การผลิตเจลาตินจากหนังสัตว์ใหญ่โดยใช้โพรทีโอไลติกเอนไซม์ที่สกัดจากยางมะละกอ

นายสิทธิรักษ์ พิตรปรีชา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
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GELATIN PRODUCTION FROM LARGE ANIMAL RAW HIDE USING PROTEOLYTIC ENZYME EXTRACTED FROM PAPAYA LATEX

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for the Degree of Master of Engineering Program in Chemical Engineering

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Thesis Title	GELATIN PRODUCTION FROM LARGE ANIMAL RAW HIDE USING PROTEOLYTIC ENZYME EXTRACTED FROM PAPAYA LATEX
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Field of Study	Chemical Engineering
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สิทธิรักษ์ พิตรปรีชา : การผลิตเจลาตินจากหนังสัตว์ใหญ่โดยใช้โพรทีโอไลติกเอนไซม์ที่สกัดจากยางมะละกอ (GELATIN PRODUCTION FROM LARGE ANIMAL RAW HIDE USING PROTEOLYTIC ENZYME EXTRACTED FROM PAPAYA LATEX). อ. ที่ปรึกษา: รศ.ดร. ศิริพร ดำรงค์ศักดิ์กุล, 115 หน้า. ISBN 974-53-2940-1.

งานวิจัยนี้แบ่งเป็นสองส่วนคือ การสกัดโพรทีโอไลติกเอนไซม์อย่างหยาบจากยางมะละกอ และการผลิตเจลาตินด้วยการไฮโดรไลซิสหนังสัตว์โดยใช้โพรทีโอไลติกเอนไซม์ที่สกัดได้ ผลการศึกษาการสกัดโพรทีโอไลติกเอนไซม์ด้วยน้ำและฟอสเฟตบัฟเฟอร์ที่มีค่าความเป็นกรดเบสเท่ากับ 6 พบว่าค่าผลได้ของการสกัดเอนไซม์ไม่เปลี่ยนแปลงตามเวลาแต่ขึ้นกับอัตราส่วนระหว่างปริมาตรน้ำยางกับตัวทำละลาย และยังพบอีกว่าค่าผลได้ของเอนไซม์ที่สกัดด้วยน้ำมีค่าสูงกว่าการสกัดด้วยฟอสเฟตบัฟเฟอร์ หลังจากนั้นเอนไซม์ที่สกัดได้จะนำไปตกตะกอนในสารละลายแอมโมเนียมซัลเฟตอิ่มตัว 45% พบว่าค่าผลได้และค่ากิจกรรมของเอนไซม์ลดลงอย่างมาก จึงเลือกนำเอนไซม์ที่สกัดด้วยน้ำที่อัตราส่วน 1:1 ไปใช้เปรียบเทียบกับปาเปนเกรดการค้าในขั้นตอนการผลิตเจลาตินต่อไป โดยในการผลิตเจลาตินได้ศึกษาผลของสภาวะ ได้แก่ อุณหภูมิ ค่าความเป็นกรดเบส เวลา และ อัตราส่วนเอนไซม์ต่อหนังสัตว์ ที่มีต่อผลได้ของเจลาติน และสมบัติของเจลาติน จากการศึกษาพบว่า ในช่วงแรกของปฏิกิริยาผลได้ของเจลาตินจะเพิ่มขึ้นอย่างรวดเร็วหลังจากนั้นจะเพิ่มขึ้นอย่างช้าๆ สภาวะที่เหมาะสมในการใช้เอนไซม์สกัดอย่างหยาบและปาเปนเกรดการค้าที่ให้ค่าผลได้ของเจลาตินสูงสุด คืออุณหภูมิ 75°ซ และความเป็นกรดเบสเท่ากับ 7 ซึ่งมีผลได้ของเจลาตินประมาณ 60-70 เปอร์เซ็นต์โดยน้ำหนักของปริมาณโปรตีนในหนังสัตว์เริ่มต้น เจลาตินที่สกัดได้จากกระบวนการไฮโดรไลซิสที่ใช้เอนไซม์ทั้งสองชนิดมีความแข็งของเจลและความหนืดต่ำ อย่างไรก็ตามผลได้ของเจลาตินและสมบัติของเจลาตินที่ได้สามารถควบคุมได้โดยโดยการปรับอัตราส่วนของเอนไซม์ต่อหนังสัตว์ อุณหภูมิ และค่าความเป็นกรดเบสที่ใช้ในการไฮโดรไลซิสหนังสัตว์ ตัวอย่างเช่น การสกัดที่สภาวะซึ่งเหมาะสมกับค่ากิจกรรมของเอนไซม์สูงสุด (75°ซ, ความเป็นกรดเบสเท่ากับ 7) จะได้ผลได้ของเจลาตินที่สูง แต่เจลาตินที่ได้มีความแข็งของเจลต่ำ (55.8-58.4 กรัมบลูม) ในทางกลับกันการสกัดที่สภาวะซึ่งกิจกรรมเอนไซม์ค่อนข้างต่ำ จะทำให้ได้ผลได้ของเจลาตินที่ต่ำ และได้เจลาตินที่มีความแข็งแรงของเจลที่สูงขึ้น (166.4-187.6 กรัมบลูม) ซึ่งผลได้และคุณสมบัติของเจลาตินจากการสกัดด้วยเอนไซม์ทั้งสองจะมีค่าใกล้เคียงกัน แต่ปริมาณของเอนไซม์สกัดอย่างหยาบที่ใช้จะน้อยกว่า และราคาของเอนไซม์สกัดอย่างหยาบจากยางมะละกอมีราคาถูกกว่าปาเปนเกรดการค้ามาก

ภาควิชา.....วิศวกรรมเคมี.....ลายมือชื่อนิสิต.....

สาขาวิชา.....วิศวกรรมเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2548.....

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KEY WORD: GELATIN/ COLLAGEN/ HYDROLYSIS/ PAPAYA LATEX/ PROTEOLYTIC ENZYME/ PAPAIN

SITTIRUK PITPREECHA: GELATIN PRODUCTION FROM LARGE ANIMAL RAW HIDE USING PROTEOLYTIC ENZYME EXTRACTED FROM PAPAYA LATEX. THESIS ADVISOR: ASSOC.PROF. SIRIPORN DAMRONGSAKKUL, Ph.D, 115 pp. ISBN 974-53-2940-1.

This work is divided into two parts: crude extraction of proteolytic enzyme from papaya latex, and the use of crude extracted proteolytic enzyme to produce gelatin via the hydrolysis of raw hide. From the results of solvent extraction of proteolytic enzyme using water and phosphate buffer pH6, the yield of extracted enzyme did not change by extraction time but it was dependent on the solvent and the ratio of latex to solvent. The yield of extracted enzyme from water extraction was higher than those from phosphate buffer extraction. Following the solvent extraction, the extracted enzyme was precipitated in 45% saturated ammonium sulfate solution. The yield and activity of precipitated enzymes were considerably decreased. Therefore, crude extracted enzyme from water extraction at ratio of 1:1 was selected to be used in gelatin production comparing to the use of commercial papain. For the gelatin production, the effects of conditions which were temperature, pH, hydrolysis time and enzyme to raw hide ratio, on the yield of gelatin recovery and properties of gelatin were investigated. At the beginning of hydrolysis reaction, the yield of gelatin recovery was greatly increased, and then slightly increased. The optimum conditions of crude extracted enzyme and commercial papain hydrolysis for the highest gelatin recovery are 75°C and pH 7. The percentage of gelatin recovery is around 60-70wt%. The results showed that the gel strength and viscosity of gelatin obtained from both types of enzymatic hydrolysis are low. However, gelatin recovery and properties of gelatin could be manipulated by temperature, pH, and ratio of enzyme to raw hide. For example, at the optimum condition for enzyme activity (75°C, pH7), the high gelatin recovery could be achieved but the low gel strength gelatin (55.8-58.4 g Bloom) was obtained. On the other hand, at the condition for relatively low enzyme activity (85°C, pH7), the lower gelatin recovery was reached and gelatin with relatively higher gel strength (166.4-187.6 g Bloom) was received. The gelatin recovery and properties of gelatin obtained from crude extracted enzyme and commercial papain hydrolysis were similar. But the used amount and the cost of crude extracted enzyme from papaya latex were much less than those of commercial papain.

Department.....Chemical Engineering.....Student's signature Sittiruk Pitpreecha...

Field of study.....Chemical Engineering.....Advisor's signature.....

Academic year...2005.....

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CHAPTER I

INTRODUCTION

1.1 Background

Nowadays, gelatin is used in many traditional applications such as food industry, pharmaceutical industry, photographic industry, and cosmetic industry. For food industry, gelatin is used in desserts, meats and dairy products. It is also used in hard capsules and soft elastic capsules known as “softgels” in pharmaceutical industry. In photographic industry, gelatin is coated on photographic film and paper. For cosmetic industry, gelatin is added to facial cream, body lotion, shampoo, hair spray and sun screen. Type of gelatin applications is determined by the gel strength of gelatin which depends on protein source and manufacturing.

Gelatin is a large protein obtained from partial hydrolysis of collagen. Collagen is the most common fibrous protein found in skin, hide, bone, horn, hoof and white connective tissue of animals. The major source of raw material of gelatin manufacturing industry is from tannery industry. Tannery industry produces a large amount of wastes such as hoof, horn and bone. The solid waste can be used as raw material in gelatin production. This makes value added to the waste and reduces ecological problems. There are two typical methods of gelatin production; acid and liming processes. In acid process, collagen is hydrolyzed by acid solution and gelatin obtained from acid process is called type A gelatin. Pig skin is used as raw material for acid process and the hydrolysis time is about 10-45 hours. The liming process employs alkaline to hydrolyze collagen and gelatin obtained from liming process is called type B gelatin. Bone and hide are raw materials of liming process and the hydrolysis time is about 30-100 days. These two typical methods of gelatin production have many disadvantages such as long period of processing time, and producing a lot of waste.

Recently, the enzymatic hydrolysis for gelatin production is of interest since the processing time is short and a small amount of wastes is generated. It can be applied to all protein sources of raw material for example bone, hide, skin, fish scales and leather wastes. There are many works on enzymatic hydrolysis for protein waste treatment (Taylor *et al*., 1989; Simeonova *et al*, 1996; Aspmoa *et al*., 2005; and Ratanathammapan, 2005). They reported the methods of protein waste hydrolysis using various type of commercial proteolytic enzymes sush as pancreatin, enzeco, alcalase, neutrase, alkaline proteinase, protamax and papain. The most recent work of Ratanathammapan (2005) on the uses of papain in the gelatin production from large animal raw hide have reported that papain could be effectively used to produce gelatin with low gel strength. The gel strength of gelatin can be controlled by manipulating hydrolysis conditions and the amount of papain used. However, the production of commercial papain is not available in Thailand and the imported papain is expensive. As it is well known that papain is a plant-derived proteolytic enzyme from papaya which is a local plant of Thailand. It is therefore of interest to extract proteolytic enzyme from papaya latex in order to be used in raw hide hydrolysis.

