CHAPTER 1

INTRODUCTION

1.1 Principle and rationale

Glycyl endopeptidase (EC 3.4.22.25), one of four cysteine proteinases found in the latex of *Carica papaya*, exhibits different specificity from the others [Azarkan *et al.*, 2003 and Moussaoui *et al.*, 2001]. It constitutes 23-28% of total proteins which displays as a major protein in the latex [Barrett, 1998]. According to the restricted specificity for glycyl bond of glycyl endopeptidase [Buttle *et al.*, 1990], the enzyme is important for the synthesis of several peptides containing glycine such as sweeteners (e.g. N-Ac-Gly-Lys), flavor enhancer (e.g. Gly-Leu), delicious peptides (e.g. Gly-Asp), immunoactive tripeptide (Gly-Phe-Leu) and neuromodulator pentapeptide (e.g. enkephalin Tyr-Gly-Gly-Phe-Leu(Met)) etc.[Gill *et al.*, 1996 and López-Fandiño *et al.*, 1994a].

It has long been known that proteases can be used to catalyze peptide formation in organic media which offers several advantages, including increasing solubility of hydrophobic substrates, catalyzing reaction that are unfavorable in water, ease of recovery for some products and biocatalysts, enhancing thermal enzyme stability and reducing microbial contamination [Khmelnitsky and Rich, 1999; Laane *et al.*, 1986; Bell *et al.*, 1995]. However, the use of organic solvent often leads to loss of enzyme activity [Klibanov, 2001; Ghatorae *et al.*, 1994] and reduction of peptide yield due to low substrate solubility [Ahn *et al.*, 2001]. Moreover, it is often difficult to select a suitable solvent that is not deleterious to the enzyme used, and which would solubilise high concentration of substrates equally well [Gill and Vulfson, 1994]. In the last decade, it has been shown that good reaction rates and high equilibrium yield can be achieved when most of the substrates and products remain in the solid form. Peptide synthesis has been successfully carried out in a reaction mixture containing only 10% to 20% water/organic solvent or just pure water [Erbeldinger *et al.*, 1998a; 1998b; Halling *et al.*, 1995; Ulijn *et al.*, 2002]. Such reaction is named 'solid-to-solid' peptide synthesis which can be catalysed surprisingly efficient by enzyme such as papain [López-Fandiño *et al.*, 1994], thermolysin [Erbeldinger *et al.*, 1998b], chymotrypsin [Kim *et al.*, 2001] and subtilisin [López-Fandiño *et al.*, 1994b]. The high substrate concentrations of this system

lead to high efficiency in comparison with conventional enzymatic reaction in organic solvents [Erbeldinger *et al.*, 1998b; Ulijn *et al.*, 2000]. In addition, peptide synthesis in the solid phase is promising in terms of commercial use. The mild conditions employed in such solvent free solid-to-solid synthesis would be highly advantageous for the drug and food industries: the absence of solvents makes the process more economically and ecologically important for large scale, and enzyme-solvent interactions need not to be considered [Erbeldinger *et al.*, 1998b; 2001a]. Therefore, this solid-to-solid peptide synthesis will be applied to synthesise valuable peptide by glycyl endopeptidase. Since papaya is widely grown in Thailand, fresh latex will be collected locally and used as a source for glycyl endopeptidase purification by an aqueous two-phase system following by two-step salt precipitation. Generally, papaya latex is used for the enzyme purification, but collecting the latex is laborious. Therefore, papaya peel which is a major waste from the Thai's papaya pickle industry will also be investigated as an alternative enzyme source.

1.2. Literature review

1.2.1 Papaya cysteine proteases

1.2.1.1 Composition of cysteine proteases in fruit latex and other parts of the tree

Papaya (*Carica papaya* Linn.), a soft-stemmed and unbranched tree, is widely cultivated in tropical and subtropical regions around the world for its edible fruit and its latex. Papaya latex is a thixotropic fluid with a milky appearance that contains about 85% water. Approximately 15% of dry matter consists of both soluble and insoluble parts [Azarkan *et al.*, 2003]. The insoluble particulate fraction is tightly associated with lipase and other practically unknown compounds. On the other hand, the soluble fraction contains both the usual ingredients such as carbohydrate (~10%), salts (~10%) and lipids (~5%), and representative biomolecules for instance, cysteine proteases (~30%), several other proteins (~10%) and glutathione [Moussaoui *et al.*, 2001]. Altogether, papaya cysteine proteases makes up to 40% of the dry matter [Azarkan *et al.*, 2003] and account for more than 80% of the whole enzyme fraction [Oberg *et al.*, 1998]. Presumably, they circulate freely within the laticiferous cell with their concentration higher than 1 mM [Oberg *et al.*, 1998].

The importance of papaya latex as a source of enzyme was first recognised in 1873 and the name "papaïne" was first used in 1879. Workers around the turn of the

century used commercial products with names like "papayotin", "papoid" and "papaïn". The idea that papaïn might contain more than one protease was first suggested in 1905 and it has become the now familiar Anglicised form "papain" since 1917 [Brocklehurst and Salih, 1983]. Recently, papaya cysteine proteases from the latex comprised of 4 endopeptidase named papain, chymopapain, caricain and glycyl endopeptidase. They all belong to the peptidase family C1, known as the papain family, in the clan CA of cysteine peptidases [Barrett *et al.*, 1998]. Their amino acid sequences have been elucidated both at the protein level [Michel *et al.*, 1970; Dubois *et al.*, 1988; Jacquet *et al.*, 1989; Watson *et al.*, 1990; Ritonja *et al.*, 1989] and through sequencing corresponding cDNA clones [Cohen *et al.*, 1986; Mckee *et al.*, 1986; Revell *et al.*, 1993; Baker *et al.*, 1996; Taylor *et al.*, 1999]. Papaya proteases are all synthesised as proenzymes. The three disulfide bonds of the enzyme molecules are formed in identical ways as shown in **Figure 1.1**. Their amino acid sequences are aligned in **Figure 1.2** which exhibits a strong degree of homology. Some of their characteristics are also summarised in **Table 1.1**.

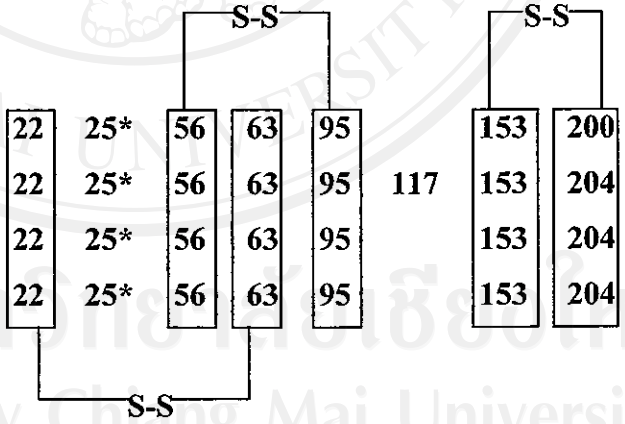

Figure 1.1 Cysteine and half-cystine residues and disulfide bonds in the four papaya cysteine proteases. 25* denotes the catalytic cysteine residues and chymopapain shows additional cysteine at 117.

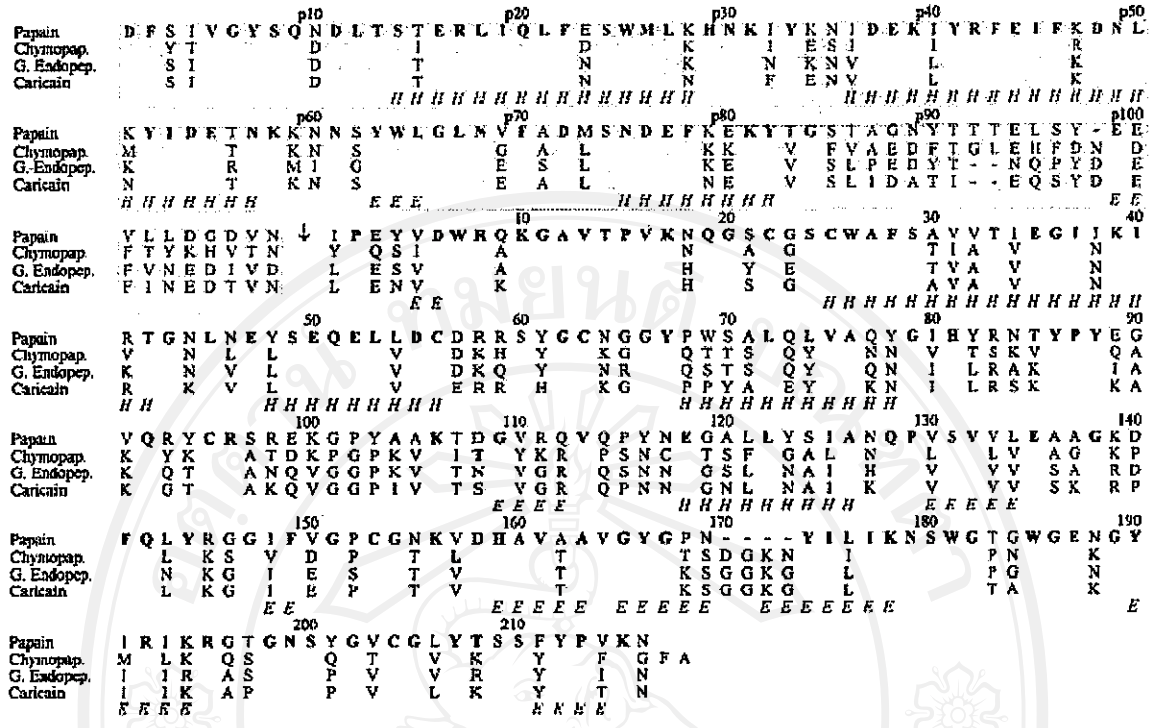

Figure 1.2 Alignment of the prosequences of papaya cysteine proteases. Residues conserved in all four sequences are in bold and for clarity, deletions are denoted by hyphens. The arrow indicates the cleavage site delimiting the proregion (shade) from the mature protein. Residues that participate in β ladders and those that contribute to α helices are indicated by the italised letters E and H, respectively. [Moussaoui *et al.*, 2001].

Research on chymopapain suggested its heterogeneous nature. Results from many studies revealed that multiple forms separated by cation-exchange chromatography have been termed chymopapain A, chymopapain B and chymopapain S [Kunimitsu and Yasunobu, 1970; Khan and Polgar, 1983; Buttle and Barrett, 1984; Baines *et al.*, 1986 etc.]. The isolation of cDNA clones has demonstrated that there are at least five isoforms of chymopapain in *C. papaya* [Taylor *et al.*, 1999]. For this reason, the terminology of chymopapain has been extensively discussed [Polgar, 1984; Brocklehurst *et al.*, 1983; 1984; 1985; Barrett and Buttle, 1985]. Nonetheless, it has been found that polyclonal antisera raised against chymopapain (a chromatographic "B" form) reacted with all the peaks of chymopapain from cation-exchange column. This suggested that the multipleforms are the products of a single gene, and that differences between them are

Table 1.1 Characteristics and properties of cysteine proteases from papaya latex.

Aspects	Papain	Chymopapain	Caricain	Glycyl endopeptidase
EC number *[1]	3.4.22.2	3.4.22.6	3.4.22.30	3.4.22.25
Previous Name *[1,2,3]	Papaya peptidase I Papain peptidase I	Chymopapain A Chymopapain B Chymopapain S	Papaya peptidase A Papaya peptidase II Papaya Proteinase A Papaya Proteinase III Papaya Proteinase 3 Papaya proteinase Ω	Papaya peptidase B Papaya peptidase β Papaya Proteinase IV Papaya Proteinase 4 Chymopapain M Gly-specific protease
Year of discovering *[1,2]	1917	1941	1967	1979
Amino acid residues	212 *[4]	218 *[5,6]	216 *[7]	216 *[8]
MW (Da) *[1]	23429	23650	23280	23313
6 residues N-terminal	IPEYVD *[2]	YPQSID *[5,6]	LPESVD *[7]	LPENVD *[8]
Number of free thiol group/molecule	1 (Cys_{25})	2 (Cys_{25}, Cys_{117})	1 (Cys_{25})	1 (Cys_{25})
% identical of *[1]				
papain	100	58	68	67
chymopapain	58	100	65	70
caricain	68	65	100	81
glycyl endopeptidase	67	70	81	100
$A_{280,1\%,cm}$	25.0 *[9]	18.3 *[10]	18.3 *[10]	16.5 *[11]
ε (M^{-1} cm^{-1})	5.6×10^4 *[12]	4.26×10^4 *[10]	4.19×10^4 *[10]	3.75×10^4 *[1] 3.85×10^4 *[13]
Isoelectric point (pI)	8.75 *[1,14,15]	10.2-10.6 *[1] 10.4 *[14] 10.3-10.4 *[15]	11.7 *[1] 11.4 *[14] 11.0 *[15]	>10 *[1] 11.6 *[14] 11.0 *[15]
% Relative protein	8 *[16,17] 10 *[18,19]	26-30 *[1] 20 *[17]	14-26 *[1] 40 *[17]	23-28 *[1,13] 12 *[17]

*[1] Barrett, 1998; *[2] Brocklehurst and Salih, 1983; *[3] Brocklehurst *et al.*, 1984; *[4] Michel *et al.*, 1970; *[5] Jacquet *et al.*, 1989; *[6] Watson *et al.*, 1990; *[7] Dubois *et al.*, 1988; *[8] Ritonja *et al.*, 1989; *[9] Glazer and Smith, 1961; *[10] Robinson, 1975; *[11] Buttle *et al.*, 1989; *[12] Brocklehurst *et al.*, 1973; *[13] Buttle, 1994; *[14] Sumner *et al.*, 1993; *[15] Goodenough and Owen, 1987; *[16] Azarkan *et al.*, 2003; *[17] Nitsawang *et al.*, 2006a; *[18] Pendzhiev, 2002; *[19] Taylor *et al.*, 1999.

probably the result of post-translational events. These modifications possibly occur during commercial processing of the latex [Buttle and Barrett, 1984; Barrett and Buttle, 1985]. Therefore, chymopapain has been proposed as a single enzyme and the term "chymopapain" can be representing the multiple chromatographic forms [Buttle and Barrett, 1985; Dekeyer *et al.*, 1994].

