 Mono Q column FPLC	71
3.4 Presence of glycyl endopeptidase in papaya peels and latex	73
3.5 Purification of glycyl endopeptidase from fresh papaya latex	77
3.6 Properties of purified glycyl endopeptidase	79
3.6.1 Substrate specificity	79
3.6.2 Optimal pH	80

	Page
3.6.3 Optimal temperature	81
3.6.4 Optimal activation before catalysis of enzyme	82
3.6.5 Lack of inhibition by cystatin	83
3.6.6 Stability of glycyl endopeptidase	84
3.6.7 Alteration of enzyme's form	85
3.7 Glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub>	88
3.7.1 Effect of substrate molar ratios	88
3.7.2 Composition of liquid phase in substrates mixture	90
3.7.3 Analysis result of reaction mixture	90
3.7.4 Optimal amount of glycyl endopeptidase	94
3.7.5 Effect of Z-Gly-OH sources	94
3.7.6 Effect of cysteine on glycyl endopeptidase catalysis	96
3.7.7 Effect of EDTA on glycyl endopeptidase catalysis	97
3.8 Various nucleophiles coupling with Z-GlyOH in glycyl endopeptidase catalysed solid-to-solid synthesis	97
3.8.1 Solid-to-solid synthesis of Z-Gly-Leu-NH <sub>2</sub>	98
3.8.2 Solid-to-solid synthesis of Z-Gly-Tyr-NH <sub>2</sub>	99
3.8.3 Solid-to-solid synthesis of Z-Gly-Tyr-OEt	99
3.8.4 Solid-to-solid synthesis of Z-Gly-Asp-OBzl and Z-Gly-Pro-NH <sub>2</sub>	100
3.9 Improvement of peptide conversion of solid-to-solid synthesis catalysed by glycyl endopeptidase	100
3.9.1 Water evaporation from reaction mixture	101
3.9.2 Particle size of reactants	102
3.9.3 Entrapment of solid substrates	105
3.9.4 Equilibrium of reaction	108
3.9.5 Addition of new enzyme solution	109
3.10 Activity of glycyl endopeptidase recovered from solid-to-solid reaction mixture	111
3.10.1 Glycyl endopeptidase activity	111
3.10.2 SDS-PAGE	112
3.10.3 HPLC analysis	113

	<b>Page</b>
<b>Chapter 4 Discussion and conclusions</b>	
4.1 Preparation of proteases from papaya peels	115
4.2 Proteolytic contents in papaya proteases from peels and latex	117
4.3 Screening of glycyI endopeptidase from papaya peel proteases	121
4.4 Purification and properties of glycyI endopeptidase from fresh papaya latex	121
4.5 Solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> catalysed by glycyI endopeptidase	124
4.6 Nucleophile selectivity of glycyI endopeptidase in solid-to-solid synthesis	127
4.7 Study on parameters improving solid-to-solid peptide conversion	129
4.8 Conclusions	132
References	135
Appendix	148
Curriculum vitae	151

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่

Copyright© by Chiang Mai University

All rights reserved

## LIST OF TABLES

Table	Page
1.1 Characteristics and properties of cysteine proteases from papaya latex.	5
1.2 Chromatographic methods for preparation of glycyl endopeptidase.	23
3.1 Comparison of extracting methods for papaya peel proteases extraction from 20 g dried (200 g fresh) papaya peels.	60
3.2 Separation of papaya peel proteases from 38 mL of crude extract obtained from 5 g of dried papaya peels.	63
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).	64
3.4 Relative amount of papaya enzymes from fruit peels and latex. The enzymes were separated by cathodic gel electrophoresis and analysed by densitometry.	75
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.	78
3.6 Selective hydrolysis of glycyl endopeptidase on the two amide synthetic substrates comparing with standard proteases and latex solution.	80
3.7 Amidase activity of papaya cysteine proteases with presence and absence of chicken cystatin.	84
3.8 Liquid phase compositions at 27 h of substrate mixture equilibrated with 1050 $\mu$ L of water at 40°C.	90
3.9 Initial rate and % conversion from glycyl endopeptidase catalysed solid-to-solid Z-Gly-Phe-NH <sub>2</sub> synthesis in the presence and absence of cysteine. Reactions were performed at substrate molar ratio 2:1 and using 20 mg enzyme per reaction.	96
3.10 Quantity of liquid and solid phase components in reaction mixture of glycyl endopeptidase catalysed solid-to-solid Z-Gly-Phe-NH <sub>2</sub> synthesis.	109