This work aims to utilize proteolytic enzymes from local papaya latex in gelatin production from raw hide. The crude extraction of proteolytic enzymes from papaya latex is first investigated. Then, the obtained crude extracted enzyme is used in the hydrolysis of raw hide to produce gelatin. The performance of crude extracted enzyme and the properties of obtained gelatin are investigated and compared with the case of using commercial papain.

1.2 Objectives

1.2.1 Investigate the extraction of proteolytic enzyme from papaya latex for raw hide hydrolysis

1.2.2 Compare the effects of enzyme on the yield and the physical properties of recovered gelatin from the hydrolysis reaction of raw hide.

1.3 Scope of work

1.3.1 Investigate the effects of solvent extraction on the enzyme activity and the yield of extracted enzyme from papaya latex. The extract conditions were:

- Solvent: water and phosphate buffer pH6
- Ratio of papaya latex to solvent
- Extraction time from 5 to 60 minutes

1.3.2 Investigate the effects of ammonium sulphate precipitation on the enzyme activity and the yield of extracted enzyme.

1.3.3 Investigate the influences of enzymatic conditions on the yield of recovered gelatin from the hydrolysis reaction of raw hide. The enzymatic conditions were:

- Enzyme to substrate ratios
- pH of raw hide slurry from 6 to 8
- Hydrolysis temperature from 65 to 85^{o}C
- Hydrolysis time from 0 to 90 minutes

1.3.4 Determine the physical properties of recovered gelatin such as

- Gel strength
- Viscosity
- Molecular weight
- Ash

1.3.5 Compare the cost of gelatin production using commercial papain and crude extracted enzyme from papaya latex.

CHAPTER II

THEORY

2.1 Enzyme (Fox, 1991)

The use of enzyme in food industry occurred before the discovery of enzyme. The oldest was the use of yeast in brewing and baking. Enzyme is protein that is biocatalyst, speed up chemical reactions in living organisms, but it is not consumed in those reactions. Kunhne first used the term of 'enzyme' in 1878 from the Greek meaning 'in yeast'. The most striking properties of enzyme are the catalytic power, specificity and regulation.

Catalytic power

Enzymes have enormous catalytic power, accelerating rates of reactions catalyzed by enzyme as much as 10^{14} fold over rates of reactions catalyzed by the synthetic catalysts. Enzymes accomplish these abilities in dilute aqueous solutions under mild conditions of temperature and pH. For example, enzyme urease catalyzes the hydrolysis of urea. The rate constant for the enzyme catalyzed reaction at 21^{o}C is 5.0×10^{6} mol/(liter·sec), the rate constant for H^{+} catalyzed hydrolysis reaction of urea at 62^{o}C is 7.4×10^{-7} mol/(liter·sec). Thus, the ratio of the rate of reaction catalyzed by enzyme to that by inorganic catalyst is about 10^{13}.

Specificity

Enzymes usually react with specific substrates. Specificity of enzyme can be divided into two distinct hypotheses: 'lock and key' hypothesis and induced fit hypothesis. The 'lock and key' hypothesis proposes that enzymes have a particular shape of active site into which the substrates are fitted exactly. For the induced fit hypothesis, the active site of an enzyme might be modified as the substrate interacts with the enzyme. Enzymes have different degrees of specificity. The lowest type of specificity is bond specificity. This group includes a number of degradative enzymes

such as peptidases, phosphatases, and esterase. The second type of specificity is group specificity where the range of substrates is much more restricted. The third type is the most specific, known as absolute specificity, enzyme catalyses a reaction with only a single substrate.

Regulation

Regulation of enzyme does not involve only inhibitory effects, but activating effects. In the reaction, the substrate interacts with the enzyme to form enzyme-substrate complex then product is released. One of these products becomes the substrate for the next enzyme, and continues through several more enzymes. When a higher concentration of final product begins to build up, it feeds back to the first enzyme and reacts at the allosteric site. The shape of enzyme cannot be changed so that the substrate does not fit into the active site, therefore no more reactions can take place.

2.1.1 Enzyme activity (Deutscher, 1990)

Standardized unit and terms have been developed for the reporting of enzyme activity. In describing activity three items should be included: the amount of substrate converted or product formed in some standard unit, usually the micromole; the time of the reaction, usually minutes; and the amount of enzyme. Thus, enzyme activity unit is often quoted as μmol/min. The value should be stated as amount of enzyme, which catalyzes the transformation of one mass unit of substrate per minute under defined condition of pH and temperature. The amount of activity per unit of enzyme would be given as units/mg and is called the specific activity.

2.1.1.1 Effect of temperature on enzymatic activity

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases when the temperature is raised. 10^{o}C rises in temperature can increase the activity of most enzymes by 50% to 100%. Variations in reaction temperature as small as 1^{o}C or 2^{o}C may introduce changes of 10% to 20% in the results. In the case

of enzymatic reactions, many enzymes are denatured by heat at high temperature. As shown in Figure 2.1, the reaction rate of enzyme increases with temperature to a maximum level, and then suddenly decreases with increase of temperature. It is because most enzymes become denatured at above 40^{o}C, most enzyme determinations are carried out below that temperature.

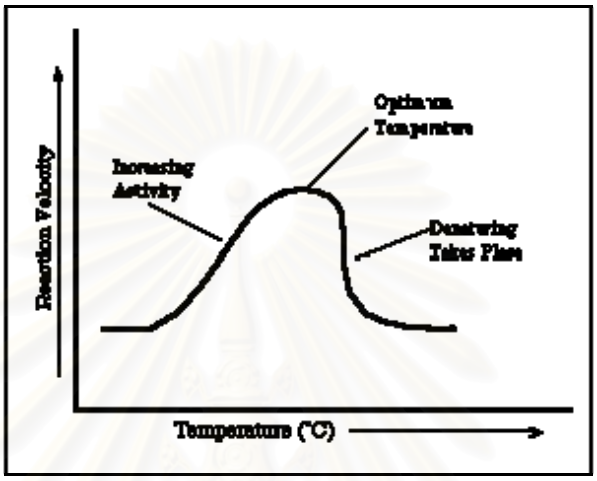

Figure 2.1 The effect of temperature on reaction rate of enzyme
(http://www.worthington-biochem.com/introBiochem/images/ie21.gif)

2.1.1.2 Effect of pH on enzymatic activity

Enzymes are affected by changes in pH. The optimum pH, where the enzyme is most active, is illustrated in Figure 2.2. At extremely high or low pH values, activity of most enzymes is lost. In general, the suitable pH is also a factor in the stability of enzymes. As with activity, there is also a region of pH optimal stability for each enzyme.

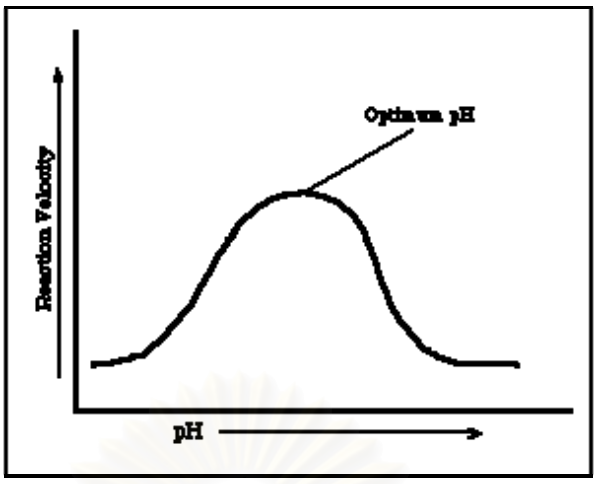

Figure 2.2 The effect of pH on reaction rate of enzyme
(http://www.worthington-biochem.com/introBiochem/images/ie22.gif)

2.1.1.3 Effect of inhibitor on enzymatic activity

Enzyme inhibitors are substances, which alter the catalytic action of the enzyme and consequently slow down, or in some cases, stop catalysis. There are three common types of enzyme: inhibition-competitive, non-competitive and substrate inhibition.

Competitive inhibition is occurred when the substrate and a substance resembling the substrate are both added to the enzyme. A theory called the ‘lock and key theory’ of enzyme catalysts can be used to explain why competitive inhibition is occurred. The lock and key theory uses the concept of an active site. The concept holds that one particular portion of the enzyme surface has a strong affinity for the substrate. The substrate is held and converted to the reaction products. The enzyme and the substrate are considered as the lock and the key, respectively. The key is inserted in the lock and it is turned. The door is opened and the reaction proceeds. However, when an inhibitor that resembles the substrate is presented, it will compete with the substrate for the position in the enzyme lock. When the inhibitor wins, it gains the lock position but is unable to open the lock. Hence, the reaction is slowed down because the inhibitor occupies some of the available enzyme sites.

Non-competitive inhibitors are considered to be substances that are added to the enzyme and alter the enzyme in a way that it cannot accept the substrate.

Substrate inhibition will sometimes occur when excessive amounts of substrate are presented. Figure 2.3 shows the reaction velocity decreasing after the maximum velocity has been reached. Additional amounts of substrate added to reaction mixture after this point actually decreases the reaction rate. There are so many substrate molecules competing for the active sites on the enzyme surfaces that they block the sites and prevent any substrate molecules from occupying them. This causes the reaction rate to drop.

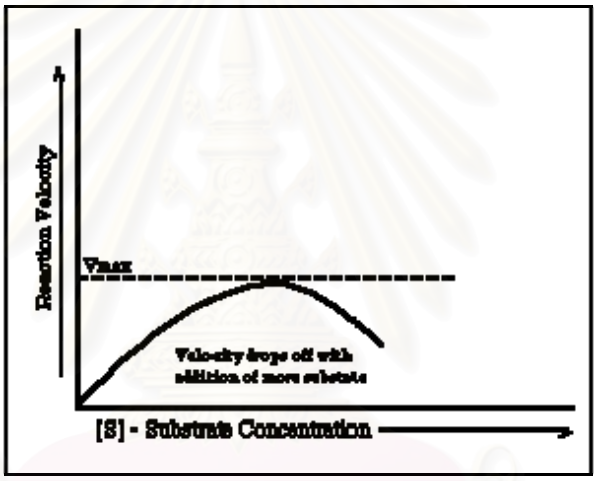

Figure 2.3 Reaction rate of enzyme when substrate become rate-inhibiting (http://www.worthington-biochem.com/introBiochem/images/ie19.gif)

2.1.2 Denaturation of enzymes

For an enzyme to maintain activity, it has to maintain its native structure. Activity may be lost by a change in the three-dimensional structure (denaturation) or by the cleavage of peptide bonds (degradation). Cleavage of peptide bonds is brought by attack of proteinases, although under the extreme of pH and temperature the enzyme may be completely degraded.