Most of the literatures on the cysteine proteases of *C. papaya* relate to enzymes isolated from the commercially available dried latex. However, some studies of proteases from other parts of papaya trees have also been documented. The crude papain can be prepared from press juices from leaves, stalks, flower stems and bark of young papaya trees by precipitating with organic solvent or ammonium sulfate [Balls and Thompson, 1940]. Fresh latex from stem, leaves and petioles of the growing plant contains papain, caricain and multiple forms of chymopapain A [Brocklehurst *et al.*, 1985]. However, the last enzyme does not present in the fruit latex [Buttle and Barrett, 1984]. The major component in non-fruit (leaf base, petioles and main stem) latex is the chymopapain, whereas the amounts of other three cysteine proteases are greatly reduced [Mckee and Smith, 1986]. The cysteine proteases from papaya fruit peels, named crude papain, have also been prepared without any study on properties of the enzyme [Arimura, 1989; Espin and Islam, 1998].

1.2.1.2 Separation by ion-exchange/FPLC

In 1982, Pharmacia introduced an innovative chromatographic method called fast protein liquid chromatography (FPLC) that uses experimental conditions intermediate between classical column chromatography and HPLC. Since lower pressures are used in FPLC than in HPLC, a wider range of column supports is possible. Chromatographic techniques incorporated in an FPLC system are ion exchange, gel filtration, affinity, hydrophobic interaction, reversed phase, and chromatofocussing [Boyer, 2000].

In process of ion exchange, ions that are electrostatically bound to an insoluble and chemically inert matrix are reversibly replaced by ions in solution. Polyanions and polycations therefore bind to anion and cation exchangers, respectively. Various proteins bind to the ion exchanger with different affinities. At elution process, those proteins with relatively low affinities for the ion exchanger move through the column faster than the proteins that bind to the exchanger with higher affinities. The contents of the column

effluent may be directly monitored through column-mounted detectors according to its UV absorbance at a specific wavelength, often 280 nm for proteins because the aromatic side chains of His, Phe, Trp and Tyr have strong absorbances at this wavelength, its fluorescence, its radioactivity, its refractive index, its pH, or its electrical conductivity. The purification process can be improved by washing the protein-loaded column using the "gradient elution". Here, the salt concentration and/or pH is continuously varied as the column is eluted so as to release sequentially the various proteins that are bound to the ion exchanger. This procedure generally leads to a better separation of proteins than does elution of the column by a single solution or "stepwise elution". The most widely used of these is the linear gradient, in which the concentration of the eluant solution varies linearly with the volume of solution passed. Ion exchangers consist of charged groups covalently attached to a support matrix. Some commercially available ion exchangers in common use are given below.

Anion exchanger	Mono Q (Pharmacia)
	DEAE-Sephadex (Pharmacia)
	DEAE cellulose (Whatman)
Cation exchanger	Mono S (Pharmacia)
	SP-Sepharose (Pharmacia)
	CM-cellulose (Whatman)
	CM Bio-Gel (Bio Rad)

Chromatography on ion-exchange supports, especially cation exchanger, has been widely used in separation of papaya cysteine proteases. Due to their common sizes and charges, the papaya enzymes can not be purified by single chromatography. The ion-exchange column has traditionally been used in accompany with covalent chromatography in papaya enzyme purification. CM-cellulose incorporated with agarose mercurial column can separate caricain and a new enzyme called papaya peptidase B (glycyl endopeptidase) from papaya latex or commercial chymopapain [Lynn, 1979; Polgar, 1981]. Chromatography on CM-cellulose followed by Thiol-Sepharose column can separate the multi-fractions of chymopapain [Khan and Polgar, 1983] and successfully purified chymopapain and caricain [Dubois *et al.*, 1988]. In addition, this method can also separate chymopapain from other two nearly unknown protein bands (identified later as chitinase and glycyl endopeptidase) [Dubois *et al.*, 1988].

Cation exchanger S-Sepharose column accompanied with affinity chromatography on immobilised alanyl-phenylalaninaldehyde semicarbazone (Sepharose-ECH-Ala-PheSc) column can provided the fully active and free of contaminant chymopapain from commercially dried latex [Buttle *et al.*, 1990a].

Separation of three papaya enzymes, papain, chymopapain and caricain, can also be carried out by precipitating with ammonium sulfate followed by separation via Mono S column chromatography [Buttle and Barrett, 1984; Zucker *et al.*, 1985].

Four cysteine proteases from papaya latex can be accomplishedly purified by using two ion-exchanger columns, S-Sepharose and Mono S, incorporated with hydrophobic interaction Shodex HIC PH-814 column [Dekeyser *et al.*, 1994].

Mono Q column attached to FPLC can separate the enzyme from spray dried papaya latex by eluting with 1,3-diaminopropane buffer pH 10.8 with linear gradient of NaCl [Goodenough and Owen, 1987]. Papaya chitinase did not bind to the column, whereas caricain, mixture of chymopapain isoforms, and papain were eluted at fraction number 13, 15 and 20, respectively (**Figure 1.3**). On the other hand, Mono S column does not elute papain from the ion exchange material or completely separate the other proteins [Goodenough and Owen, 1987].

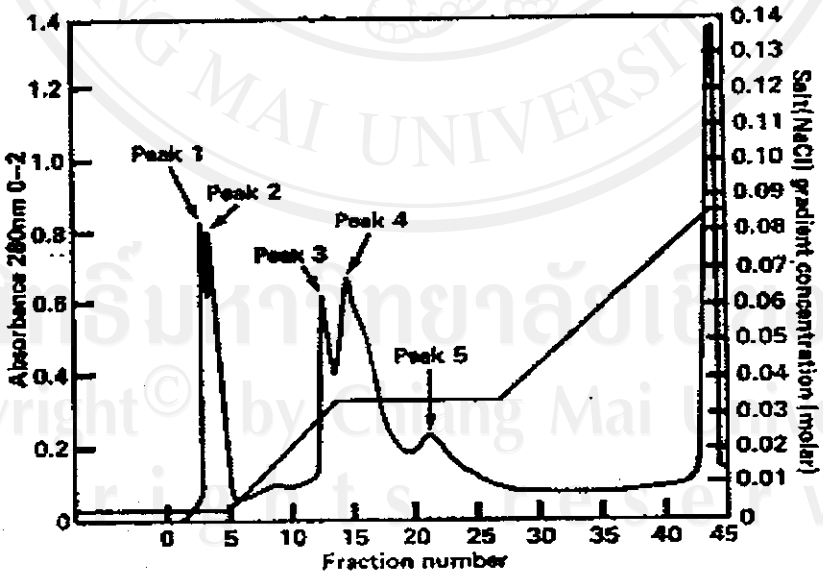

Figure 1.3 Separation of papaya proteases using a Mono Q anion-exchange column attached to the Pharmacia FPLC: peak 1; chitinase (denoted in the paper as papaya proteinase B), peak 2; impurities, peak 3; caricain, peak 4; mixture of chymopapain isoforms, and peak 5; papain [Goodenough and Owen, 1987].

1.2.1.3 Cathodic polyacrylamide gel electrophoresis pattern

Gel electrophoresis, the migration of ions on the gel in an electric field, is among the most powerful and conveniently used methods of macromolecular separation. The gels in common use, polyacrylamide and agarose, have pores of molecular dimensions whose sizes can be specified. The molecular separations are therefore based on gel filtration as well as the electrophoretic mobilities of the molecules being separated. Since the molecules in a sample cannot leave the gel, the electrophoretic movement of larger molecules is impeded relative to that of smaller molecules.

In polyacrylamide gel electrophoresis (PAGE), gel are made by the free radical-induced polymerization of acrylamide and the cross linking agent *N,N'*-methylene-bis-acrylamide in the buffer [Voet and Voet, 2004]. The free radical is usually result from the chemical decomposition of ammonium persulfate ($S_2O_8^{2-} \longrightarrow 2SO_4^{-\bullet}$). *N,N,N',N'*-tetramethyl ethylenediamine (TEMED), a catalyst and a free radical stabiliser, is regularly added to the gel mixture at concentration 5% of the total acrylamide present. The gel is typically cast as a thin rectangular slab in which several samples can be simultaneously analysed in parallel lanes that is a good way of comparing similar samples.

Native PAGE like cathodic and anodic gels are different from SDS-PAGE that the latter produces linear polypeptide chains coated with negatively charged SDS molecule. As a consequence, protein mobility in SDS-PAGE depends primarily on size. In the native PAGE process, mobility is influenced by both charge and size of the protein samples because they are not treated with the detergent. So, the proteins in this gel still retain its conformation and none denaturing. It should be noted that electrophoresis separates on the same basis as ion exchange chromatography. The first proteins to elute from anion exchange gels will be the slowest moving on a normal acrylamide electrophoresis gel at the same pH buffer used.

Because of the four papaya cysteine proteases have very similar in molecular weight of 23 kDa approximately, cathodic PAGE is therefore commonly used to monitor these enzymes after purification rather than SDS-PAGE. **Figure 1.4** shows a typical of mobility patterns of papaya proteases in SDS and cathodic PAGE. It is notable that chymopapain and glycyl endopeptidase have practically the same electrophoretic properties (**Figure 1.4B**). This made it difficult to distinguish the two from one another and delay the discovery of glycyl endopeptidase [Dekeyser *et al.*, 1994].

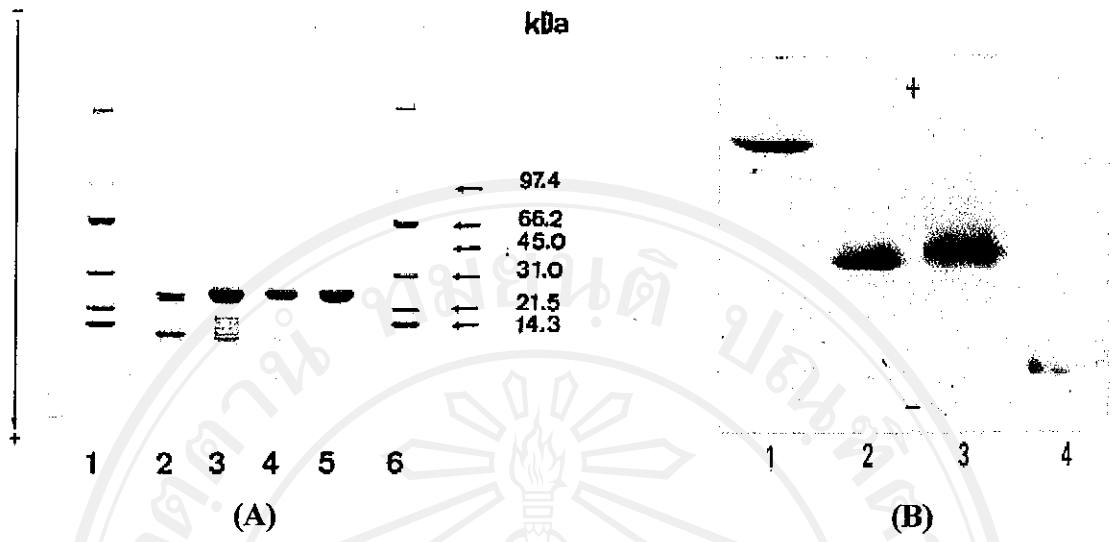

Figure 1.4 Gel electrophoresis of the four papaya cysteine proteases after purification by chromatographic methods.