Table	Page
3.11 Activity of glycyl endopeptidase in reaction mixture for Z-Gly-Phe-NH <sub>2</sub> 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.	112
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.	128



## LIST OF FIGURES

Figure	Page
1.1 Cysteine and half-cystine residues and disulfide bonds in the four papaya cysteine proteases. 25* denotes the catalytic cysteine residues and chymopapain shows additional cysteine at 117.	3
1.2 Alignment of the prosequences of papaya cysteine proteases. Residues conserved in all four sequences are in bold and for clarity, deletions are denoted by hyphens.	4
1.3 Separation of papaya proteases using a Mono Q anion-exchange column attached to the Pharmacia FPLC: peak 1; chitinase (denoted in the paper as papaya proteinase B), peak 2; impurities, peak 3; caricain, peak 4; mixture of chymopapain isoforms, and peak 5; papain.	8
1.4 Gel electrophoresis of the four papaya cysteine proteases after purification by chromatographic methods.	10
1.5 Catalytic mechanism for hydrolysis of peptide bond by cysteine proteases.	13
1.6 Schematic drawing of simplest version of propapain-papain transition.	14
1.7 Reaction producing inactive forms of protein thiol group.	14
1.8 Three dimension strand structure of glycyl endopeptidase; ball & stick representing the catalytic Cys25 (green), catalytic His159 (pink), three disulfide bond residues (yellow), Leu1 at N-terminal (red), and Asn216 at C-terminal (blue).	17
1.9 Terminology of the cleavage site of glycyl endopeptidase, and also papaya cysteine proteases.	18
1.10 Structures of (a) Sepharose-glutathione-S-S-2-Py gel, (b) Sepharose-2-hydroxypropyl-S-S-2'-Py gel, and (c) 2,2'-dipyridyl disulfide (2-Py-S-S-2-Py).	22
1.11 Protease catalysed thermodynamically controlled and kinetically controlled synthesis of peptides.	24
1.12 Comparison of a kinetically and a thermodynamically (or equilibrium) controlled peptide synthesis.	25

Figure	Page
1.13 Comparison of enzymatic peptide synthesis in organic media (upper row) or in solid-to-solid system (lower row). Initial and equilibrium compositions are represented at the left and the right column, respectively. All areas equal concentration in w/w.	29
1.14 Green aspects of solid-to-solid biocatalysis.	30
1.15 Schematic overview of precipitation driven biocatalysis. The three sub-processes involved are: the dissolution of substrates, the enzyme catalysed reaction and the precipitation of the reaction product (s).	31
1.16 Schematic representation of solid-to-solid reaction. If $Z_{\text{sat}} < K_{\text{eq}}$ , the product precipitates and equilibrium will be reached only when the solid substrates completely run out (and completely dissolve).	34
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 $\text{Na}_2\text{EDTA}$ (Δ).	61
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 $\text{Na}_2\text{EDTA}$ (Δ).	61
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 $\text{Na}_2\text{EDTA}$ (Δ).	62
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% saturated ammonium sulfate (D), and spray drying with addition of 20% ammonium sulfate (E).	64
3.5 Optimal pH at 37°C on casein hydrolyses of papaya peel proteases (■) and papaya latex proteases (▲).	65
3.6 Optimal temperature on casein hydrolyses in Tris-HCl buffer pH 8.0 of papaya peel proteases (■) and papaya latex proteases (▲).	66
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.	67