Enzymes are stabilized by hydrogen bonds, hydrophobic interactions, internal salt bridges, and disulphide bonds in some cases. A change in the magnitude of any of

these could cause denaturation. Denaturation can be brought about by numerous factors, including heat, extremes of pH, organic solvents, inorganic and organic solutes, detergents and radiation.

Heat

Enzymes are denatured by an increase in temperature, and denaturation is completed over a narrow range of temperature. The temperature at which denaturation occurs varies for different enzymes and depends on their structure. Most enzymes from mesophilic organisms are inactivated at temperature above 40^{o}C. Thermophillic organisms which grow at temperature greater than 55^{o}C, and sometimes as high as 90^{o}C, have enzymes that show greater heat stability. From some studies of heat stability of enzyme, they emphasize the importance of hydrophobic interaction and salt bridge in stabilizing enzyme against heat denaturation.

Change in pH

Enzymes are denatured by an extreme of pH. This is generally as a result of protonation and deprotonation of certain amino acid residues, which may cause the disruption of salt bridges and hydrogen bonds. In addition, placing a charge on an internal residue, such as tyrosine, may increase its polarity and disrupt hydrophobic interaction.

Organic solvent

The effect of organic solvents such as ethanol, acetone, chloroform or phenol on enzymes is generally to promote or cause denaturation. These solvents interact with non-polar groups on the enzyme, which are presented in the interior. The enzyme becomes unfolded and denatured. Although most monohydric alcohols at concentrations greater than 10% tend to denature enzymes, a number of polyhydric alcohols, such as glycerol and ethylene glycol, tend to have a stabilizing influence. Enzymes can often be stored below 0^{o}C after adding of glycerol without loss of enzymatic activity.

Solutes

Two organic solutes which have been used in protein denaturation studies are urea and guanidinium chloride. Both urea and guanidinium chloride increase the solubility of non-polar groups, such as certain amino acid side chine, in water. This can decrease the strength of hydrophobic interactions by one third. Anions have an effect on denaturation which is related to their ability to disrupt the structure of water. Their ability to do this is in decreasing order, SCN^- > ClO_4^- > I^- > Cl^- > citrate > acetate > SO_4^{2-}. It is interesting to note that guanidinium thiocyanate is a more powerful denaturant than guanidinium chloride and that guanidinium sulphate shows negligible denaturing properties. These differences are due to the combined effect of the guanidinium cation and the other anions. Although most proteins are denatured by 8M urea or 6M guanidinium chloride, some proteins which are stabilized by disulphide bridges or divalent metal ions require additional agents for denaturation.

Detergents

Detergents show strong denaturing properties and they are effective at much lower concentrations than urea or guanidinium salts. For example, SDS is able to induce complete denaturation at concentration of less than 10 mM, whereas urea or guanidinium are higher. Enzymes become denatured due to the strong binding of the detergent to the enzyme.

2.1.3 Proteolytic enzymes (Walsh, 2002)

The International Union of Biochemistry and Molecular Biology (IUB) has developed a nomenclature for enzymes, the EC numbers; each enzyme is described by a sequence of four numbers, preceded by ‘Enzyme Number’ (EC no.). The first number broadly classifies the enzyme based on its mechanism. Enzymes can be classified into six groups, as showed in Table 2.1.

Table 2.1 Major groups of enzyme

Group	Reaction catalyzed
EC 1 Oxidoreductases	To catalyze oxidation/reduction reactions
EC 2 Transferases	To transfer of a function group from one substrate to another
EC 3 Hydrolases	To form two products from a substrate by hydrolysis
EC 4 Lyases	Non-hydrolytic addition or removal of group from substrates
EC 5 Isomerases	Intermolecule rearrangment changes within a single molecule
EC 6 Ligases	Join together two molecules by synthesis of new C-O, C-S or C-C bonds with simultaneous breakdown of ATP

Hydrolases group (EC 3) is a group of enzymes which catalyze hydrolysis reaction. There are many subclasses of enzyme in hydrolases group such as: esterases, glycosylases, etherase, and peptidase.

The proteolytic enzymes or proteases or peptidases are enzymes which hydrolyze peptide bonds of protein. They are the most important group of industrial enzymes currently in use. They are usually used in the food and detergent industries and more recently have been employed in leather processing.

Classification of proteases

The International Union of Biochemistry and Molecular Biology (1984) has recommended to use the term peptidase (subclass EC 3.4) for the subset of peptide bond hydrolases. Peptidases are divided into exopeptidases that act only near a terminus of a polypeptide chain and endopeptidases that act internally in polypeptide chains. The usage of peptidase is recommended with protease as it was originally used as a general term for both exopeptidases and endopeptidases. It should be noted that previously, in Enzyme Nomenclature (1984), peptidases were restricted to the exopeptidases. The term proteases were used for the endopeptidases.

The proteases are divided into sub-subclasses on the basis of catalytic mechanism, and specificity is used only to identify individual enzymes within the amino groups. Sub-subclasses of protease are serine proteases, cysteine proteases, aspartic proteases, and metallo proteases.

Serine proteases (EC 3.4.21) are characterized by the presence of three essential amino acids: histidine (His-57), aspartic acid (Asp-102), and especially serine (Ser-195) at the active site (Figure 2.4). This class comprises two distinct families. The chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and the substilisin family which includes the bacterial enzymes such as subtilisin. Most serine proteases have a molecular mass between 18 and 35 kDa and the maximum activity is occurred between pH 7 and 11. The first step in the catalysis is the formation of an acyl-enzyme intermediate between the substrate and the essential serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the ser-hydroxyl of the enzyme. The deacylation which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the serine residue. The histidine residue provides a general base and accept the OH group of the reactive Ser. From an applied perspective, the bacterial subtilisins are the subgroup of serine proteases of greatest industrial significance. Subtilisins are particularly produced by selected *bacilli* that found widespread and uses as detergent additives.

Cysteine proteases (EC 3.4.22) include the plant proteases such as papain, actinidin or bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases. They are characterized by the presence of cysteine and a histidine residue at the active site, which forms a catalytic dyad essential for biological activity. Like the serine proteinases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential cysteine (Cys-25) and His-159 (papain numbering) play the same role as Ser-195 and His-57 respectively. The

nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighbouring imidazolium group of His-159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps and then a water molecule is not required. In industrial application, they are used mainly in food industry.

Aspartic proteases (EC 3.4.23) are a group of acidic proteases that contain an aspartic acid residue at the catalytic site which is presented in Figure 2.4. Most of aspartic proteases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin chymosin rennin (animal stomach-derived aspartic proteases), and Microbial aspartic proteases which produced mainly by *Aspergillius*, *Penicillium*, *Rhizopus* and *Mucor* spp. A second family comprises viral proteinases such as the protease from the AIDS virus (HIV) also called retropepsin. Aspartic proteases show maximum activity at pH between 3 and 4, and have isoelectric points between 3 and 4.5. The generally molecular masses are around 30-45 kDa. In contrast to serine and cysteine proteases, catalysis by aspartic proteinases do not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate. The best-known application of aspartic proteases is in cheese manufacture.

Metallo proteases (EC 3.4.24) are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. The catalytic mechanism leads to the formation of a non covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group. Most metalloproteases show the maximum activity at neutral to alkaline pH. The best known of this group is microbial thermolysin, a very heat-stable neutral protease.

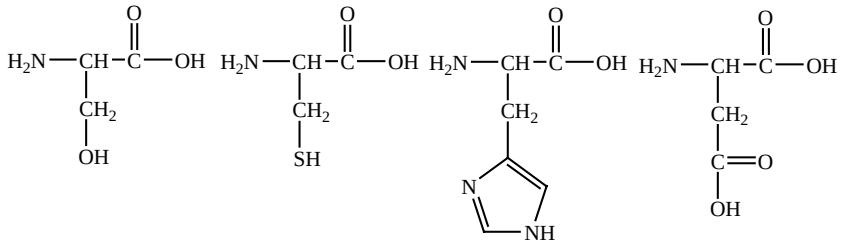

Figure 2.4 Structure of amino acids essential for the catalytic activity of proteases (Walsh, 2002).

2.2 Papaya (*Carica papaya*)

Carica papaya is widely cultivated in tropical and subtropical regions all around the world, including Thailand, for its edible fruits and for enzyme stored in its laticifers. Laticifers are displayed in all aerial part of the tree forming a dense network of articulated and anastomosing structures. Damaging the papaya tree inevitably servers its laticifers, eliciting an abrupt release of latex. Papaya latex is a thixotrotic fluid with a milky appearance that contains about 15% of dry matter. Forty percentage of this dry matter is constituted by enzymes, mainly cysteine endopeptidases since they are more than 80% of the whole enzyme fraction. The papaya endopeptides thus constitute a potential danger for the plant. They are stored in the laticifers as inactive forms which rapidly convert to active enzyme after the release of latex from plant. Because it contains several cysteine endopeptidases that has found many applications in the food (meat tenderizer and beer chill-proofing) and pharmaceutical industries. Cysteine endopeptidases that contain in papaya latex such as papain (EC 3.4.22.2), chymopapain A and B (EC 3.4.22.6), and papaya peptidase A (EC 3.4.22.30).

In the past, the number of proteinases presented in papaya latex are not clearly known, resulting in the use of dried instead of fresh latex as a source of enzyme.

2.2.1 Papain (EC 3.4.22.2)

Papain is an endolytic cysteine protease. It is a single polypeptide chain containing 212 amino acid residues, with a molecular weight of 23 kDa. It has three disulphide bonds which help structural conformation, and one free functional sulfhydryl group (-SH) of cysteine residue at active site. The amino acid sequence of papain is presented in Figure 2.5. The mechanism of protein hydrolysis is occurred with Cys-25 (cysteine position 25) and His-159 (histidine position 159) in the enzyme chain. The optimum working conditions are at 65-80^{o}C and pH 6-7. In native state of papain, it exhibits low enzyme activity as its free sulfhydryl group is blocked. Papain is activated by reducing agents such as cysteine, sulfide, sulfite, HCN and EDTA, and is inactivated by heavy metals ion and H_2O_2.