(A): SDS-PAGE in which lane 1 & 6, molecular mass standard; lane 2, papain with papaya cystatin; lane 3, chymopapain with papaya cystatin; lane 4, caricain and lane 5, glycyl endopeptidase [Akarzan *et al.*, 2003].

(B): Cathodic PAGE in which lane 1, papain; lane 2, glycyl endopeptidase; lane 3, chymopapain and lane 4, caricain [Buttle *et al.*, 1989].

The cathodic PAGE has been used for verifying purity of papaya enzyme since 1980s. A system of 7.5% gel pH 4.3 with the acidic β-alanine was used for separating the new enzyme (glycyl endopeptidase) and caricain from commercial chymopapain (Sigma) [Polgar, 1981]. The bands showed that caricain run much farther than glycyl endopeptidase and papain, respectively. In addition, bands of glycyl endopeptidase and chymopapain appeared to be homogeneous.

Cathodic PAGE of purified papain, chymopapain and caricain from commercial dried papaya latex, employing 12.5% gel with Tris-alanine buffer pH 4.5, revealed that the migration patterns of enzymes were similar to that of the first gel [Zucker *et al.*, 1985]. Unfortunately, the running pattern of the three enzymes was not compared with whole papaya latex. As a consequence, accurate numbers of protein bands from the latex were still unknown.

When using PAGE system with 15% gel (pH 4.3) in β-alanine-HOAc buffer pH 4.3, more protein bands, especially heterogeneous bands of chymopapain, from chromatographic elution of commercial chymopapain (Sigma) have been found. This study also signified the position of papaya proteinase B (glycyl endopeptidase) band, which runs slower than any other except papain, due to it possesses the lowest efficiency for hydrolysing L-BAPNA [Mckee and Smith, 1986].

Soon after, Goodenough and Owen [Goodenough *et al.*, 1986; Goodenough and Owen, 1987] using the same condition with of Mckee above obtained the similar enzymes' mobility profile. Moreover, chymopapain was obviously separated into 2 bands. Molecular weight 28 kDa of papaya proteinase β (glycyl endopeptidase) was reported by using SDS-PAGE. Unfortunately, a vast number of later experiments of N-terminal and molecular weight analyses have proved that this protein band belongs to papaya chitinase [Azarkan *et al.*, 1997; Subroto *et al.*, 1999; Nitsawang and Kanasawud, 2006].

Employing the 15% gel with acidic β-alanine pH 4.3, similarly to those of two previous groups, chymopapain fraction showed 3 apparently separated bands [Dubios *et al.*, 1988]. Re-chromatography with affinity column of this fraction resulted in only one band of highly active chymopapain (other two bands are solved later as glycyl endopeptidase and chitinase).

Purification and characterisation of glycyl endopeptidase in 1989 provided more obvious result [Buttle *et al.*, 1989]. The four papaya proteases were purified by specific columns and verified them by running on 12.5% gel with electrode buffer pH 4.5 which was adapted from method of Thomas and Hodes [1981]. The enzyme mobility of this work is shown in **Figure 1.4B** indicating that glycyl endopeptidase and chymopapain have the same electrophoretic properties.

Therefore, the migration pattern of papaya enzymes on the cathodic PAGE in order to increase the mobility is now proved as papain, chitinase, chymopapain, glycyl endopeptidase and caricain, respectively. Numerous works use the cathodic PAGE for verifying papaya cysteine proteases with slightly varying the gel concentration and pH of buffer used, most of them are 15% gel and β-alanine-HOAc buffer pH 4.5 [Dekeyser *et al.*, 1994; Monti *et al.*, 2000; Pendzhiev, 2002; Nitsawang *et al.*, 2006a; 2006b].

1.2.1.4 Catalytic mechanism and form of enzyme

Papaya cysteine proteases catalyse the hydrolysis of various covalent bonds, including peptide, amide, ester and thiol ester. Their activities are dependent on the establishment of an active site ion pair between Cys-25 and His-159. These active site residues are located at the interface of the clef on opposite domains of enzymes. It is accepted that the active site of cysteine proteases consist of a thiolate-immidazolium ion pair, Cys-S^-/His-Im^+H [Polgar, 1974; Lewis *et al.*, 1981]. The existence of two ionisable groups is consistent with a bell-sharp form for the pH dependency of activity. The pH versus activity profile for an enzyme is generally a reflection of the ionisation of active site residues. The acid limb, with a pK_a around 3.4-4, is usually attributed to the ionisation of the Cys-25. On the other hand, the basic limb with a pK_a about 8.5–8.7, is typically considered for the ionisation of His-159. Papain and glycyl endopeptidase are usually active over a pH range of 4-8.5 and 4-9.5, respectively, that the ionic groups are present as the thiolate-immidazolium ion pair [Migliorini *et al.*, 1986; Thomas *et al.*, 1994].

The overall mechanism of hydrolysis involves a number of steps represented in **Figure 1.5** for the hydrolysis of peptide bond. The first step is the non-covalent binding of the enzyme (I) and substrate to form the Michaelis complex (II). This step is followed by acylation of enzyme (IIIa), with the first product ($R'NH_2$) is formed and released. In the following step, the acyl-enzyme (IIIb) reacts with a water molecule in deacylation step resulting in the release of carboxy product (RCOOH). Many intermediates (THI) and/or transition states (TS) are believed to exist along these pathways.

Naturally, papaya cysteine proteases are present in three forms (active, reversibly inactive and irreversibly inactive form) characterised by the possession of thiol group at catalytic site. Active form contains a free thiol group (-SH) at Cys-25. While reversibly inactive form, this thiol group is formed a disulfide bond with another thiol group of Cys-22 or Cys-63 in the molecule becoming propapain as shown in **Figure 1.6** [Brocklehurst and Kierstan, 1973; Kierstan *et al.*, 1982]. In addition, this catalytic site thiol group can form the disulfide bond with several thiol compounds such as free cysteine, glutathione (GSH) and protein sulfhydryl. It can also be reversibly oxidised to sulfenic acid, -SOH (**Figure 1.7**). Treatment of these reversibly inactive enzymes with a mild reducing agent, for instance, cysteine, 2-mercaptoethanol as well as dithiothreitol (DTT) produces the active cysteine proteases. Optimum activation was found to occur

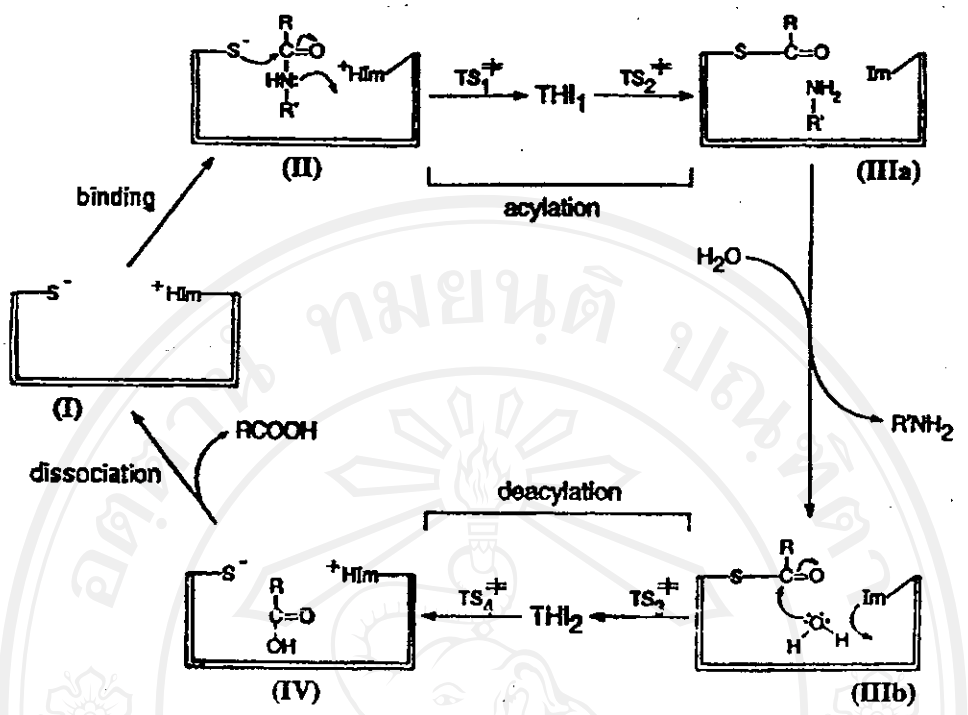

Figure 1.5 Catalytic mechanism for hydrolysis of peptide bond by cysteine proteases [Storer *et al.*, 1994].

upon simultaneous application of a thiol compound like cysteine and a heavy metal-binding agent like EDTA, or by the addition of BAL (2,3-dimercaptopropanol), an agent which combines the functions of both thiol compound and metal binder [Arnon, 1970]. It should be noted that formation of intermolecular disulfide bond between the 2 molecules of enzyme (papain-S-S-papain) is not possible [Glazer and Smith, 1965]. For the irreversibly inactive form, the thiol group at the active site has been highly oxidised to sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$). These oxidised state groups cannot be reversed to the active enzyme by any reducing agent. It has been reported that the total potential proteolytic activity in freshly collected papaya latex represents a fraction of only active proteases [Silva *et al.*, 1997].

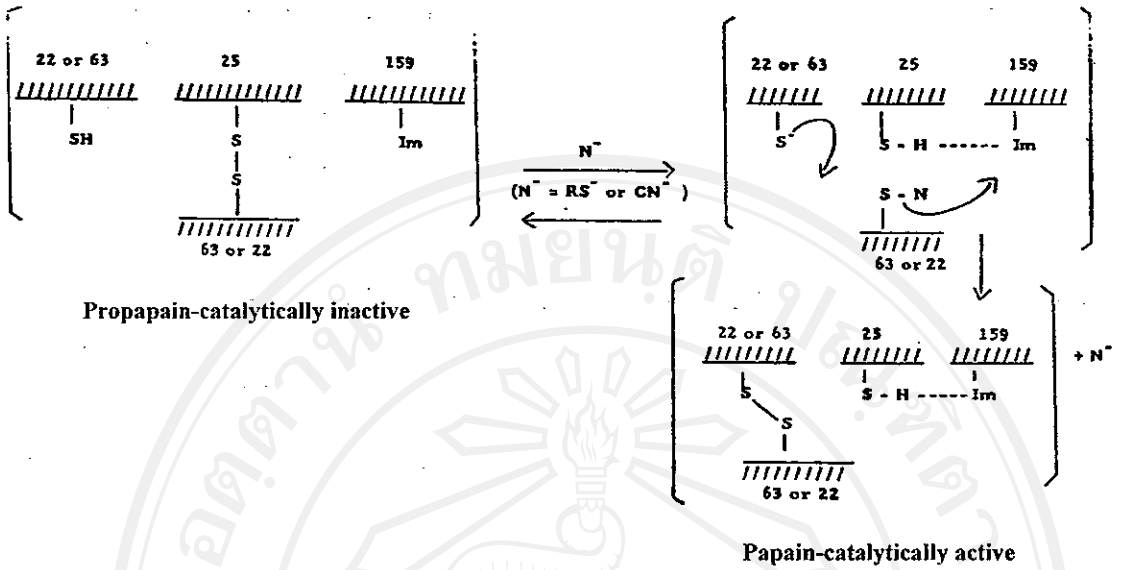

Figure 1.6 Schematic drawing of simplest version of propapain-papain transition [Brocklehurst and Kierstan, 1973].

Reversibly oxidized forms of protein sulfhydryls

1 electron oxidation

(1) protein-SH + $R^{\bullet}$ ⇒ RH + protein-$S^{\bullet}$ (thiyl radical)

2 electron oxidation

(2) protein-S⁻ + RSSR ⇒ RS^- + protein-S-SR (disulfide)
(3) protein-SH + N_2O_3 ⇒ HNO_2 + protein-SNO (nitrosothiol)
(4) protein-SH + HOOH ⇒ H_2O + protein-SOH (sulfenic acid)
(5) protein-SH + HOOH + GSH ⇒ $2H_2O$ + protein-SSG (S-thiolated)
(6) protein-SH + $R^{\bullet}$ + GSH + O_2 ⇒ $2H_2O$ + RH + $O_2^{\bullet -}$ + protein-SSG

Irreversibly oxidized forms of protein sulfhydryls

2 electron oxidation

(7) protein-SOH + (O_x) ⇒ protein -SO_2H (sulfinic acid)
(8) protein -SO_2H + (O_x) ⇒ protein -SO_3H (sulfonic or cysteic acid)

Figure 1.7 Reaction producing inactive forms of protein thiol group [Thomas and Mallis, 2001].