Figure	Page
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.	68
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.	68
3.10 Separation of proteins by cathodic gel electrophoresis, stained with Coomassie Brilliant Blue (A) and <i>in situ</i> verifying their proteolytic activities (B).	70
3.11 Separation of proteins by anodic gel electrophoresis, stained with Coomassie Brilliant Blue (A) and <i>in situ</i> verifying their proteolytic activities (B).	71
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 (....).	72
3.13 Separation of proteins by cathodic gel electrophoresis, stained with Coomassie Brilliant Blue.	73
3.14 Protein band intensity of standard papain in lane 5 of <b>Figure 3.13</b> . Band No. 1; papain, No. 2 and 3; chitinase and chymopapain, respectively.	76
3.15 Protein band intensity of latex proteases in lane 4 of <b>Figure 3.13</b> . Band No. 1; papain, No. 2; chitinase, No.3; chymopapain, No. 4; glycyl endopeptidase and No. 5; caricain.	76
3.16 Protein band intensity of papaya peel proteases in lane 2 of <b>Figure 3.13</b> . 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.	77
3.17 Cathodic gel electrophoresis of papaya cysteine proteases which lane 1; standard papain from Sigma (5.5 µg protein), lane 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).	79
3.18 The pH-activity profile of glycyl endopeptidase.	81

Figure	Page
3.19 The temperature-activity profile of glycyl endopeptidase in sodium phosphate buffer pH 7.5 at temperature ranging between 20 and 80°C.	82
3.20 Effects of activator and incubation time on glycyl endopeptidase activity.	83
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/w (□).	85
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/w (□).	85
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.	86
3.24 Change in total recoverable (■), directly active (○) and reversibly inactive (□) activities of 50% solid spray dried papain in in phosphate buffer pH 7.5, incubated at 40°C.	87
3.25 Change in total recoverable (■), directly active (○) and reversibly inactive (□) activities of 16% solid clarified papaya latex solution, incubated at 40°C.	87
3.26 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> , with the substrate molar ratios (Z-Gly-OH : H-Phe-NH <sub>2</sub> ) at 1:1 (■), 1.05:1 (○), 1.1:1 (▲), 1.3:1 (+), 1.5:1 (x) and 2:1 (*).	89
3.27 A typical glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> before (A) and after (B) mixing of reactants with the substrate molar ratio 2:1.	89
3.28 Chromatographic profile at reaction progress of solid-to-solid condensation between Z-Gly-OH and H-Phe-NH <sub>2</sub> at the substrate molar ratio 2:1, 20 mg of both glycyl endopeptidase and solid cysteine.	91
3.29 Chromatographic profile at reaction progress of solid-to-solid condensation between Z-Gly-OH and H-Phe-NH <sub>2</sub> at the substrate molar ratio 1:1, 20 mg of both glycyl endopeptidase and solid cysteine.	92

Figure	Page
3.30 Accurate mass spectra of peptide product, Z-Gly-Phe-NH <sub>2</sub> (A) and by-product, Z-Gly-Phe-OH (B) from glycyl endopeptidase catalysed solid-to-solid peptide synthesis.	93
3.31 Effect of glycyl endopeptidase (GE) amount on the initial rate of solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> .	94
3.32 Comparison in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> by using Z-Gly-OH from Bachem (●) and Novabiochem (○) and H-Phe-NH <sub>2</sub> at molar ratio of 2:1, 20 mg of enzyme and solid cysteine.	95
3.33 A typical appearance of Z-Gly-OH powders from Bachem (left) and Novabiochem (right).	95
3.34 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> . Two reactions were compared with the presence (■) and absence (□) of 1 mg EDTA in the mixture.	97
3.35 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Leu-NH <sub>2</sub> , with 20 mg of both glycyl endopeptidase and solid cysteine per reaction. The substrate molar ratios (Z-Gly-OH:H-Leu-NH <sub>2</sub> ) were varied at 1:1 (■), 1.1:1 (×) and 1.5:1 (○).	98
3.36 Time course of glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-NH <sub>2</sub> , with 20 mg of glycyl endopeptidase and solid cysteine per reaction. The substrate molar ratios (Z-Gly-OH:H-Tyr-NH <sub>2</sub> ) were varied at 1:1 (■) and 1.1:1 (Δ).	99
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 (x) and 1.5:1 (○).	100
3.38 The separation of reaction mixture in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> ; the reaction mixture before (A) and after (B) incubation in a water bath 40°C for 25 h.	101