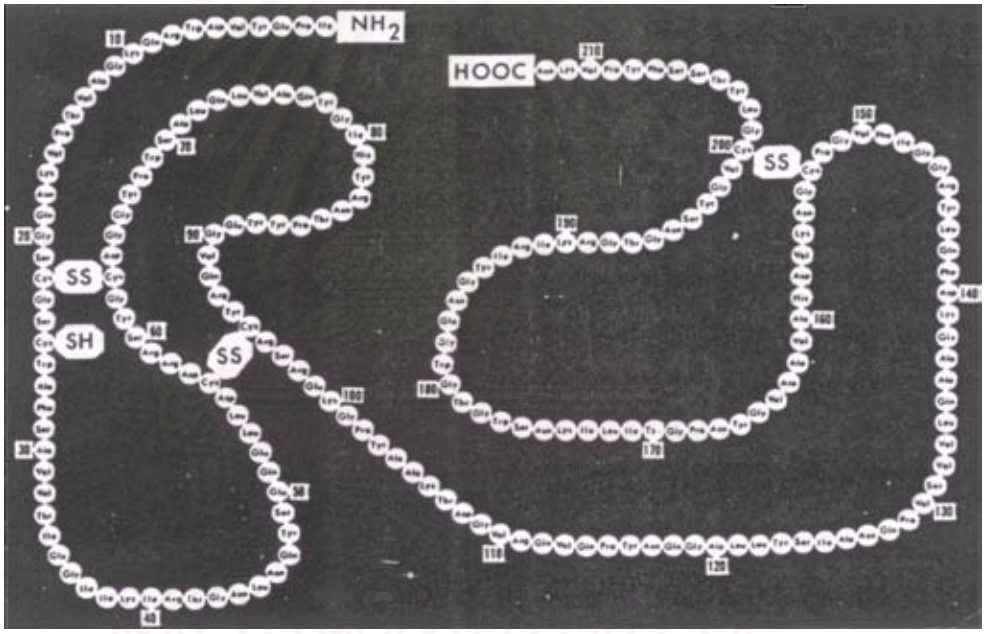

Figure 2.5 Amino acid sequence of papain (Arnon, 1970)

2.2.2 Chymopapain (EC 3.4.22.6)

Chymopapain is the major endolytic cysteine protease of papaya latex but its enzyme activity is a half of papain activity. The molecular weight of chymopapain is about 34 to 36 kDa. Chymopapain is classified as chymopapain A and B, of which the properties are shown in Table 2.2. Properties of chymopapain are similar to papain such as optimum pH and temperature, activators, and inhibitors. It is stable at pH 2 and 12. Stability of pH and temperature solubility in water of chymopapain is better than papain.

2.2.3 Papaya peptidase A (EC 3.4.22.30)

Papaya peptidase A or caricain has a molecular weight of 24 kDa. Its protein composition is the same as chymopapain but less complex. Papaya peptidase A is stable at pH 2 and may be called 'chymopapain II'.

Table 2.2. Properties of papain, chymopapain, and papaya peptidase A (Arnon, 1970 and Lynn, 1979)

Properties	Papain	Chymopapain		Papaya peptidase A
		Chymopapain A	Chymopapain B	
pI	8.75	10.1	10.4	>11
Molecular weight	20,700-24,000	36,400±1,500	34,500±1,500	24,000
Numbers of sulfhydryl group in molecule	1 SH	2 SH	2 SH	1 SH
Shape of precipitate	N.A.	Rods	Needles	N.A.
Other names	-Papaya peptidase I	-Chymopapain S	-Chymopapain B_1-B_3	-Caricain -Papaya peptidase II

2.3 Animal skin (Mclaughlin *et al*., 1945)

Animal skin is made up of a number of distinct tissues and contains a number of distinct organs. The tissues may be divided into the following classes: epithelial, connective, muscular, nervous, glandular, fatty, and the blood tissues. The organs include voluntary and involuntary muscles, fat glands, sweat glands, nerves and blood vessels. The solid matter of the skin is about 90-95% of proteins and in fresh skin the protein content is about 35% of the total weight.

The physiologic functions of the skin are very important. One of the main functions of skin is to keep the temperature of the body constant. The body temperature is controlled by sweat glands (permitting loss of heat) and fat glands (retaining body heat). The skin is one of the principal excretory organs of the body and protects it against bacterial invasion and mechanical damage. Another function of skin is the color filters that protect underlying tissues from ultraviolet rays from sunlight.

Animal skin can be divided into two layers: derma or corium, and epidermis. Flesh attaches to skin with varying quantities of adipose tissue, yellow connective tissue, blood vessels, nerves, and voluntary muscle. During preparation for tanning process, flesh must be removed from skin. The derma, corium, or true skin can be divided into upper and lower portion. The upper portion is composed of glands, muscles, and hair follicles. Since this layer is composed of those organs that concern with the management of body temperature, it is termed ‘thermostat’ layer. The lower portion of the derma is usually termed the ‘reticular’ layer, since the interlacing collagen fiber of the lower portion presents a net-like appearance. The main protein constituent of both dermal layers is collagen, which is arranged in interlacing bundles of fibers or fibrils. Epidermis is the same as derma but the principal composition of epidermis is keratin.

Raw hide is the treated skin of large animal, where flesh is removed. The composition of raw hide is showed in Table 2.3.

Table 2.3 The composition of raw hide (Sharphouse, 1980)

Water	Protein	Fats	Mineral salts	Other substances
64%	33%	2%	0.5%	0.5%

Structural protein			Non-structural protein	
Collagen	Keratin	Elastin	Abumins globulins	Mucins mucoids, etc.
29%	2%	0.3%	1%	0.7%

2.4 Collagen (Friess, 1998)

Collagen is a major structure of protein in skin, tendon, bone, cornea, dentin, horn, hoof, and white connective tissues in animals. Bones and teeth are collagen that is composed of mineral crystals. Collagen provides structure to our bodies, protecting and supporting the softer tissues and connecting them with the skeleton. An important characteristic of collagen is its very large swelling capacity in aqueous acid and alkaline systems in the absence of high salt concentration. The action of hot water converts collagen to gelatin. The name collagen is derived from Greek, and meant ‘glue former’.

The primary structure of collagen is the polypeptide α-chain that composed of more than 1000 amino acids such as glycine, proline, hydroxyproline and hydroxylysine. The amino acids are arranged in a helix forming sequence. Glycine (Gly) has the smallest side group and its repetition at every third position on the sequence allows close package of the chain into a helix, which leaves little space for residues in core. About 35% of non-glycine positions in the repeating unit Gly-X-Y are occupied by proline in the X-position, and proline or hydroxyproline in Y-position (see Figure 2.6). There are only minor differences between the collagen from difference species. The polypeptide α-chains formed left-handed helices with 3.3 residues per turn and a pitch of 8.7 Å. This is classified as the secondary structure of collagen.

The tertiary structure of collagen refers to the fundamental unit originally know as tropocollagen: three polypeptide chains intertwined to form a right-handed triple-helix with pitch of approximately 86 Å. The rod-shaped triple-helix has an average molecular weight of approximately 300 kDa, a length of 3,000 Å with a diameter of 15 Å (see Figure 2.7). The different chains are bonded to each other by H-bridges described NH from the glycine and CO from residues of the other chains. These H-O bonds are perpendicular on the axis of the collagen chains. The other bonds are H-bridges of hydroxyl-groups of hydroxyprolines and H-bridges with water molecules. All these bonds stabilize the triple-helical structure of collagen.

On the fourth level of structure, the triple-helical molecules stagger longitudinally and bilaterally into fibrils with distinct periodicity, a length of 400 Å. The collagen molecules aggregate through fibrillogenesis into microfibrils consisting of four to eight collagen molecules and further into fibrils. These collagen fibrils organize into fiber, which on their part can form even larger fiber bundle (see Figure 2.7). The systematic packing of the triple-helices lends strength and resilience to the collagen fibers. Additional mechanical and chemical stability derives from intra- and intermolecular crosslinks. Intramolecular crosslinks form between two α-chains in non-helical section of the same molecule by aldol condensation of two aldehydes. Intermolecular crosslinks is occured between the telopeptide region of one collagen molecule and the helical region of a adjacent molecule by aldimine formation between aldehyde residues and amino groups.