1.2.2 Glycyl endopeptidase

1.2.2.1 History

The first indication that papaya latex may contain an endopeptidase with specificity markedly different from those of the three previously purified enzymes, papain, chymopapain and caricain, came with the use of Z-Gly-$OPhNO_2$ [Lynn, 1979, Polgar, 1981] detected a chromatographic fraction that demonstrated activity toward Z-Gly-$OPhNO_2$ and Z-Lys-$OPhNO_2$ but not against Z-Tyr-$OPhNO_2$ and Bz-Arg-pNA (BAPNA). This new enzyme called "papaya peptidase B" referring to that fourth component after papaya peptidase A (caricain) possessed N-terminal sequence Leu-Pro-Glu-Ser and 24 kDa [Lynn, 1979]. This new enzyme contains 1 essential thiol group at catalytic site similarly to papain and papaya peptidase A. The peptidase B was found to run on cathodic PAGE much farther than papain, but slower than caricain [Polgar, 1981]. The term "peptidase" may preferably be later substituted by "proteinases" in accordance with the proposals of the Enzyme Commission [Polgar, 1984]. After discovering by Lynn [1979] and Polgar [1981], there had been doubted for existence of the fourth papaya proteases and its characteristic was still in query [Mckee and Smith, 1986; Goodenough *et al.*, 1986; 1987].

Confirmation of the existence of the fourth papaya endopeptidase awaited the serendipitous discovery that an endopeptidase with no activity on BAPNA, could be separated with high yield from the other papaya endopeptidases by selective affinity and cation-exchange chromatography. This enzyme was initially characterised and named "papaya proteinase IV" which referred to the most basic cysteine protease of papaya latex, caricain, as papaya proteinase III [Buttle *et al.*, 1989]. Further characterisation of papaya proteinase IV was later revealed [Ritonja *et al.*, 1989; Buttle *et al.*, 1990b; 1990c]. This new enzyme was also called "chymopapain M" (M denoting a monothiol-containing cysteine protease within the chymopapain elution band) [Topham *et al.*, 1990]. The name "glycyl endopeptidase" was recommended by IUBMB in 1992. Despite, the name chymopapain M is inappropriate in that glycyl endopeptidase share no more sequence similarity to chymopapain than it does to the other cysteine endopeptidase from papaya. Moreover, the restricted specificity demonstrated by glycyl endopeptidase contrasts markedly with the catholic nature of the specificity of chymopapain [Barrett *et al.*, 1998]. Glycyl endopeptidase is once commercially available proteinase Gly-C from Calbiochem-Novabiochem (UK) Ltd.

1.2.2.2 Physicochemical properties

Glycyl endopeptidase is clearly a member of the papain family of cysteine peptidases [Rawling and Barrett, 1993]. It is synthesised as pro-enzyme like other papaya proteases [Moussaoui *et al.*, 2001]. This enzyme is an abundant protein in the latex of papaya. Quantification, based on single radial immunodiffusion or cathodic PAGE-densitometry, shows that enzyme constitutes 23-40% of the total protein considering as a mature enzyme in the latex. It is a basic protein with pI probably above 10 and it exists as a single unglycosylated polypeptide with MW 23313 Da of 216 residues. Its amino acid sequence shows similarity to the other three papaya endopeptidases, being 81%, 70%, and 67% identical to caricain, chymopapain and papain, respectively [Ritonja *et al.*, 1989]. The comparable characteristics of all four papaya cysteine proteases are displayed in **Table 1.1**. The C-terminal 62 residues are identical to those that are encoded by a partial cDNA sequence from a papaya leaf tissue library [Mckee et al., 1986]. The enzyme possess $A_{1\%,\ 280}$ of 16.5 therefore gives an extinction coefficient at 280 nm of $3.85 \times 10^4\ M^{-1}cm^{-1}$ [Buttle *et al.*, 1989].

The crystal structure confirms the overall of enzyme is similar to papain. The highly distinctive substrate specificity of glycyl endopeptidase stems largely from the substitution of Gly23 and Gly65 (as found in papain and conserved in all other known papain-like enzymes) by Glu and Arg, respectively. This dramatically alters the S_1 subsite, as the side chains of Glu23 and Arg65 form a barrier across the binding pocket and sterically exclude residues with large side chains. Molecular modeling has suggested that these substitutions are mainly responsible for the lack of inhibition by cystatin B or stefin B due to unfavourable interaction between enzyme and the inhibitor. Moreover, the residues clashing with part of the N-terminal region of cystatin B, and Glu23 of glycyl endopeptidase are also being involved in a steric clash with the hairpin loop of the inhibitor [O'Hara *et al.*, 1995].

Independent confirmation of the importance of Glu23 and Arg65 to the restricted substrate specificity and inhibitor profile of glycyl endopeptidase has come from site-directed mutagenesis of the equivalent residues in cathepsin B [Fox *et al.*, 1995]. It has been reported that crowding of the active-site cleft also prevents autocatalytic activation of the pro-enzyme to be mature glycyl endopeptidase [Baker *et al.*, 1996]. As other family C1 of cysteine peptidases, glycyl endopeptidase contains

essential Cys25 and His159 at opposite side of the active site cleft. It also contains 3 disulfide bonds like other papaya cysteine proteases (**Figure 1.8**).

Glycyl endopeptidase is similar to other enzymes in family C1 which the pro-region is inhibitor for its own mature enzyme and often of other enzymes in the family. The K_i for the inhibition of glycyl endopeptidase by its pro-peptide being $8.6x10^{-7}$ M, whereas inhibition of the other three papaya cysteine proteases is even stronger [Taylor *et al.*, 1995]. It has also been reported that the pro-region of glycyl endopeptidase inhibits the cysteine protease in digestive tract of insect pests [Visal *et al.*, 1998].

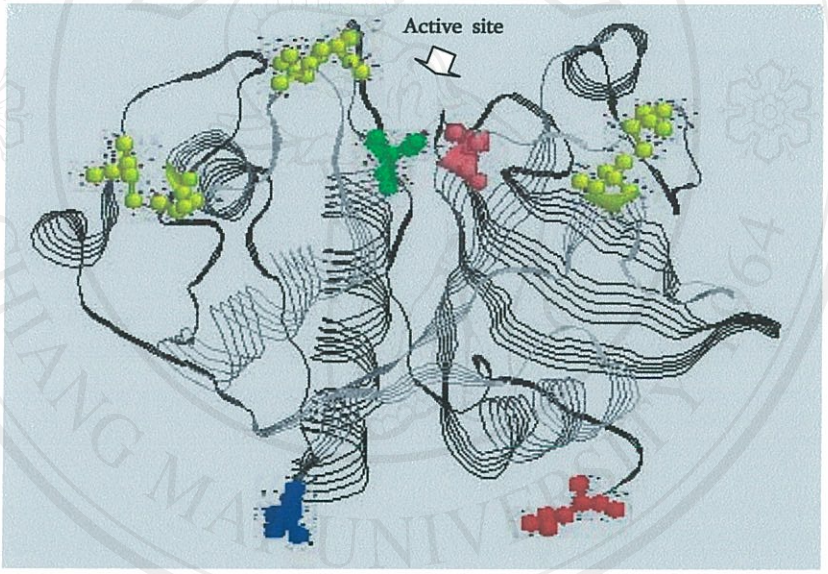

Figure 1.8 Three dimension strand structure of glycyl endopeptidase; ball & stick representing the catalytic Cys25 (green), catalytic His159 (pink), three disulfide bond residues (yellow), Leu1 at N-terminal (red), and Asn216 at C-terminal (blue). Source: Brookhaven Protein Databank (Code 1GEC).

The S_1 subsite in other members of papain family is a wide and unrestricted pocket which relatively little influence on the substrate specificity. In contrast to glycyl endopeptidase, the highly conserved Gly23 and Gly65 in other enzymes are replaced by Glu23 and Arg65. Their side chain and associated hydrogen bond form a barrier to entry

into the binding pocket resulting in satisfactorily account for the specificity for Gly at the S_1 subsite [Buttle, 1994; O'Hara *et al.*, 1995]. The active site cleft containing 7 subsites for binding the substrate is shown in **Figure 1.9**.

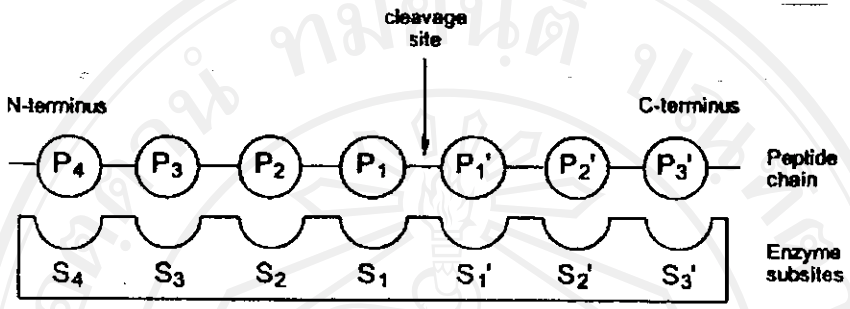

Figure 1.9 Terminology of the cleavage site of glycyl endopeptidase, and also papaya cysteine proteases (according to Schechter and Berger, 1967).

1.2.2.3 Enzymatic properties

Glycyl endopeptidase appears to hydrolyse only glycyl bonds (Gly at P_1) efficiently. It does not cleave the usual substrate of papaya cysteine proteases like BAPNA. Therefore, assay for the enzyme activity widely was performed using small molecule substrates of N-blocked esters and amides of glycine, including Z-Gly-$OPhNO_2$ [Lynn, 1979; Polgar, 1981], Boc-Gly-$OPhNO_2$ [Buttle *et al.*, 1989; Thomas *et al.*, 1994], Ac-Phe-Gly-pNA [Thomas *et al.*, 1994; 1995], Boc-Ala-Ala-Gly-NHMec and Boc-Ala-Ala-Gly-pNA [Buttle *et al.*, 1990b; Buttle, 1994]. The kinetic constants, k_{cat}/K_m, for the cleavage of Boc-Ala-Ala-GlypNA and Boc-Ala-Ala-GlyNHMec by glycyl endopeptidase at pH 6.8 have been reported of 4.23 and 31.2 $mM^{-1}sec^{-1}$, respectively [Buttle *et al.*, 1990b], while it hydrolysed Ac-Phe-Gly-pNA at pH 6.0 giving 1.497 $mM^{-1}sec^{-1}$ [Thomas *et al.*, 1994]. The strict selectivity for glycyl bonds was demonstrated by the substitution of Ala for Gly in the –NHMec substrate, the presence of the methyl side chain of Ala leading to a 60-fold and 30-fold reductions in k_{cat}/K_m and k_{cat}, respectively [Buttle *et al.*, 1990b]. Against Boc-Ala-Ala-Gly-pNA, glycyl endopeptidase has a pH optimum of 7.0-7.5 [Buttle, 1994], similarly with the maximal hydrolysis of Ac-Phe-Gly-pNA which was found at pH 6.5-7.5 [Thomas *et al.*, 1994].

The compilation of known cleavage sites of proteins by glycyl endopeptidase shows that 42 bonds cleaved, 38 were glycyl bonds [Buttle *et al.*, 1990b; Buttle, 1994; Barrett *et al.*, 1998]. As with most of the enzymes in family C1, there is a preference for hydrophobic side chains at P_2 and P_3, particularly aliphatic ones. Charged residues in the vicinity of the scissile bond do not appear to be favoured for glycyl endopeptidase [Buttle, 1994].

The strict specificity of glycyl endopeptidase has found extensive application in the preparation of protein fragments and in peptide mapping and sequencing [e.g. Ritonja *et al.*, 1990; 1996; Križaj *et al.*, 1992; Brouwer *et al.*, 1995; Chang *et al.*, 1996; Lenarčič *et al.*, 1997; De La Cruz *et al.*, 2000]. Moreover, glycyl endopeptidase appears to cleave proficiently in the vicinity of disulfide bonds, a half-cystine being accommodated well in P_2. The enzyme is therefore a very useful reagent for designation of disulfide bridges in proteins [Bernard *et al.*, 1993].

Glycyl endopeptidase is not inhibited by most cystatins, contrasting with other three papaya cysteine peptidases which form a tight inhibitory complex with the cystatin. Resistance of glycyl endopeptidase involves the substitution of Gly23 and Gly65 as mentioned above in structural property [O'Hara *et al.*, 1995]. This property is not unique among the family C1 enzymes or papain family because both stem bromelain and fruit bromelain are also being refractory to inhibition [Barrett *et al.*, 1998]. However, glycyl endopeptidase cleaves and inactivates some type II cystatins, such as chicken cystatin and human cystatin C [Buttle *et al.*, 1990c]. This capacity appears to be unique among the family C1 enzymes. The one exception to the lack of inhibition by cystatin is moderate inhibition by papaya cystatin (K_i = 3.44 nM) [Song *et al.*, 1995]. The low rates of inactivation by iodoacetate and iodoacetamide are also unusual property, but not unique, of glycyl endopeptidase [Buttle *et al.*, 1990c; Buttle, 1994].