Figure	Page
3.39 Effects of tube size and centrifugation on glycyI endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> . Substrate molar ratio (Z-Gly-OH : H-Phe-NH <sub>2</sub> ) was 2:1. The reaction was carried out in the tubes of 1.5 mL (■) and 0.5 mL with (o) or without (x) centrifugation.	102
3.40 Time course of glycyI endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> 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.	103
3.41 Time course of glycyI endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Leu-NH <sub>2</sub> 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.	103
3.42 Time course of glycyI endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Tyr-NH <sub>2</sub> 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.	104
3.43 Time course of glycyI 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.	104
3.44 Effect of grinding solid cysteine on glycyI endopeptidase catalysed peptide synthesis. Substrate molar ratio (Z-Gly-OH : H-Phe-NH <sub>2</sub> ) of 2:1, added solid cysteine was ground (□) or unground (■).	105
3.45 Time course of glycyI endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> 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 (○).	106
3.46 Time course of glycyI endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Leu-NH <sub>2</sub> 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 (○).	107

Figure	Page
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 (○).	107
3.48 Glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> . Substrate molar ratio (Z-Gly-OH : H-Phe-NH <sub>2</sub> ) 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 (+).	108
3.49 Effect of adding fresh enzyme after conversion had stopped in glycyl endopeptidase catalysed solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> 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 (□).	110
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 (□).	110
3.51 Inactivation of glycyl endopeptidase while catalysing solid-to-solid synthesis of Z-Gly-Phe-NH <sub>2</sub> with substrate molar of 2:1; progress of synthesis (—■—), and residual amidase activity of enzyme (---Δ---).	111
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).	113
3.53 Absorption at 280 nm of glycyl endopeptidase recovered from the reaction mixture of solid-to-solid Z-Gly-Phe-NH <sub>2</sub> 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).	114



**Figure****Page**

- 4.1 Three dimensional structure of glycyI 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).

132

## ABBREVIATIONS AND SYMBOLS

AB	product peptide
Ac	acetyl
Ac-Phe-Gly-pNA	<i>N</i> -acetyl-L-Phenylalanylglycine- <i>p</i> -nitroanilide
Ahx-Gly-Phe-NHCH <sub>2</sub> CN	6-aminohexanoyl-glycine-phenylalanine-aminoacetonitrile
AOH	acyl donor
AS	ammonium sulfate
BH	nucleophile
Boc	<i>t</i> -butyloxycarbonyl
Boc-Ala-Ala-Gly-NHMec	<i>t</i> -butyloxycarbonyl-L-alanyl-L-alanyl-L-glycine-7-(4-methyl)coumaryl-amide
Boc-Ala-Ala-Gly-pNA	<i>t</i> -butyloxycarbonyl-L-alanyl-L-alanyl-L-glycine- <i>p</i> -nitroanilide
Boc-Gly-OPhNO <sub>2</sub>	<i>t</i> -butyloxycarbonyl-L-glycine- <i>p</i> -nitrophenyl ester
Bz	benzoyl
Bz-Arg-pNA	<i>N</i> -benzoyl-DL-arginine- <i>p</i> -nitroanilide
DL-BAPNA	<i>N</i> -benzoyl-DL-arginine- <i>p</i> -nitroanilide
$\Delta G$	gibbs free energy change (Jmol <sup>-1</sup> )
GSH	glutathione
HOAc	acetic acid
K <sub>eq</sub>	concentration based equilibrium constant (M <sup>-1</sup> )
K <sub>th</sub>	thermodynamics equilibrium constant
Log P	partitioning between octanol and water
R <sub>m</sub>	relative mobility
pI	isoelectric point
PAGE	polyacrylamide gel electrophoresis
PEG	polyethyleneglycol
2-Py-S-S-2-Py	2,2'-dipyridyl disulfide
S <sub>AOH</sub>	molar solubility of acyl donor (M <sup>-1</sup> )

$S_{\text{BOH}}$	molar solubility of nucleophile ( $M^{-1}$ )
$S_{\text{AB}}$	molar solubility of peptide product ( $M^{-1}$ )
TFAc	trifluoroacetyl
Z	benzyloxycarbonyl
Z-Gly-O $\text{PhNO}_2$	benzyloxycarbonyl-L-glycine- <i>p</i> -nitrophenyl ester
$Z_{\text{sat}}$	saturated mass action ratio
$Z_{\text{th}}$	thermodynamics mass action ratio

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่  
 Copyright© by Chiang Mai University  
 All rights reserved