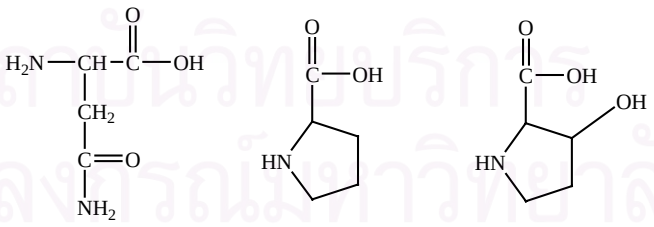

Figure 2.6 The regular amino acids in collagen

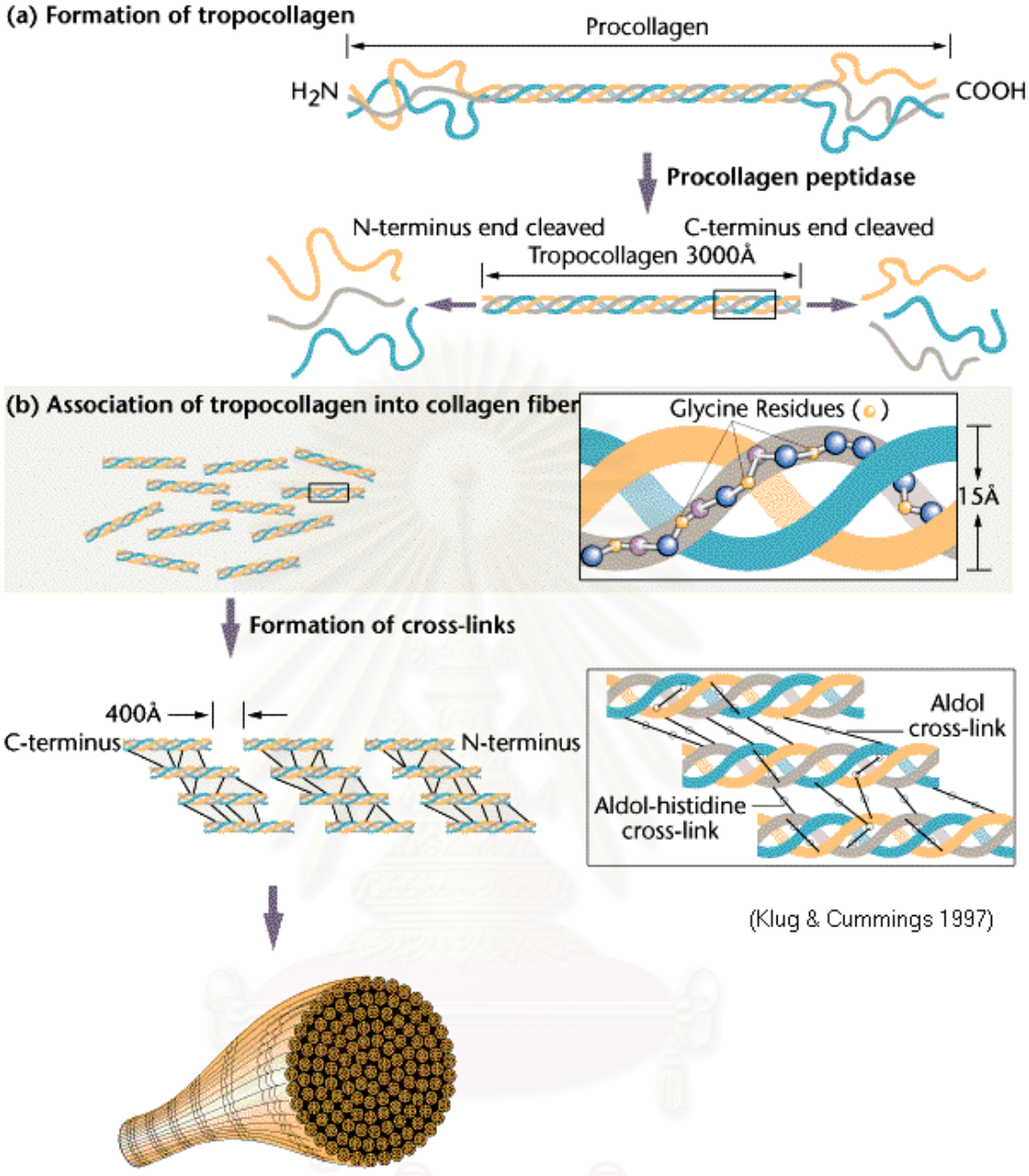

Figure 2.7 Model of triple helix structure of collagen.
(www.mun.ca/biology/scarr/Collagen_structure.gif)

2.5 Gelatin (Kroschwitz *et al*., 1992)

Gelatin is a protein obtained by partial hydrolysis of collagen. A typical chemical formulation of gelatin is presented in Figure 2.8. Type A gelatin is produced by acid processing of collagen containing materials and type B gelatin by alkaline or lime processing. Because gelatin is obtained from collagen by a controlled partial hydrolysis and does not exist in nature, gelatin is classified as a derived protein. Animal glue and gelatin hydrolysate (referred to liquid protein) are products obtained by a more complete hydrolysis of collagen. They contain lower molecular-weight fractions of gelatin.

The type of gelatin application are based on the properties of gelatin such as reversible gel-to-sol transition of aqueous solution, viscosity of warm aqueous solutions, ability to act as a protective colloid, water permeability and insolubility in cold water, but complete solubility in hot water. These properties are utilized in the food, pharmaceutical, and photographic industries.

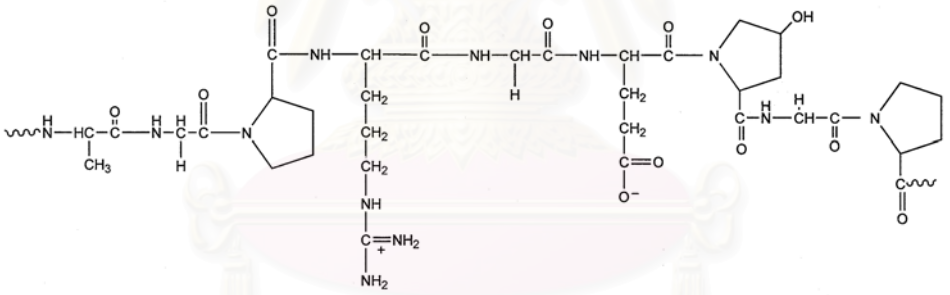

Figure 2.8 A typical chemical formulation of gelatin. (www.sbu.ac.uk)

2.5.1 Chemical composition and structure

Gelatin is not a single chemical substance. The main constituents of gelatin are large and complex polypeptide molecules of the same amino acid composition as the parent collagen, converting a broad molecular weight distribution range. In different grades of gelatin, the molecular weight can be varied from 20,000 to 250,000.

The analysis shows the amino acids of gelatin from 0.2% tyrosine to 30.5% glycine. The five most common amino acids are glycine 26.4-30.5%, praline 14.8-18%, hydroxyproline 13.3-14.5%, glutamic acid 11.1-11.7%, and alanine 8.6-11.3%. The remaining amino acids in decreasing order are arginine, aspartic acid, lysine, serine, leucine, valine, phenylanine, threonine, isoleucine, hydroxylysine, histidine, methionine, and tyrosine.

The α-chain form in gelatin solution behaves like a random-coil polymer, whereas the gel form may contain as much as 70% helical conformation. The remaining molecules in nonhelical conformation link helical regions together to form the gel matrix. Helical regions are thought to contain both inter- and intramolecular associations of chain segments. From electron microscope, the structure of the gelatin gel is a combination of fine and coarse interchain networks. The ratio of fine and coarse interchain networks depends on the temperature during the polymer-polymer and polymer-solvent interaction leading to bond formation. The gel strength of gelatin is approximately proportional to the square of the gelatin concentration.

Dried gelatin can be stored in airtight containers at room temperature for many years. It decomposes above 100^{o}C for complete combustion can be occurred at the temperatures above 500^{o}C. When dry gelatin is heated in air at relatively high humidity, >60% rh, and at moderate temperatures, above 45^{o}C, it gradually lose its ability to swell and dissolve. Aqueous solutions or gels of gelatin are highly susceptible to microbial growth and breakdown by proteolytic enzymes. Stability is a function of pH and electrolytes and generally stability decreases with increasing temperature because of hydrolysis.

2.5.2 Manufacturing process

In the past, bone and ossein have been supplied by India and South America. In the 1990s, slaughterhouses and meat-packing houses are an important source of bones. The supply of bone has been greatly increased since the meat-packing industry introduced packaged and fabricated meats because the growth of fast-food restaurants. Dried and rendered bones yield about 14-18% gelatin, whereas pork skins yield about 18-22%. The typical gelatin manufacturing process can be divided into two methods: acid process (for type A gelatin) and liming process (for type B gelatin).

Type A gelatin is made from pork skin, having grease as a marketable by-product. The process includes macerating of skin, washing to remove extraneous matter, and swelling in 1-5% hydrochloric, phosphoric, or sulfuric acid for 10-30 hours. Then four to five extractions are made at temperatures increasing from 55-65^{o}C for the first extraction to 95-100^{o}C for the last extraction. Each extraction is about 4-8 hours. Grease is then removed, the gelatin solution filtered and deionized. The solution is concentrated to 20-40% solids by continuous vacuum evaporation. The viscous solution is chilled before extruded into noodles and then dried at 30-60^{o}C. In drying process the noodles is passing through the zones of successive temperature changes while conditioned air blows across the surface and through the noodle. The dried gelatin is ground and blended to specification.

Type B gelatin is usually made from bone, but also from bovine hide and pork skin. The bone is crush and degreased. Rendered bone pieces (0.5-4 cm) with less than 3% fat, are treated with cool, 4-7% hydrochloric acid from 4 to 14 days for removal mineral. The demineralized bone or ossein are washed and transferred to large tanks where they are stored in lime slurry with gentle daily agitation for 3-16 weeks. During the liming process, some deamination of collagen occurs with evolution of ammonia. This primary process results in low isoelectric ranges for type B gelatin. After washing for 15-30 hours for removal the lime, the ossein is acidified to pH 5-7 with an appropriate acid. Then the extraction processing for type B gelatin is followed. Throughout the manufacturing process, cleanliness is important to avoid contamination by bacteria or proteolytic enzymes.

2.5.3 Physical and chemical properties

Gelatin is a vitreous and brittle solid, faintly yellow in color. Commercial gelatin is produced in mesh sizes ranging from coarse granules to fine powder. In Europe, gelatin is also produced in thin sheets for uses in cooking. Dried commercial gelatin contains about 9-13% moisture and is essentially tasteless and odorless with specific gravity between 1.3 and 1.4. Most physical and chemical properties of gelatin are measured on aqueous solutions and depended on the source of collagen, method of manufacture, conditions during extraction and concentration, thermal history, pH and chemical nature of impurities or additives. Those properties are as followes.

Gelation

The most useful property of gelatin solution is the capability to form heat reversible gel-sols. When an aqueous solution of gelatin with a concentration greater than about 0.5% is cooled to about 35-40^{o}C, at first the viscosity increases and then a gel is formed. The gelation process proceeds through three stages: 1. rearrangement of individual molecular chains into order, helical arrangement, or collagen fold, 2. association of two or three ordered segments to create crystallites and 3. stabilization of the structure by lateral interchain hydrogen bonding within the helical regions. The rigidity or jelly strength of the gel depends on the concentration, the intrinsic strength of the gelatin sample, pH, temperature and additives.

Because the economic value of gelatin is commonly determined by jelly or gel strength, the test procedure for its determination is important. Commercially, gel strength of gelatin is determined by standard tests which measure the force required to depress the surface of a prepared gel by distance of 4 mm using a flat-bottomed plunger (12.7 mm in diameter). The force applied may be measured in the form of the quantity of fine lead shot required to depress the plunger and is recorded in grams. The measurement is termed the Bloom strength. The conversion temperature for gelatin is determined as a setting point, sol to gel, or melting point. Commercial gelatins melt between 23 and 30^{o}C, with the setting point being lower by 2-5^{o}C.