Glycyl endopeptidase tends to contaminate chymopapain preparations, even in commercial chymopapain [Buttle *et al.*, 1990a]. A convenient method for detecting this contamination takes advantages of the different behaviour of these two enzymes towards chicken cystatin, activity against blocked-GlypNA or azocasein which rather limited hydrolysis by glycyl endopeptidase [Buttle *et al.*, 1989].

1.2.2.4 Preparation

Similarity in physicochemical properties of the papaya cysteine endopeptidases makes it difficult to purify them by traditional ion-exchange and size-exclusion techniques. This is particularly true for glycyl endopeptidase and chymopapain, which are common in their size as well as charge properties over a range of pH values. This led past researchers to believe that glycyl endopeptidase was a one form of chymopapain and delayed the discovery and classifying this enzyme as the fourth papaya proteases.

The first purification of glycyl endopeptidase was reported by Buttle *et al.* [1989] that relied heavily on affinity chromatography on Sepharose-Ahx-Gly-Phe-$NHCH_2CN$ (Ahx-, 6-aminohexanoyl) 4 mL bed volume column. The ligand was a reversible inhibitor of papain and glycyl endopeptidase, but only low affinity for chymopapain and caricain. An 80 mg portion of the latex protein from clarified 0.5 g dried papaya latex was used. After applying, chymopapain and caricain were washed with elution buffer (50 mM sodium citrate in water/ethanediol, pH 4.5). The column was then equilibrated with 50 mM hydroxyethyl disulfide for 1 day and glycyl endopeptidase and papain were later eluted with elution buffer containing hydroxyethyl disulfide. This mixture was then applied to Mono S column for separating glycyl endopeptidase and papain by using 1 M sodium acetate with Na^+ gradient. Both enzymes were recovered with the active site thiol protected as mercaptoethanol mixed disulfide, thus reducing autolysis and improving stability. This method provided glycyl endopeptidase contaminating with 3% chymopapain and 1% by each of papain and caricain [Buttle *et al.*, 1989]

The second method using the basis of enzyme bound to the same immobilised active site-directed affinity ligand, Ahx-Gly-Phe-$NHCH_2CN$, as the first purification. This method was simplified from the first one by taking advantage of the conclusion that inactivation of glycyl endopeptidase by iodoacetate was much slower than those of the other papaya proteases [Buttle *et al.*, 1990c] and the conclusion that reaction between iodoacetate and cysteine residue in each proteases was not very different. About 200 mg latex protein was dissolved in 12 mL of 50 mM sodium phosphate buffer, containing 1 mM EDTA, in water/ethanediol, pH 6.8. Papain, chymopapain and caricain were then selectively inactivated by iodoacetate. After applying to the affinity column, glycyl endopeptidase as the mercury derivative was then eluted from the column with elution buffer containing 10 mM $HgCl_2$. The mercurial form of enzyme was generally stable and

is rapidly activated by cysteine. This method yielded 10 mg purified enzyme contaminating with <0.01% chymopapain and caricain and 0.07% papain [Buttle, 1994].

The third method was developed by Thomas et al. [1994]. The procedure used is similar to, but not identical with, that described by Buttle *et al.* [1989] in the first method. After chymopapain and caricain were eluted, the Sepharose- Ahx-Gly-Phe-$NHCH_2CN$ column bounded with the rest two proteases was applied with sodium citrate buffer containing 2-Py-S-S-2-Py (2,2′-dipyridyl disulfide, **Figure 1.10**) and stopped overnight. The same solution was used to elute the fraction containing a mixture of papain-S-S-2-Py (-S-S-2-Py = 2-pyridyl disulfide) and glycyl endopeptidase-S-S-2-Py which were separated well by Mono S column. The enzyme-S-S-2-Py was converted to the active form by treatment with 20 mM cysteine [Thomas *et al.*, 1994].

The subsequent purification took advantage of the failure of glycyl endopeptidase to covalently bind to Sepharose-glutathione-S-S-2-Py, which bound the other papaya cysteine proteases [Thomas *et al.*, 1994]. Papaya latex (15 g) was dissolved in sodium acetate buffer saturated with 2-Py-S-S-2-Py. The clarified solution was applied to S-Sepharose Fast Flow cation-exchange column. The glycyl endopeptidase-S-S-2-Py fraction was eluted with Na^+ gradient after chymopapain and lysozyme. The fractions rich glycyl endopeptidase-S-S-2-Py were collected to precipitate the enzyme with 85% saturation ammonium sulfate. The pellet was dissolved in 40 mM cysteine containing 1mM EDTA to convert the enzyme mixed with 2-pyridyl disulfide into the active form. After precipitating and dissolving again, the free glycyl endopeptidase was mixed with Sepharose-glutathione-S-S-2-Py gel. Of this, the filtrate through the sintered-glass funnel was the free and high purity of glycyl endopeptidase which was subsequently precipitated with 85% saturated ammonium sulfate [Thomas *et al.*, 1994]. The failure of glycyl endopeptidase to react with glutathione-S-S-2-Py gel permits its separation from chymopapain contaminant, which does bond to the gel. However, it does not allow the separation of glycyl endopeptidase from the oxidised form due to both active and oxidised enzymes pass through the gel [Thomas *et al.*, 1994; 1995].

The purification method introduced by Thomas *et al.* [1995] improved the previous method to obtain fully active form of enzyme. This procedure is similar to above purification [Thomas *et al.*, 1994] but the covalent chromatography ligand was substituted by Sepharose-2-hydroxypropyl-S-S-2′-Py gel. This is due to the enzyme is unable to react with glutathione-S-S-2-Py gel resulted from steric restrictions in its S_1

subsite [Thomas *et al.*, 1994], but can bind to the 2-hydroxypropyl-S-S-2′-Py gel. The two gel structures are shown and compared in **Figure 1.10**. After carried out as procedure described above, the glycyl endopeptidase was mixed with Sepharose-2-hydroxypropyl-S-S-2′-Py in order to obtain Sepharose-2-hydroxypropyl-S-S-enzyme. The glycyl endopeptidase was then released by 40 mM cysteine in Tris-HCl buffer pH 8.0. After precipitation with 85% saturated ammonium sulfate, the glycyl endopeptidase solution was applied to Mono S HR column to analyse the contaminated chymopapain.

The separation of papaya latex enzymes using cation-exchange incorporated with hydrophobic interaction chromatography have been reported [Dekeyser *et al.*, 1994; Azarkan *et al.*, 2006]. The clarified solution of 1 g latex [Dekeyser *et al.*, 1994] or approximately 7 g papaya latex [Azarkan *et al.*, 2006] was loaded onto SP-Sepharose Fast Flow column. The fractions containing glycyl endopeptidase and chymopapain pools were collected and applied to Shodex HIC and Mono S columns, respectively [Dekeyser *et al.*, 1994]. Alternatively, the fractions were collected and concentrated by ultrafiltration before applying onto a Fractogel EMD Propyl 650 column. The column was pre-equilibrated with a 50 mM Tris-HCl buffer pH 8.0 containing 2 M ammonium sulfate. The purified chymopapain was eluted in the avoid volume [Azarkan *et al.*, 2003]. Glycyl endopeptidase was later eluted by gradually decreasing ammonium sulfate.

Other purification method with different in the basis has also been reported [Nitsawang and Kanasawud, 2005]. The papaya proteases including chitinase were separated by aqueous two-phase system incorporated with salts precipitation. Detail of the purification of glycyl endopeptidase is given in **Chapter 2**. All above purification methods of glycyl endopeptidase are summarised in **Table 1.2**.

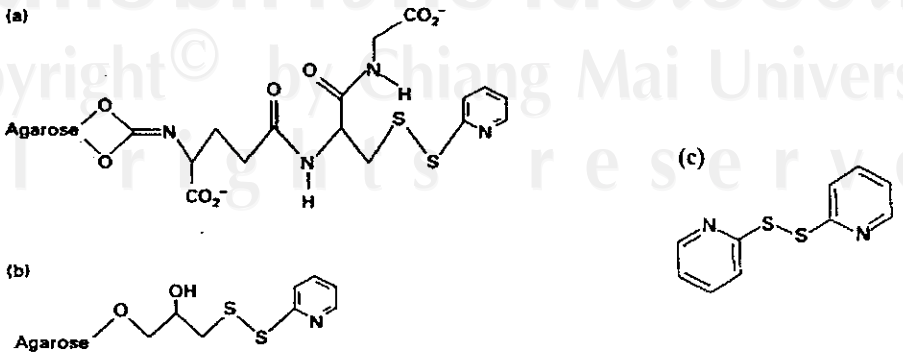

Figure 1.10 Structures of (a) Sepharose-glutathione-S-S-2-Py gel, (b) Sepharose-2-hydroxypropyl-S-S-2′-Py gel, and (c) 2,2′-dipyridyl disulfide (2-Py-S-S-2-Py).

Table 1.2 Chromatographic methods for preparation of glycyl endopeptidase.

Method	Starting material	Step of purification	Noted	Ref.
1	80 mg protein portion of 0.5 g dried latex	1. Affiniy chromatography on Sepharose-Ahx-Gly-Phe-$NHCH_2CN$ column 2. Cation-exchange chromatography on Mono S column	1. Obtaining 5% protein yield with 90% active 2. Contaminating with 3% chymopapain 1% papain 1% caricain	Buttle *et al.*, 1989
2	200 mg latex protein	1. Selective inactivated other proteases by iodoacetate 2. Affiniy chromatography on Sepharose-Ahx-Gly-Phe-$NHCH_2CN$ column	1. Obtaining 5% protein yield with 90% active 2. Contaminating with <0.01% chymopapain <0.07% papain <0.01% caricain	Buttle, 1994
3	250 mg spray dried latex	1. Affinity chromatography on Sepharose-Ahx-Gly-Phe-$NHCH_2CN$ column 2. Binding enzyme with 2-Py-S-S-2-Py 3. Cation-exchange chromatography on Mono S column	Obtaining more active enzyme than the 1st method	Thomas *et al.*, 1994
4	15 g spray dried	1. Binding enzyme with 2-Py-S-S-2-Py before applying to cation-exchanger S-Sepharose column 2. Precipitation with 85% sat. AS* 3. Covalently binding on other enzymes with Sepharose-GSH-S-S-2-Py gel	Obtaining both active and oxidised enzyme	Thomas *et al.*, 1995
5	15 g spray dried latex	1. Binding enzyme with 2-Py-S-S-2-Py before applying to cation-exchanger S-Sepharose column 2. Precipitation with 85% sat. AS* 3. Covalently binding on glycyl endopeptidase with Sepharose-2-hydroxypropyl-S-S-2'-Py gel 4. Cation exchange chromatography on Mono S column	Obtaining only fully active enzyme	Thomas *et al.*, 1995
6	1 g spray dried latex	1. Cation-exchanger S-Sepharose column 2. Hydrophobic chromatography on Shodex HIC column 3. Cation exchange chromatography on Mono S column	(not reported)	Dekeyser *et al.*, 1994
7	7 g lyophilised or spray dried latex	1. Cation exchange chromatography on SP-Sepharose column 2. Ultrafiltration before applying on hydrophobic chromatography on Fractogel EMD Propyl 650 column	(not reported)	Azarkan *et al.*, 2006

*AS = ammonium sulfate

1.2.3 Thermodynamically and kinetically controlled peptide synthesis

Enzymatic peptide synthesis may be thermodynamically or kinetically controlled. In the former mode, the reaction is a reverse of hydrolysis reaction and in the later, there is a competition after acyl-enzyme formation between two nucleophilic reactants. The acyl-enzyme intermediate becomes deacylated by the nucleophiles to form the product peptide or by water which leads to hydrolysis (**Figure 1.11**).