Solubility

In most commercial applications, gelatin is used as a solution. Gelatin is soluble in water and in aqueous solutions of polyhydric alcohols such as glycerol and propylene glycol. Gelatin can also dissolve in highly polar, hydrogen-bonding organic solvents such as acetic acid, and formamide. Gelatin is practically insoluble in less polar organic solvents such as acetone, ethanol, ether, benzene, and most other nonpolar organic solvents. Many water soluble organic solvents are compatible with gelatin, but interfere with gelling properties. Dried gelatin absorbs water exothermally. The rate and degree of swelling is a characteristic of the particular gelatin. Swelled gelatin granules dissolve rapidly in water above 35^{o}C. The cross-linking of gelatin matrix by chemical method is used extensively in photographic products and resulting in permanent hardening which reduces the solubility of gelatin.

Amphoteric character

The amphoteric character of gelatin is due to the functional groups of the amino acids and the terminal amino and carboxyl groups created during hydrolysis. In strongly acidic solution the gelatin is positively charged and migrates as a cation in an electric field. In strongly alkaline solution, it is negatively charged and migrates as an anion. The intermediate point, where net charge is zero and no migration occurs, is known as the isoelectric point (IEP, pI) and is designated in pH units. A related property, the isoionic point, can be determined by utilizing a mixed-bed ion exchange resin to remove all nongelatin cations and anions. The resulting pH of the gelatin solution is the isoionic point and expressed in pH units. The isoionic point is reproducible, whereas the isoelectric point depends on the salts presented. The isoionic point of Type A gelatin is varied in pH range from 7-10, and type B gelatin pH range from 4.8-5.2 depend on liming condition. The isoionic point can also be estimated by determining a pH value at which a gelatin solution exhibits maximum turbidity. Many isoionic point references are recorded as isoelectric points even though the latter is defined as a pH at which gelatin has net charge of zero and thus shows no movement in the electric field.

Viscosity

The viscosity of gelatin solutions is depended on gelatin concentration, temperature, molecular weight of the gelatin, pH, additives and impurities. In aqueous solution and temperature above 40^{o}C, gelatin exhibits Newtonian behavior. At temperatures between 30-40^{o}C, non-Newtonian behavior is observed, probably due to linking together of gelatin molecules to form aggregates. The viscosity of gelatin solution is determined by using a capillary viscometer and concentration of gelatin solution is at 6.67 or 12.5% solids (60^{o}C). The viscosity of gelatin solutions increases with increasing gelatin concentration or decreasing temperature. The viscosity of gelatin solution is reached the minimum value at the isoionic point.

Colloid and emulsifying properties

Gelatin is an effectively protective colloid that can prevent crystal, or particle, aggregation, thereby stabilizing a heterogeneous suspension. The protective colloid property is important in photographic applications where it stabilizes and protects silver halide crystals while still allowing for their normal growth and sensitization during physical and chemical ripening processes. It acts as an emulsifying agent in cosmetics and pharmaceuticals involving oil-in-water dispersions. The anionic or cationic behavior of gelatin is important when used in conjunction with other ionic materials.

Coacervation

A phenomenon associated with colloids wherein dispersed particles separate from solution to form a second liquid phase is termed coacervation. Gelatin solutions form coacervates with the addition of salt such as sodium sulfate, especially at pH below the isoionic point. In addition, gelatin solutions coacervate with solutions of oppositely charged polymers or macromolecules such as acacia. This property is useful for microencapsulation and photographic applications.

Swelling

The swelling property of gelatin is important in photographic film processing and the dissolution of pharmaceutical capsules. That pH and electrolyte content affected swelling has been explained by the simple Donnan equilibrium theory. This explains why gelatin exhibits the lowest swelling at its isoelectric point (pI). At pH below the isoelectric point, anions can control swelling, whereas above the isoelectric point, cations primarily affect swelling. These effects probably involve breaking hydrogen bonds, resulting in increased swelling. In photographic products, the swelling of the gelatin layer is controlled by coating conditions, drying conditions, chemical cross-linking and the composition of the processing solutions. Conditioning at 90% rh and 20^{o}C for 24 hours greatly reduces swelling of hot dried film coating. The ratio of lateral to vertical swelling is important in the photographic industry since it can cause curling of photographic papers or films when changes in the humidity or moisture content take place.

2.5.4 Applications of gelatin

Food products

In food industry, gelatin formulations are made with water or aqueous polyhydric alcohols as a solvent for candy, marshmallow or dessert preparations. In dairy products and frozen foods, the protective colloid property of gelatin prevents crystallization of ice and sugar. Gelatin products having a wide range of Bloom and viscosity values are utilized in the manufacture of food products, specific properties being selected depending on the needs of the application. The use of gelatin in ice cream has greatly diminished. In sour cream and cottage cheese, gelatin inhibits water separation. In meat products, such as canned hams, various luncheon meats, jellied beef and other similar products, gelatin in 1-5% concentration helps to retain the natural juices and enhance texture and flavor. Use of gelatin to form soft, chewy candies, so-called gummy candies, has increased worldwide gelatin demand. The largest use of edible gelatin in the United States is in the preparation of gelatin desserts. For this use, gelatin is sold either premixed with sugar and flavoring or as unflavored gelatin pockets. Most edible gelatin is type A, but type B is also used.

Pharmaceutical products

Gelatin is used in pharmaceutical industry for the manufacture of soft and hard capsules. Elastic or soft capsules are made with a rotary die from two plasticized gelatin sheets which form a sealed capsule around the material being encapsulated. The gelatin for soft capsules is low bloom type A 170-180 g, type B 150-175 g or a mixture of type A and B. Hard capsules consisting of two parts are first formed and then filled. Medium-to-high bloom type A 250-280 g, type B 225-250 g or the combination of type A and B gelatin are used for hard capsules. Usage of gelatin as coating for tablets has increased dramatically. In a process similar to formation of gelatin capsules, tablets are coated by dipping in colored gelatin solutions, thereby giving the appearance and appeal of capsule, but with some protection from adulteration of the medication.

Photographic products

Gelatin has been used for over 100 years as a binder in light-sensitive products. The useful functions of gelatin in photographic film manufacture are a protective colloidal properties during the precipitation and chemical ripening of silver halide crystals, setting and film-forming properties during coating, and swelling properties during processing of exposed film or paper. Photographic gelatins are manufactured to standard specifications since the testing is time-consuming and costly. Photographic products may have up to twenty gelatin layers grouped into three categories: 1. light-sensitive silver halide-bearing layers (2-10 μm thickness referred to as emulsion layers) 2. surface, spacer, filter or protective layers (1-2 μm thickness) and 3. backing, antihalo or anticurl layers coated on the opposite side of the film substrate from the emulsion layer.

Since the early 1970s, the photographic industry has switched from so-called active gelatins derived from hides to inert type derived from bones. The latter are very low or void of nature restrainers, reduction and sulfur sensitizers. Other changes in techniques have been brought about by abandoning the lengthy noodle wash technique. It is used to remove salts after silver halide precipitation in favor of precipitating, coagulating or derivatizing gelatin. The precipitate is washed by

ultrafiltration techniques. The new coating techniques allow simultaneous coating of several layers at one time at speeds 10 times as fast as before. The shot-time high temperature processing may require new cross-linking agents that are unlike the aldehydes and metal salts previously used. Many new hardeners are extremely fast-acting and are metered into the solution during the coating operation. It is quite common to use a derivatized gelatin, such as phthalated gelatin, to precipitate silver halide. Gelatin used in the auxiliary layers must be able to withstand high temperature processing and allow high speed coating.

Gelatin is also used in so-called subbing formulations to prepare film bases such as polyester, cellulose acetate, cellulose butyrate and polyethylene-coated paper base for coating by aqueous formulations. Solvents such as methanol, acetone or chlorinated solvents are used with small amounts of water. Gelatin containing low ash, low grease and having good solubility in mixed solvents is required for these applications. In certain lithographic printing, light-sensitive dichromated gelatin is used. Light causes permanent cross-linking of gelatin in the presence of the dichromate; this phenomenon is used to make relief images for printing. Dichromated gelatin coatings are commonly used in production of high quality holographic images. In this application the light sensitivity of the image-receiving medium is less important than the image-resolving power. Gelatin coating in photographic products are further tested for brittleness, scratch resistance, friction, swelling rate, drying rate, curling tendency, dry adhesion, wet adhesion and pressure sensitivity. These properties are becoming more critical with the development of more sophisticated cameras, printing and processing equipment. Photographic technology offers a rapidly changing, highly sophisticated, very competitive market for photographic gelatin manufacturers.

2.6 Protein purification (Murray, 1990)

There are many techniques of protein purifications and analysis, but the removal of low molecular weight solutes and concentration of protein solutions are the most frequently performed. The variety of applications and the multitude of techniques render the selection of the optimum method for each case. The techniques most often applied to purify and analyze protein include centrifugation, salt precipitation, dialysis, ultrafiltration, lyophilization, and electrophoresis.

Centrifugation

Centrifugation is one of many techniques for the two-phase separation (liquid-liquid or liquid-solid) by acceleration. Centrifugation of very large protein molecules, multienzyme complex, and subcellular organelles in preparative ultracentrifuges can be used for concentration and desalting of particles. Almost complete recovery of this method is feasible, and depending on the molecular weight of the particle and the viscosity of the solution. Desalting and concentration can be achieved in a short time.

Salt precipitation

The solubility of protein depends on the salt concentration in the solution. At low concentrations, salt stabilizes various charged groups on protein molecules, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as ‘salting-in’. However, as the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is less and less water available to solute protein. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This protein precipitation in the presence of excess salt is known as ‘salting-out’. The common salts for precipitation are ammonium sulfate and sodium chloride that are cheap and high soluble.

Dialysis

Dialysis is one of the oldest method technique for removal of low molecular weight solutes or exchange of buffer. This method is based on the properties of a semi permeable membrane separating the protein solution from the dialysis buffer. It allows passage of molecules below a certain molecular weight, molecular weight cutoff, while macromolecules cannot pass the membrane pores. The process of dialysis is driven by the difference in concentration of solutes between the two sides of membrane. As the equilibrium concentration is approached, the diffusion of solutes becomes equal in two directions. The diffusion of solutes is dependent on the temperature and viscosity of the solution. Although a higher temperature increases the rate of diffusion, in most cases the stability of protein requires dialysis to be conducted 4-8^{o}C. A protein solution with too high viscosity usually has to be diluted prior dialysis because the rate of diffusion is drastically reduced with increasing viscosity of solution. Dialysis can be accelerated by increasing the ratio of membrane area to volume of the solution. Furthermore, membrane may be contaminated with heavy metal. In some case, wetting of the membrane and washing with the intended buffer solution may be employed as a pretreatment.