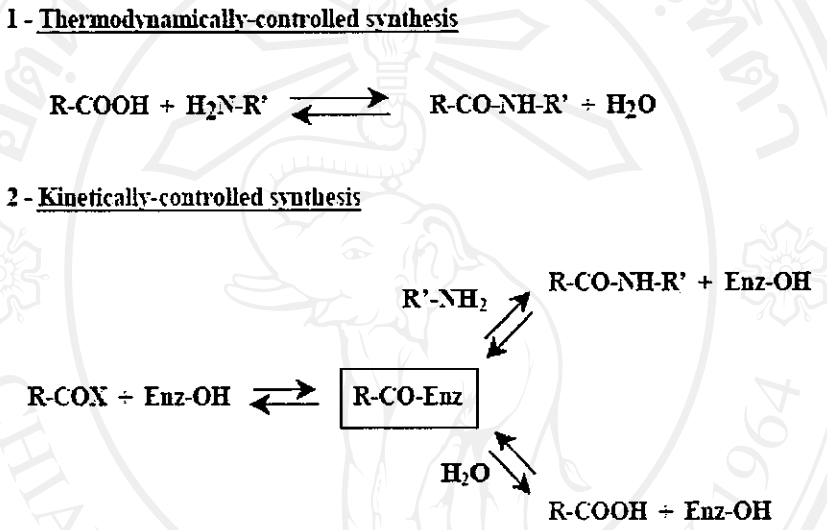

Figure 1.11 Protease catalysed thermodynamically controlled and kinetically controlled synthesis of peptides [Lombard *et al.*, 2005].

The thermodynamically (or equilibrium) controlled synthesis may be promoted by any of the four classes of proteases. The thermodynamic analysis of the reaction shows the existence of two consecutive steps: the proton transfer which gives the unionised forms -COOH and -NH_2 (exergonic process) and the condensation (endergonic process) [Lombard *et al.*, 2005]. It is believed that the formation of the acyl intermediate is a very slow process and the synthesis rate should depend on this fact. In general, thermodynamically controlled synthesis is limited by the reaction equilibrium. A substantial influence on this equilibrium is water, one of the reaction products. Therefore, reduction of water concentration shifts the whole reaction equilibrium

towards peptide bond formation. For this reason, a numerous research concentrated mainly on the question of how to reduce the water amount.

Apart from thermodynamically controlled synthesis, kinetically controlled synthesis also depends on the water content. Competition between hydrolysis and aminolysis are results from enzyme specificity, nucleophiles concentration, mode of carboxylic group activation, pH and reaction medium [Kullman, 1987; Lombard *et al.*, 2005]. The main difference between kinetically and thermodynamically controlled peptide synthesis is that the kinetically controlled method usually offers faster reaction progress, incorporated with less amount of enzyme used [Erbeldinger, 1999]. The kinetically controlled synthesis is more complicated than the thermodynamically controlled mode, since also secondary hydrolysis of the product peptide has to be considered. This means that the yield eventually goes down and that the product needs to be recovered at its maximum yield (**Figure 1.12**). Unlike thermodynamically controlled peptide synthesis, only cysteine or serine proteases can be used in this controlled system [Jakubke 1985; 1987]. Only these types of proteases contain a reactive thiol group or serine hydroxyl in their catalytic site. This is important to catalyse the reaction via the acyl intermediate. Research in the filed of kinetically controlled peptide synthesis tends to use reaction systems in solvent. Anhydrous organic media tackle the main problem, hydrolysis of the acyl intermediate and of the product peptide, and gives good yields. Many investigations about the influence of solvents and water concentration or water activity have been reported since the 1980s [for example Halling 1987].

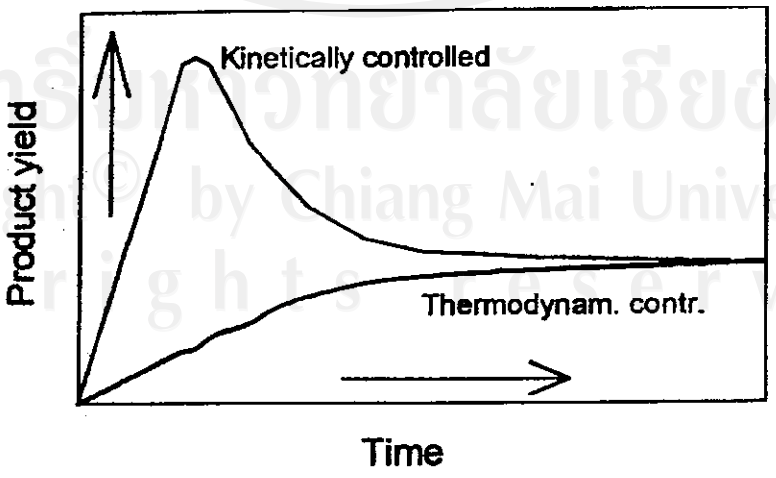

Figure 1.12 Comparison of a kinetically and a thermodynamically (or equilibrium) controlled peptide synthesis [Erbeldinger, 1999].

The "enzymatic solid-to-solid peptide synthesis" described in this thesis is clearly considered as thermodynamically controlled synthesis. It is also obviously an alternative to the reaction in organic media [Erbeldinger, 1999]. This reaction is mostly an equilibrium controlled method with minimisation of the water content. Keeping the advantages of enzymatic reactions, but avoiding the disadvantanges of organic solvents, are the reasons for using this method. The theoretical thermodynamic of this solid-to-solid reaction will be given in detail in the following.

1.2.4 Solid-to-solid peptide synthesis

1.2.4.1 Historical background

Proteases catalysing formation of peptide bond in organic solvents is well recognised and documented, since it offers several advantages over conventional chemical synthesis. The most important advantages are the enzyme's high chemo-, regio- and stereo specificities [e.g., Halling, 2000; Klibanov, 2001]. Benefits include the avoidance of racemisation, dispensation with side-chain protection, and the use of nonhazardous reaction conditions. This enzymatic approach has been successfully applied to the synthesis of a variety of peptides, for example sweetener peptides (aspartame [Nakaoka *et al.*, 1998], alitame [Kim and Shin, 2001], and sweet lysine peptide [Gill *et al.*, 1996]), bitter taste peptide [Trusek-Holownia, 2003; Morcelle *et al.*, 2006], neuropeptides (kyotorphin [Kim *et al.*, 2001], enkephalins [Clapés *et al.*, 1995; Ye *et al.*, 1998], and dinorphin [Jakubke *et al.*, 1985]), chiral drugs [Garćia-Junceda *et al.*, 2004], and RGD (Arg-Gly-Asp-OH) sequence of adhesive proteins [So *et al.*, 2000]. Other numerous enzymatic syntheses of biologically active peptides have also been extensively reviewed by several authors, for example, Gill *et al.* [1996] and Klibanov [2001]. Although considerable advances have been made through the applications of enzyme in aqueous-organic and low-water organic media, a number of problems have hampered the wider synthetic applications of this methodology. In particular, the limited solubility of substrates in those solvents results in the low productivities as compared to chemical approaches [Ahn *et al.*, 2001, Feliu *et al.*, 1995]. In addition, there are limitations to the use of organic solvents, particularly in the production of peptides for uses as food additives [Schoemaker *et al.*, 2003].

It has been reported that these drawbacks could be largely avoided by performing the initial reaction mixture as mainly solid suspended in a low-volume of

saturated liquid phase and in which the final reaction mixture consists of mainly solid product. Such reaction system was originally started by two independent studies in the early 1990s. One approach was to systematically reduce or replace organic solvent content in the reaction mixture [Kuhl *et al.*, 1990; 1992]. The second started with highly concentrated system by heating the substrate mixture to form heterogeneous eutectic mixture prior to start the reaction [Gill and Vulfson, 1993]. The two independent studies achieved very similar results with reasonable initial rates, high yields and nearly maximal ratio of product to reaction mixtures.

The first group described chymotrypsin catalysed the synthesis of blocked-Ala-Phe-Leu-NH_2 in different organic solvents with salt hydrates. The solvents ranged from trichloroethylene to the nonpolar hexane. The most effective reaction took place with yields over 80% conversion in 2 h. Poor solubility of peptides in most solvents has often been seen as a restriction in the selection of conditions for peptide coupling. The study showed that the enzymatic peptide synthesis can proceed effectively even when most of the reactants are undissolved [Kuhl *et al.*, 1990; 1992]. Further studies concentrated on the effect of mixing in mainly undissolved reaction mixture with ultrasonication and fluidisation. The evidence revealed that the chymotrypsin and thermolysin catalysed peptide coupling can be accomplished in the presence of only low amount of water without any other solvent. It should be noted that the source of water was used in only bound form ($Na_2SO_4 \cdot 10H_2O$) [Kuhl *et al.*, 1995]. This mainly undissolved reaction system was later called "solid-to-solid conversion" [Halling *et al.*, 1995].

In the other approach, various short peptides were synthesised by coupling of acyl donor, as the highly viscous liquid like L-Phe-OEt or the solid reactant like blocked-TyrOEt, with several acyl acceptors of amino amides and ethyl ester. The explanation for this reaction is the formation of "semi-liquid eutectic" obtained upon mixing the substrates together with small quantities of "adjuvants" and immobilised protease that the reaction mixture was called "heterogeneous eutectic mixture". The term adjuvant refers to organic liquids added in order to affect dispersion of substrates and to modify the physical properties of the reaction medium [Gill and Vulfson, 1993]. The additive adjuvants ranged from water to organic solvents of several compositions in amounts ranging from 10 to 30% [Gill and Vulfson, 1994].

However, there are differences in the preparation of the reaction mixture between the two groups. The first group prepared the mixture by adding the water and

enzyme directly to the solid substrates [Kuhl *et al.*, 1990; 1992; 1995; Halling *et al.*, 1995]. On the other hand, the second group described the formation of eutectic mixture by heating the substrates and additives up to 60°C prior to starting the reaction by adding the enzyme [Gill and Vulfson, 1993; 1994]. Therefore, the first approach seems more attractive for industrial applications (particularly for continuous system) because they do not use organic additives or pre-heating of substrates.

1.2.4.2 General aspects

Solid-to-solid peptide synthesis is an enzyme-catalysed synthesis in a low water medium. However, unlike other reactions in low water media, no organic solvents are involved and the initial reaction mixture consists just of solid substrates mixed with enzyme solution in water. The term "solid-to-solid synthesis" describes the appearance of the typical reaction mixtures in which consist of solid substrates with added liquid (often pure water) around 10% w/w and mainly solid product. However, it could give the mistaken impression that the enzymatic reaction itself occurs in the solid phase. In contrast the reaction requires a liquid phase where the actual biotransformation taking place, even though this may be of very small volume [Erbeldinger *et al.*, 1998a]. With the product precipitating rapidly during formation, the overall visual impression of such reaction mixture is a solid or highly viscous suspension [Erbeldinger *et al.*, 2001a]. In solid-to-solid reaction system, the solid reactants in the initial reaction mixture are nearly all converted to solid product. As a consequence, very high productivity can be obtained and significant reduction in reactor volume which in turn reduces production cost. The method combines advantages of synthesis in organic solvent, such as high yields, with those of using water as a solvent, such as good reaction rates, cheap, environmentally acceptable, preferred medium for enzymes [Erbeldinger *et al.*, 2001b; Ulijn, 2001].

A comparative study of enzyme catalysed peptide synthesis, either in organic solvents or in solid-to-solid system was reported. It was shown that the latter system gave both higher reaction rates and higher conversion [Ulijn *et al.*, 2000]. Moreover, the content enzyme added to the substrate mixture was relatively high (up to 10% w/w) because the smaller reaction volume but higher concentration level with 10-100 times when comparing to conventional reaction in organic solvent [Ahn *et al.*, 2001]. This is due probably to the most volume in the latter is predominant by the solvent. Typical volumes of reactants and product in enzymatic syntheses via solid-to-solid system and in

organic solvent are shown in **Figure 1.13**. Apart from the advantages described above, it was demonstrated that solid-to-solid reactions also produce significantly less heat and have better atom efficiency than conventional methods in organic solvents. All of the positive features described above make this enzymatic solid-to-solid peptide synthesis an attractive green chemistry option as explanation by Ulijn and Halling (**Figure 1.14**) [2004].

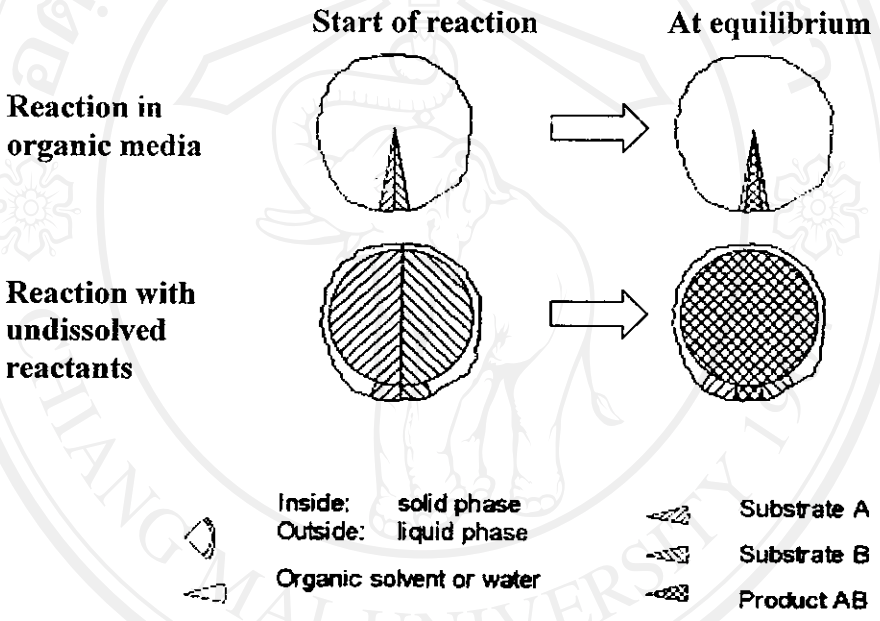

Figure 1.13 Comparison of enzymatic peptide synthesis in organic media (upper row) or in solid-to-solid system (lower row). Initial and equilibrium compositions are represented at left and right column, respectively. All areas are equal concentration in w/w [Erbeldinger *et al.*, 1998].

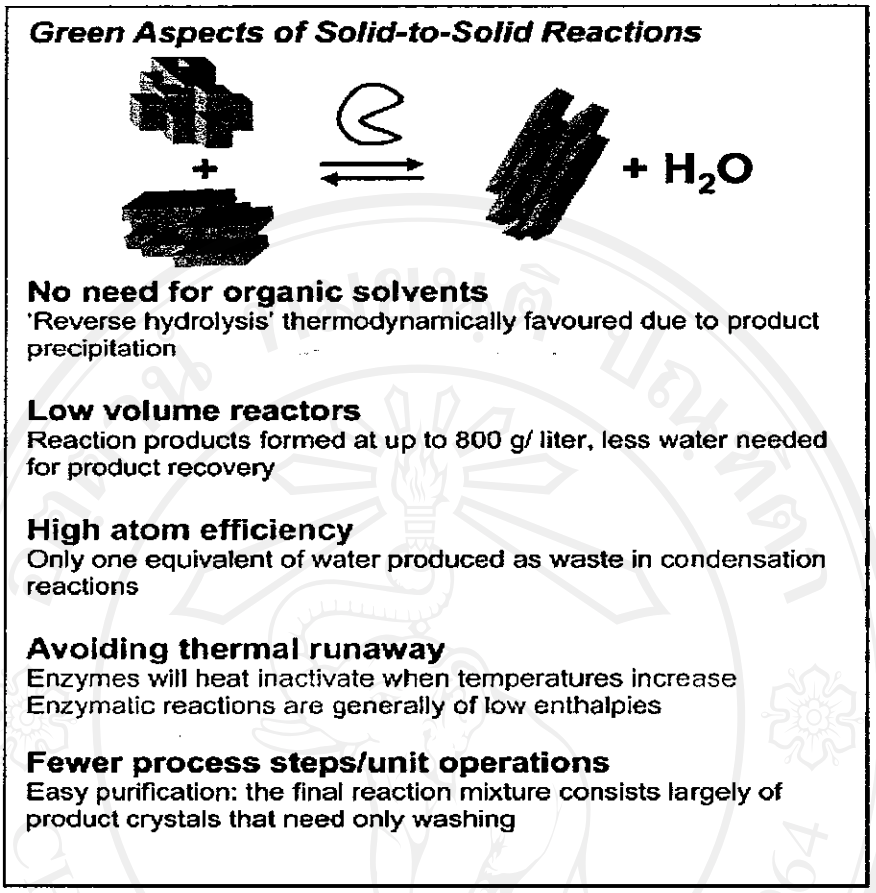

Figure 1.14 Green aspects of solid-to-solid biocatalysis [Ulijn and Halling, 2004].

The overall rate of solid-to-solid reactions is governed by three sub-processes (**Figure 1.15**). These are the dissolution of the substrates, the enzyme catalysed reaction and the precipitation of the reaction product. All of these steps occur at the same time [Ulijn *et al.*, 2003]. In such method, the driving force for the very good yield obtained is the precipitation of the reaction products. Hence, the term "precipitation driven biocatalysis" has also been used to describe this reaction system [Ulijn *et al.*, 2003]. Besides the term solid-to-solid, a variety of names have been reported describing essentially the same reactions. They are described either as "aqueous two-phase systems with precipitated substrate and/or product" [Kasche and Galunsky, 1995], "suspension-to-suspension" [Eichhorn *et al.*, 1997] or "heterogeneous eutectic mixture" [Gill and Vulfson, 1994]. All of these entire reaction systems, including solid-to-solid conversion, are covered by the name precipitation driven biocatalysis.

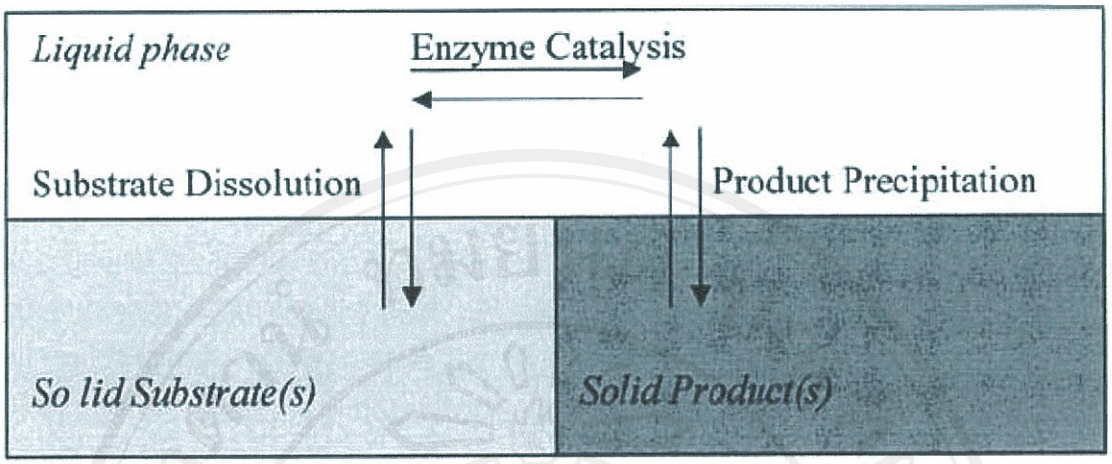

Figure 1.15 Schematic overview of precipitation driven biocatalysis. The three sub-processes involved are: the dissolution of substrates, the enzyme catalysed reaction and the precipitation of the reaction product (s) [Ulijn *et al.*, 2003].

Precipitation driven reaction of peptide synthesis shows very favourable displaying when compared to other low-water systems [Ulijn *et al.*, 2000]. Several aspects of this reaction system are significantly different from those in the solution. The presence of solid substrates has important consequences for the reaction kinetics and thermodynamics and it requires different strategies for reaction, including effect of organic solvents, pH control and form of enzyme used.

1.2.4.3 Switch like thermodynamics of reaction

Thermodynamics of reaction exhibit a key feature of solid-to-solid system. Halling *et al.* [1995] was the first group attempting to explain some aspects by considering the physical picture of the reaction system and some description of equilibrium behavior were obtained. An important aspect of these predominantly solid reactant systems is that the thermodynamics of the reaction exhibit a particular behaviour, giving either very high of very low yields, depending on whether product precipitation is favoured or not [Halling *et al.*, 1995]. While in many cases the reaction were successful, much lower yields have been observed in biocatalysis with undissolved substrates where the product does not precipitate [Diender *et al.*, 1998; Schroen *et al.*, 1999]. These observations can be explained in terms of the "switch like

thermodynamics" of system with solid substrates present [Eichhorn *et al.*, 1997; Ulijn *et al.*, 2001; 2003] as explained in what follows by Ulijn *et al.* [2001; 2003].

For a reaction with suspended substrate and precipitating product, the overall reaction can be described in terms of solids (and water) only. For a reverse hydrolysis reaction, this leads to equation **(1)**:

$$AOH_{(s)} + BH_{(s)} \rightleftharpoons AB_{(s)} + H_2O \qquad (1)$$

where, AOH is an acyl donor, BH is a nucleophiles, and AB is the reaction product.

It is useful to consider two thermodynamic constants. The thermodynamic equilibrium constant (K_{th}) of the chemical reaction is given by equation **(2)**:

$$K_{th} = \frac{a_{AB}^{eq} a_{w}^{eq}}{a_{AOH}^{eq} a_{BH}^{eq}} \qquad (2)$$

where, at equilibrium, a's are thermodynamic activities of the reactants and a_w is the water activity.

The thermodynamic mass action ratio (Z_{th}) (the ratio of thermodynamic activities of the pure solid product over the solid substrates) is defined by equation **(3)**:

$$Z_{th} = \frac{a_{AB}^{s} a_{w}}{a_{AOH}^{s} a_{BH}^{s}} \qquad (3)$$

Provided the same reference standard state (for example the pure solid) is used in K_{th} and Z_{th} for each reactant, it can be combines these to give the free energy of the solid-to-solid conversion (for conditions when water is in its standard state) according to equation **(4)**:

$$\Delta G_{solid-solid} = -2.3RT \log\left(\frac{K_{th}}{Z_{th}}\right) \qquad (4)$$

The equation will be approximately correct whenever a dilute aqueous phase is present. If $\Delta G_{solid\text{-}solid}$ is negative, product precipitation can be expected, while, if it is positive, no product precipitation will be observed.

However, both K_{th} and Z_{th} are difficult to measure. In terms of directly measurable parameters, equations **(2)** and **(3)** can be replaced by **(5)** and **(6)**.

$$K_{eq} = \frac{[AB]}{[AOH][BH]} \qquad (M^{-1}) \qquad \textbf{(5)}$$

$$Z_{sat} = \frac{S_{AB}}{S_{AOH}S_{BH}} \qquad (M^{-1}) \qquad \textbf{(6)}$$

when K_{eq} is the equilibrium constant and Z_{sat} is the saturated mass action ratio.

As the reaction proceeds, both the concentrations of AOH and BH will remain constant at their limiting solubility values S_{AOH} and S_{BH}, while product AB will begin to accumulate in the liquid phase. Two different situations can then arise.

1) If [AB] is able to reach its equilibrium value as defined by equation **(5)**, the reaction will proceed no further and most of AOH and BH will still be present as solid in the reaction mixture. Thus, in this case the overall reaction yield will be very low.

2) Alternatively, if before reaching the equilibrium defined by equation **(5),** AB reaches it solubility limit, it will begin to precipitate from solution. Solid AB will then continue to precipitate until all of the solid form of reactant AOH **or** BH has dissolved. The concentration of this reactant will then fall below the solubility limit until the equilibrium constant defined by equation **(5)** can be reached. In this case, the overall yield is necessarily very high, since the reaction proceed until all the excess solid of one (or both) of the reactants is used up.

When $Z_{sat} > K_{eq}$, product AB is able to reach its equilibrium value in the liquid phase corresponding to case **1)** above. In contrast, only when $Z_{sat} < K_{eq}$ the solubility limit of AB is reached before establishing equilibrium as described in case **2)** and as a consequence, it does the precipitation driven reaction proceed toward quantitative yield. **Figure 1.16** illustrates the latter situation diagrammatically. This explains that very high yields are observed.

An example of such assessment, a study of the feasibility of precipitation driven synthesis of amoxicillin has been reported [Diender *et al.*, 1998]. At every pH measured, Z_{sat} (called R_s in that paper) was about one order of magnitude larger in value than the experimental K_{eq} and consequently product precipitation was not expected and also not

observed experimentally in a reaction with suspended substrates [Diender *et al.*, 1998]. The report of neutral peptide synthesis which product precipitation in solid-to-solid reaction was highly observed, it could be calculated that Z_{sat} were always between two and six orders of magnitude smaller than the reference K_{eq} for amide synthesis. Hence, conversion of solid substrates to solid product was favourable [Ulijn *et al.,* 2003].

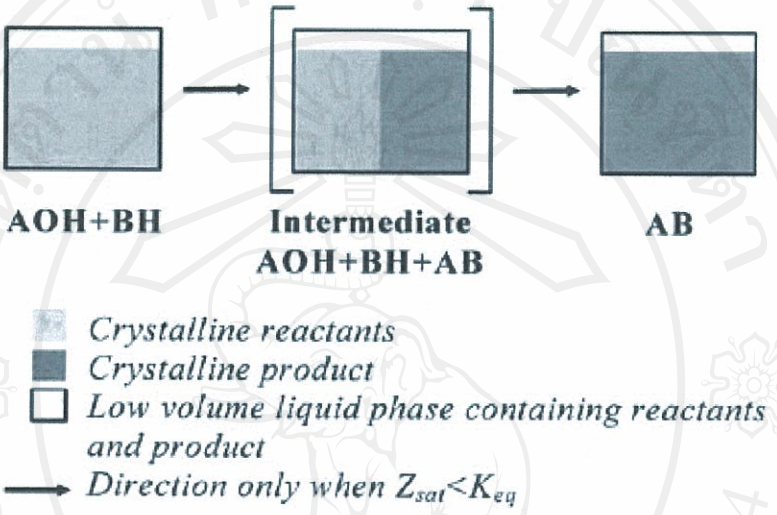

Figure 1.16 Schematic representation of solid-to-solid reaction. If $Z_{sat} < K_{eq}$ the product precipitates and equilibrium will be reached only when the solid substrates completely run out (and completely dissolve). If $Z_{sat} > K_{eq}$ the product accumulates in the liquid phase only and no more solid substrate will be dissolved to proceed the reaction at equilibrium. The intermediate state will always be thermodynamically unstable with respect to near complete conversion in one direction or other [Ulijn *et al.,* 2001; 2003].