Ultrafiltration

Dialysis and ultrafiltration techniques are based on separation of molecules according to size by employing a semipermeable membrane with a defined range of pore sizes. Process of ultrafiltration is driven by force, pressure (pressurized, inert gas), vacuum, or gravity to drive solutes through membrane. It is a very versatile technique with applications ranging from small-scale laboratory concentration and desalting experiments up to industrial ultrafiltration.The optimal membrane should be a rigid, stable structure able to withstand the working pressure of the technique. The membrane should have the narrowest, the sharp molecular weight cutoff possible to allow rapid concentration and desalting without loss of protein. Applications of ultrafiltration are not only for concentration and desalting but separation of proteins differing in size. The main problem of ultrafiltration is the concentration of protein in the vicinity of the membrane which may cause a decrease in flow rates.

Lyophilization

Lyophilization concentrates protein solution by drying of materials in the frozen state, while the solvent is removed by sublimation. This technique has high capacity and is very easy to perform. The system required for lyophilization consists of the sample manifold with a cooled condensation trap and a high-capacity vacuum pump. To enable rapid removal of the solvent and to prevent addition stress to the container due to expansion during freezing the container should be filled to only one-quarter of its volume. Additionally, a high surface area-to-volume ratio is helpful to quickly freeze the liquid. Rapid freezing is important for the stability of the protein to be lyophilized since slow freezing may result in freezing out of solution components, leading to concentration gradients and substantial shift of pH that can lead to denaturation of the proteins. Therefore, requirements for lyophilization may not be fulfilled at all time, and simple evaporation of the solvent may be denature many heat sensitive proteins.

Electrophoresis

Electrophoretic separation techniques are at least as widely distributed as chromatographic method. Most of the procedures are limited to rather small volume of protein solution. They are carried out in free solutions as in capillary and free system, or in stabilizing media such as thin-layer plates, film or gel. Substances, which are exclusively negatively or positively charged, are easy to run. Under the influence of an electrical field charged molecules and particles migrate in the direction of the electrode bearing the opposite charge. During this process, the substances are usually in aqueous solution. Because of varying charges, mass, and velocity of proteins, proteins are separated into each fraction. The electrophoresis mobility, which is a measure of the migration velocity, is a significant and characteristic parameter of a charged molecule or particle. It is dependent on the pK values of the charged groups and the size of the molecule or particle. It is influenced by the type, concentration and pH of the buffer, the temperature and the field strength as well as by the nature of the supported material.

2.7 Protein determination (Sikorski, 2001)

There are many techniques for protein determination, such as gravimetric, refractometric, turbidimetric, spectrophotometric, colorimetric, but only a few can be used in food analyses. This is because the limitation of technique depends not only on the protein content in a product, but also on contents of lipids, saccharides, and other accompanying substances which may interfere with the target substance during testing. The techniques most often applied to determine protein content in food products include Kjedahl, Lowry method and Bradford method.

2.7.1 Kjeldahl method

Kjeldahl method is frequently applied to determine the protein content, by using nitrogen-to-protein conversion factor from the nitrogen content of sample. Sulfuric acid is used for sample digestion. Digestion is complete when mixtue turn clear (light green color), usually after 20 to 30 minutes of heating. Using a catalyst can facilitate sample digestion; the order of effectiveness for metal oxide catalysts is $Hg > Se > Te > Ti > Mo > Fe > Cu > V > W > Ag$. During the acid digestion, the sample is heated with concentrated sulfuric acid, which causes dehydration and charring. Above a sample decomposition temperature, carbon, sulfur, hydrogen, and nitrogen are converted to carbon dioxide, sulfur dioxide, water, and ammonium sulfate. Ammonium sulfate is neutralized with alkali to form ammonia and then distilled and trapped using 4% boric acid. Ammonium borate is titrated with standard acid in the present of a suitable indication. The Kjeldahl technique measures sample nitrogen as ammonia and the value of nitrogen is converted to crude protein content by multiplying by a Kjeldahl factor, present in Table 2.4.

Table 2.4 Conversion factors for calculation of protein.

Food	Factor
Egg and egg products	6.68
Milk and daily products	6.38
Meat, fish and shellfish	6.25
Maize	6.25
Rice	5.95
Soya bean	5.71
Flour	5.70
Gelatin	5.55
Collagen	5.37

2.7.2 Lowry method

Lowry method is based on the reaction between peptide bonds of protein and copper under alkaline conditions producing Cu^{+}, which reacts with the Folin reagent. The phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of the tyrosine, tryptophan, cysteine, histidine and peptide bonds. The reactions result in a strong blue color with maximum absorbance at 745-750 nm. The method is best used on solution with concentrations in the range 0.01-1.0 mg/ml of protein. The protein content can be calculated from the absorbance value by using Beer and Lambert's law. In Lowry method, calibration curve of standard protein is used to determine the concentration of protein solution.

2.7.3 Bradford method

Thy Bradford assay relies on blinding of dye Coomassie Blue G250 to protein. The dye appears to bind most readily to arginyl and lysyl residues of protein. This specificity can lead to variation in the response of the assay to different proteins. The free dye can exist in three different ionic forms for which the pK_a values are 1.15, 1.82, and 12.4. The Bradford assay is based on the equilibrium between three forms of dye. The more cationic red and green forms of the dye, which predominate in acidic assay reagent solution, have the maximum absorbance at 470 nm and 650 nm,

respectively. On the other hand, the more anionic blue form of the dye, which binds to protein, has a maximum absorbance at the wavelength of 590 nm. The protein quantity can be estimated by determining the amount of dye in the blue ionic form. In this method, the absorbance of the solution is measured at the wavelength of 595 nm. Obviously, this assay is less accurate for basic or acidic proteins.

CHAPTER III

LITERATURE REVIEW

In this chapter, the literature reviews are summarized into 2 parts as follows:

1. Extraction of proteolytic enzymes from papaya latex.
2. Enzymatic hydrolysis for gelatin production from various sources of collagen.

1. Extraction of proteolytic enzymes from papaya latex.

Burke *et al.* (1974) have studied the extraction of papain from papaya latex using sodium chloride, followed by affinity chromatography of the redissolved precipitate. Precipitation of protein from the latex extraction was necessary to separate the papain from other components that interfered the binding of papain to the affinity column. During affinity chromatography, the affinity column was overloaded to insure the absence of impurities, which were capable to bind to the affinity column. It has been estimated that about 5% of the soluble protein in the latex was papain. However, only about half of this 5% was active papain. After affinity chromatography, active papain was recovered about 2-3% of the soluble latex protein. It was 90% pure active papain. Molecular weight of papain obtained was detected by acrylamide gel electrophoresis. The electrophoresis result showed a single protein band (at 22 kDa) and a trailing shadow that was less than 5% of the main band. It was identified as papain.

Lynn *et al.* (1979) reported of the purification method of papain and chymopapain from papaya latex. Papaya latex was ground in activation buffer, and then centrifuged. The supernatant was applied to agarose-mercurial column. Enzymes were bound to agarose-mercurial. Enzyme was eluted with buffer containing 0.05 mM $HgCl_2$ and identified as papain and chymopapain. Their sulphydryl groups were available for attachment to the Hg moiety of the affinity packing. They were further purified by ion exchange chromatography on a CM-52 carboxymethyl cellulose column in 10^{-2} M acetate buffer using a linear gradient of 0-0.07 M NaCl. After dialysis, the unbound esterolytic fraction isolated was clarified by centrifugation and applied to CM-52 column.

Papaya peptidase A was eluted with NaCl in acetate buffer. In enzyme activity assay, it was apparent that activity of papain was highest, the one of chymopapain was a half of papain, and the one of papaya peptidase A was lowest. The similarities of modes of reactions were also apparent at similar pH values (6.6). Papain, chymopapain, and papaya peptidase A were confirmed by gel-electrophoresis with 7.5% gels.

Mahaphon *et al*. (1987) have studied on the proteolytic activity of papain from three varieties of papaya: Go-Goa, Khag Nuan and Wat Plange. Papain was extracted from latex of green fruit by crude extraction with phosphate-cysteine disodium ethylene dinitrilotetraacetate buffer, pH 6. The supernatant was collected and its activity was assayed using casein substrate. It was found that the average specific activities of papain from Go-Goa, Khag Nuan and Wat Plange were 9.4096×10^4, 8.0713×10^4 and 7.3808×10^4 unit/g fresh latex, respectively. From these results, Go-Goa had the highest specific activity of papain and Wat Plange had the lowest. The specific activity of papain from three varieties of papaya did not depend on size and age of papaya fruit.

Wichernroj (1988) has studied on the production and purification of papain form papaya latex. 100 g of fresh latex was ground with sand. Distilled water was used as the solvent to extract papain. Ammonium sulfate was added to filtrate for precipitation. The precipitate was collected by centrifugal technique and dried in an oven. Dried precipitate was dissolved with distilled water and dialyzed in water bath. It was further purified by sephadex column and then precipitated with acetone. The net yield of solid from 100 g of dried latex was only 32 wt%, and the specific activity of papain was 3.6610 C.D.U./mg protein.

Apichasikijdamrong *et al*. (1990) have investigated the method of papain extraction from waste papaya skin. Papain was extracted from papaya skin with phosphate buffer and precipitated with 95% ethanol. The precipitate was dried by freeze drying and vacuum drying method. It was found that the suitable extraction time was 4 minutes, and the suitable ratio between weight of papaya skin to volume of phosphate buffer was 100 g of papaya skin to 100 ml of phosphate buffer solution. The specific activity of papain after vacuum drying was higher than that after freeze drying. This was because process time of freeze drying was longer than that of vacuum drying. The extraction yield was 4 g of crude enzyme from 100 g of papaya skin dry basis.

Wuttiwiangtham (1993) reported a method of purification and characterization of papain from papaya latex (*Carica papaya* Linn.), cv. Khag Dam. Proteases were purified by precipitation in ammonium sulfate and sodium chloride. Papain was separated from other proteases by sephadex gel filtration. The yield of papain was only 2.34 wt% from total protein in latex. It showed maximum activity for hydrolysis of casein at 80^{o}C pH 7.5. The optimal storage temperature of purified papain was at -20^{o}C. The purified papain showed a single band in polyacrylamide gel electrophoresis corresponding with standard papain (22 kDa).

He also reported the stability of fresh and dried papaya latex at 4^{o}C and -20^{o}C for 10 months. It was apparent that the best stability was formed in dried latex stored at -20^{o}C, following by latex stored at -20^{o}C, dried latex stored at 4^{o}C and fresh latex stored at 4^{o}C. It was shown that the high stability of latex correlated with low temperature for storage. It was also reported that the stability of purified papain solution stored at 4^{o}C for 6 month was less than that stored at -20^{o}C. It might be because papain solution stored at 4^{o}C was auto proteolysed more than that stored at -20^{o}C.