1.2.4.4 Solvent selection

In solid-to-solid peptide synthesis, small proportion of liquid phase in the reaction mixture is necessary for reaction taking place. The liquid phase during course of two reactions of L-Phe-Leu-NH_2 and Z-Tyr-Leu-NH_2 syntheses was analysed by polarising microscopy (PM) and Fourier transform-infrared microscopy (FTIRM) [López-Fandiño *et al.*, 1994b]. Their results leaved no doubt that the reaction takes place in a liquid phase. The requirement of a liquid phase was confirmed by using completely different approach [Kuhl *et al.,* 1995]. Investigation of peptide yield as a function of

added water found that a minimum amount of water is essential to maintain the catalytic activity of enzyme in the Z-Phe-OH and Leu-NH_2 system. Equilibrium was achieved around 80% conversion in the range of 1-10 mmol added water per 0.1 mmol substrates (about 1-80% w/w). It could also be concluded that no peptide coupling takes place without any water added. [Kuhl *et al.*, 1995]. If the substrates were rigorously dried beforehand, there is no catalytic activity of enzyme without any added of water [Erbeldinger *et al.*, 1998a; 1998b]. Nevertheless, a slow but significant reaction was observed when the substrates were not dried before using. It was suggested that the very small amount of water, existed in the solid substrates, is sufficient to form the liquid phase necessary for reaction. However, it also has to be considered that water is one of the products of peptide synthesis, so that more will accumulate with time [Erbeldinger *et al.*, 1998a].

The liquid phase in the solid-to-solid mixture can be generated by two ways. Firstly, the liquid can be added directly to generate the system. Most of these reactions were carried out with small amount of water [Kuhl *et al.*, 1995; Halling *et al.*, 1995; Eichhorn *et al.*, 1997; Erbeldinger *et al.*, 1998a; 1998b; 1999] or an other organic solvent or a mixture of organic solvent and water (sometime referred both to as "adjuvants") [Gill and Vulfson, 1994; López-Fandiño *et al.*, 1994a; 1994b]. The liquid phase can also be produced from two solid reactants even without further addition [Gill and Vulfson, 1993]. However, most studies have been of systems in which a small amount of water or organic solvent has been added.

The influence of organic solvents as additives was intensively investigated [1993] and summarised [López-Fandiño *et al.*, 1994a; 1994b] that the addition of any liquid, either water or organic solvents, enables the formation of the liquid phase or improves the reaction rate in eutectic systems. It has been shown that the yield of solid-to-solid synthesis does depend strongly on the solvent chosen. As a rule of thumb, rather hydrophilic solvents (low log P) give the best results for both peptides and sugar fatty acid ester [Gill and Vulfson, 1994; Cao *et al.*, 1999]. This behaviour was related to the poor solubility of the synthesised product in these solvents [Cao *et al.*, 1999].

A comparison of the solid-to-solid synthesis of Z-L-Phe-L-Leu-NH_2 in ten different solvents (but same water activity): water, methanol, *tert*-amyl alcohol, 2-propanol, ethyl acetate, acetonitrile, tetrahydrofuran, toluene, n-hexane and dichloromethane, only the last solvent whose product precipitation was not observed

[Ulijn, *et al.*, 2002]. The result revealed that the highest yields in terms of solid products were found in solvents where both substrate and product solubility are minimised. It can be concluded that the solid-to-solid Z-L-Phe-L-Leu-NH_2 synthesis is feasible in a large number of solvents with different physical properties, as the theoretically derived that the favourability of solid-to-solid conversion is solvent independent [Halling *et al.*, 1995; Erbeldinger *et al.*, 1998a]. However, product yields depended on the solvent characteristics that the highest products were obtained where both product and substrate solubility were lowest [Ulijn *et al.*, 2002].

From most of the literatures, it appears that water is an excellent solvent for solid-to-solid biocatalysis, leading nearly complete conversion to peptide product [e.g. Halling *et al.*, 1995; Krix *et al.*, 1997; Eichhorn, 1997]. It must be noted that however most of these used thermolysin as a catalyst. The choice of substrate is then restricted to hydrophobic amino acids because of the enzyme specificity. As a consequence, the synthesised peptides will exhibit poor solubility in water, resulting in high yield of product [Ulijn *et al.*, 2003].

1.2.4.5 Form of enzyme

In case of solid-to-solid reaction systems the enzyme is required to operate in a liquid phase containing very high substrate concentrations. Various forms of enzymes have been reported for using in solid-to-solid reaction. Papain, proteinase K, pronase E, chymotrypsin, subtilisin and thermolysin were adsorbed onto 100 μm Celite in the synthesis of *N*-acetyl dipeptide amides. The residual substrates and the immobilised enzymes were recovered by filtration and could be reused several times [López-Fandiño *et al.*, 1994a; 1994b]. Immobilised subtilisin, thermolysin, papain and chymopapain on Celite were employed to catalyse the synthesis of delicious peptide fragments [Jorba *et al.*, 1995] and bioactive oligopeptides [Gill and Valivety, 2002]. The syntheses displayed good operational stability and a mild solvent extraction step was sufficient to allow enzyme recovery in a highly active form ready for reuse [Jorba *et al.*, 1995]. However, loss of peptide products was found when using immobilised chymotrypsin on Eupergit C or C 250 [Kasche and Galunsky, 1995]. The authors pointed out that the precipitation of product inside the catalyst particles depends strictly on the size of the pores. Silica matrices were present to be useful support in solid-to-solid peptide synthesis of rather hydrophobic peptide catalyzed by thermolysin and chymotrypsin [Basso *et al.*, 2000].

When the more polar dipeptide product such as kyotorphin was attempted to synthesis by chymotrypsin adsorbed onto Celite R-640®, most of the precipitated product adsorbed onto the support surface thus leading to unsatisfactory reaction yield due to difficult recovery procedure [Ulijn *et al.*, 2003]. In most of the literature, lyophilised enzyme powders have been extensively used and catalysed solid-to-solid reactions effectively. The lyophilised thermolysin has successfully catalysed the synthesis of Z-Phe-Leu-NH_2 under fluidisation [Kuhl *et al.*, 1995], sonicating [Halling *et al.*, 1995] and orbital shaking condition [Ulijn *et al.*, 2002]. The solid-to-solid productions of Z- and Boc-Ala-Phe-Leu-NH_2 were accomplished by chymotrypsin powder under sonication and fluidisation [Krix *et al.*, 1997]. A vast number of dipeptides [Eichhorn *et al.*, 1997] and the peptides containing nonproteinogenic amino acid [Krix *et al.*, 1997] were also synthesised by thermolysin powder via the solid-to-solid reaction. More recent, lyophilised thermolysin catalysed the syntheses of a great number of peptides were achieved in which the reaction mixtures were just immersed in a water bath after mixing well with a spatula [Erbeldinger *et al.*, 1998; 1999; 2001]. The main advantages of using free enzymes are some of added enzymes can be dissolved in the small volume of the liquid phase and ease of use. The enzyme can also be simply washed away from the product crystals with water [Erbeldinger *et al.*, 2001a; Ulijn *et al.*, 2002].

1.2.4.6 pH effect on catalytic reaction

Little enzymology has been done in solid-to-solid media, even with water as the solvent. One important factor in this system is acid-base behaviour, which is usually dominated by the effects of substrates and products themselves. The discontinuity in pH change during solid-to-solid reaction is caused by the depletion of the solid phase of one of the substrates. Similar discontinuity will be found when a product start to crystallise, if the predominant dissolved product species is an anion or cation [Diender *et al.*, 2000]. It is obvious that the pH of reaction mixture will not influence only the rate of enzymatic reaction, but importantly also the solubility and hence the dissolution of crystallisation of the reactants [Ulijn *et al.*, 2003]. Unfortnately, it is difficult to measure the pH of the liquid phase in which the reaction occurs, because it is normally completely trapped between the solid reactant and product crystals [Erbeldinger *et al.*, 2001c]. Hence, there have been reported the mathematical model to predict or explain pH shift during the reaction [Lee *et al.*, 1998; Diender *et al.*, 2000; Erbeldinger *et al.*, 1999; 2001c].

However, control of pH in the reaction mixture is a general rule to ensure prevention of pH changes. There are two species solid substances for using in pH control; substrates themselves and inorganic salts [Ulijn *et al.*, 2003]. It is not advisable to control pH with organic buffers because of the very high (up to 10 M) substrate concentrations employed. The amount of buffering compound(s) needed to control the pH is likely to be too high in terms of their weight or volume. The use of organic buffers may also complicate the recovery of the final product [Erbeldinger *et al.*, 2001b].

pH control by substrates

In many cases no efforts were made to control the pH. Indeed, when substrate solubilities are high, the pH of the reaction mixture is set by the ionisation of the acid and basic substrates. The presence of aqueous buffers will not have a significant effect [Erbeldinger *et al.*, 1998b]. Protected amino acids have pK_a values of around 3-4 for free acids and 7-8 for amines. This provides that the concentration of other ions can be neglected; the initial pH can be derived from a charge balance of substrates. The pH will then be in between the pK_a values which are a suitable range for most enzymes [Ulijn *et al.*, 2003]. A correlation between changes in the initial substrate ratio and the pH of the liquid phase was reported, and slightly unequal substrate ratios can have large effect on the reaction rate [Erbeldinger *et al*, 1999]. A two-fold higer initial rate could be achieved in a reaction mixture with 60% L-Leu-NH_2 (pK_a around 7.8) and 40% Z-Gln-OH (pK_a around 3.6) as compared to a reaction with equimolar substrates [Erbeldinger *et al*, 1999]. However, even the unequal substrate ratios can control pH of the reaction and provides better reaction rate. They are impossible to give high yield, especially in large scale application. This is because the excess amount reduces the overall yield and additional recycling step [Erbeldinger *et al.*, 2001c]. Therefore, alternative method for controlling pH is generally preferred by adding large amounts of acidic or basic inorganic salts.

pH control by salts

An equimolar substrate, together with high yield would be attractive for any application. The acidic or basic inorganic salts are consequently introduced to control the pH of reaction mixture. The initial rate could be increased 20-fold by adding $KHCO_3$ or K_2CO_3 in the synthesis of Z-Asp-Phe-OMe [Erbeldinger *et al.*, 2001c]. The profile of

initial rate as a function of K_2CO_3 concentration displayed a bell-shaped figure typically seen for pH effects on enzyme activity. Similar effect could be observed during the thermolysin-catalysed synthesis of Z-Gln-Leu-NH_2 when $NaHCO_3$ was used instead of $KHCO_3$ [Erbeldinger *et al*, 2001c]. However, it has been suggested that the use of a weakly basic salt like $KHCO_3$ helps prevent an overshooting of pH. Stronger salts like K_2CO_3 are likely to make the pH too basic leading to the reduction in final yields [Erbeldinger *et al.*, 2001b]. The addition of strong base like NaOH was also reported that it can offer a favourable pH in thermolysin catalysed synthesis of aspartame from Z-Asp-OH and H-Phe-OMe·HCl [Eichhorn *et al.*, 1997].

1.2.5 Objective of study

Papaya fruit consumptions produce quantitative wastes of its peels. Preparation of proteases from these waste peels is an attractive alternative from that of papaya latex. Glycyl endopeptidase is abundant cysteine protease in papaya latex and has an interesting unique specificity. It is worthy to produce this enzyme from both papaya peels and the latex. The enzyme will be used in further application by catalysis of solid-to-solid peptide synthesis. Therefore, the aims of this study are as followed:

1) To separate proteases from papaya peels and determine some significant properties of the enzymes

2) To investigate the presence of glycyl endopeptidase in papaya peels and latex

3) To purify glycyl endopeptidase from papaya latex.

4) To determine the catalytic activity of purified glycyl endopeptidase.

5) To optimise the reaction parameters for glycyl endopeptidase catalysed solid-to-solid peptide synthesis.

6) To investigate the specificity of glycyl endopeptidase on nucleophiles in solid-to-solid peptide synthesis.