Monti *et al*. (2000) reported a method of crystallizing papain from papaya latex at low temperature, which gave a high level of purity and excellent catalytic activity. It was reported that 2.43 mg papain per g latex was obtained. The papain obtained was practically pure and showed a single band when submitted to electrophoresis on polyacrylamide gel, and was identical to papain obtained by other methods. In the assay of enzyme activity, the prepared papain was activated with dithioerythritol (DTE). However, treatment with DTE did not lead to a great in the number of SH groups per mol papain, $Kcat.s^{-1}$ $(mol\ SH)^{-1}$. The most probable hypothesis to explain these results might be the presence of natural inhibitors forming a complex with enzyme and released from enzyme during DTE treatment. On the basis of these results, A 55% increase in the number of SH groups per mol papain was observed when the enzyme concentration was decreased, demonstrating the presence of an enzyme inhibitor. In brief, the papain prepared by this method is the same as the papain obtained by the classical method, with the advantage of not receiving treatment for salts in high concentration. This method allowed detecting the presence of inhibitor substances forming a complex with papain.

2. Enzymatic hydrolysis for gelatin production from various sources of collagen.

Taylor *et al*. (1989) have investigated the enzymatic treatment of offal from fleshing machines. Pig skin and cattle hide were treated with one of four different proteases (pancreatin, enzeco, alcalase and neutrase). The effect of time, temperature, type of enzyme and enzyme concentration on protein and fat recovery were reported. The digestion was carried out at 40^{o}C for 3 hours and then at 60^{o}C for 1 hour. Under studied conditions, 3% concentration of pancreatin gave the highest recovery percentage of fat and protein and the lowest amount of residue. Up to 87% of the available fat was recovered as well as a readily separated protein hydrolysate fraction. These treatments could be carried out at low temperature, over short periods of time. Yield of protein product which could be used for fertilizer or accepted by sanitation departments and yield of fat product which have a low free fatty acid content have potential values to the chemical industry.

Chomarat *et al*. (1994) have compared the efficiency of pepsin and proctase for the preparation of bovine skin gelatin. Skin samples were homogenized in 0.5 M acetic acid. Pepsin was added at 5% and proctase at 7.5% in order to obtain the same quantity of enzyme units, approximately 10^5 units/g tissue dry weight. Enzyme digestion was performed at 4^{o}C for 50 hours. Conversion of extracted collagen to gelatin was performed at 60^{o}C. The collagen of skin was solubilized by pepsin and proctase with similar yields (75% and 76% of total collagen, respectively). The relative amounts of extracted collagen converted to gelatin using pepsin and proctase were 32% and 39%, respectively. The gel strength of pepsin-prepared gelatin and proctase-prepared gelatin were 140 and 29 g Bloom, respectively. Viscosity of pepsin-prepared gelatin and proctase-prepared gelatin were 3.43 and 1.11 cP, respectively. Molecular weight distribution of gelatin was detected by SDS-PAGE. It showed a decrease of high-molecular-weight polymer in proctase-prepared gelatin as compared to pepsin-prepared gelatin. These parallel biochemical and biophysical data indicated that proctase-prepared gelatin is markedly altered compared with pepsin-derived gelatin.

Simeonova *et al*. (1996) have utilized the limed split from a leather industry. The objective of this work was to elaborate a method to process this limed split into useful products. The treatment proposed involved washing to remove the inorganic salt (sodium

and calcium salts, mainly calcium hydroxide), separation of fat, extraction of collagen in hot water solution, and additional extraction of protein from the insoluble residue after hydrolysis with alkaline proteinase (B72 from *Bacillus subtilis*). This resulted in the isolation of 3 fractions: fat-cattle tallow (4-12% of total mass of initial material), collagen hydrolysate-glue (5-10%), and protein concentrate for fodder (1-3%). After the enzyme treatment only 5% of the raw material protein remained in the insoluble residue. The enzyme hydrolysate could be added to the water extraction to raise the yield of collagen hydrolysate fraction. If this fraction was purified to produce edible gelatin, it would be better to dry and use the enzyme hydrolysate separately, as the presence of low molecular weight element would reduce the gelling ability of final product. If this method was developed into technology, it could solve both economic and environmental problems.

Morimura *et al*. (2002) have developed a procedure for the extraction of protein and production of peptides by enzymatic hydrolysis from fish bone and pig skin wastes containing collagen. Fat and inorganic compounds were removed by soaking in HCl solution and a high molecular weight protein was extracted under acidic condition (pH 3) for an hour at 60^{o}C. The molecular weight of extracted protein was determined by SDS-PAGE on 12% polyacrylamide gel. It was found that the molecular weight of extract from pig skin was greater than 100kDa. Pretreated fish bone and pig skin were hydrolyzed with one of sixteen commercial enzymes. It was found that enzyme L (from *Bacillus* species) was the best for degradation of collagen. The molecular weight of enzymatic extracts from pig skin and fish bone were about 350 Da. It could be noticed that molecular weights of enzymatic hydrolysis extracts were much smaller than the one of acidic hydrolysis extracts. The hydrolysates had a high anti-radical activity and a high potential for decreasing blood pressure, suggesting the hydrolysates could be a useful additive in food materials.

Aspmo *et al*. (2005) have studied the solubilization of cod (*Gadus Morhua* L.) viscera at natural substrate pH. Viscera was hydrolysed by endogenous enzyme alone or in combination with one of seven different commercial proteases (alcalase, neutrase, protamax, papain, bromelain, actinidin and a plant protease mix). The excess enzyme to substrate ratio was used at 5.3% (w/w dry matter). The hydrolysis reaction was carried out at 55^{o}C for 24 hours with unadjusted pH. Reaction was ended by incubated at 105^{o}C for 30 minutes to inactivate enzyme. The highest yields of hydrolysate were obtained

with alcalase and papain (95% and 90% of dry matter, respectively). The yield of dry matter obtained without commercial enzyme was about 75%. The extra yield gained by adding commercial proteases was limited and might not always be economically.

Ratanathammapan K. (2005) have studied the effect of enzymatic conditions for hydrolysis of raw hide using two commercial proteases (papain and neutrase). It was found that the optimum ratio of papain to raw hide for the highest gelatin recovery was 0.15:100, and the optimum working conditions were 70^{o}C and pH 6-7. The gel strength and viscosity of gelatin from papain hydrolysis was lower than that of food and laboratory grade gelatin. In case of neutrase, the optimum ratio was 0.075:100, and the optimum working conditions were 40-50^{o}C and pH6-7. The protein obtained from neutrase hydrolysis at all conditions could not form gel.

U.S. patent number 4,064,008 (1977) presented the improvement in treatment of collagen containing materials for gelatin extraction. The collagen source was enzymatically treated at pH 7-13 using one of proteases (alcalase, esperase, trypsin, and BPN), for 4-72 hours, at 20-40^{o}C. The extracted gelatin was filtrated and the clear filtrate was desodorized with activated carbon. The yield of extracted gelatin using esperase was the highest (about 46.6% at 25^{o}C, pH 7, and 17 hours). The working temperature of these enzyme was between 25^{o}C and 35^{o}C.

U.S. patent number 4,220,724 (1980) described the method for conditioning collagen-containing raw materials and extraction of degradation products with hot water. Enzymatic hydrolysis reaction using neutral or alkaline protease was carried out at pH between 7 and 13, for 4 to 12 hours, at 20^{o}C to 40^{o}C. It was found that the optimal condition for the highest yield of gelatin extraction using alkaline protease was at pH 11.5, 40^{o}C, for 10 hours, and without agitaion. After reaction stopped, no residue was obtained. Further processing into gelatin, glue, collagen threads, and collagen fibers could be used in a known manner.

U.S. patent number 5,877,287 (1999) has reported the method for producing gelatin which can be applied to different collagen-containing materials. The slurry from raw material was treated with acid, heat, and enzyme (esperase, Nova Denmark) in order to expose the collagen. The slurry was separated into a liquid portion and a solid residue,

and the gelatin was recovered from the liquid portion. The yield of the extracted gelatin using esperase was increased from 73 wt% (without enzyme) to 87 wt%. The gel strength of the extracted gelatin without enzyme treatment was higher than gel strength of the one with enzyme treatment (about 300 and 180 g Bloom, respectively). After ultrafiltration, gel strength of the extracted gelatin with enzyme was increased to 250 g Bloom.

U.S. patent number 5,919,906 (1999) described the production of gelatin using protease. This provided a gelatin of low viscosity (4.1 cP) without loss of gel strength (250 g Bloom). The gelatin from this production was composed of a high molecular weight fraction (HMW>250 kDa) of 4.22 wt%, a beta fraction (150-250 kDa) of 8.56 wt%, and an alpha fraction (50-150 kDa) of 38.09 wt%. In general, high gel strength correlates with high alpha fraction content of gelatin, and high viscosity correlates with high HMW content of gelatin. Gelatin of this method can be used for any imaging material.

U.S. patent number 6,100,381 (2000) presented the manufacturing method of high quality gelatin from ossein utilizing protease (protex 6L) to extract gelatin. The yield of extracted gelatin was increased to 96wt%. The extracted gelatin using protease had very high gel strength (about 335 g Bloom). It was composed of a HMW fraction of 10.01 wt%, a beta fraction of 12.06 wt%, and an alpha fraction of 38.96 wt%. The value of gel strength mainly depends on alpha and HMW fraction content of gelatin.

CHAPTER IV

EXPERIMENTAL

This chapter can be divided into three main sections: (1) Materials and reagents, (2) Equipments, and (3) Experiments. Details of each section are as follows.

4.1 Materials and reagents

1. Papaya latex

Papaya latex was kindly supplied from Kasetsart University Research and Development Institute. The latex was collected from the unripe papaya fruits (*Carica papaya*), C.V. Khag Dam. The collecting of latex should start early in the morning and finish by mid-late morning. Three or four vertical incisions on the fruit skin were made with a sharp stainless steel knife to a depth of 1-2 mm. The latex flown from the cuts was collected into a plastic or glass container. After collecting, the latex was stored at -20^{o}C before use.

2. Papain

Papain from *Carica papaya* was supplied by Fluka as white powder. The activity of papain is reported at 0.51 unit/mg and the optimum working pH is at 6-7. Before uses, the activity of papain at various pH and temperature was assayed according to the method modified from the assay of endo-protease using azo-casein (Megazyme).

3. Raw hide

Raw hide material used in this work was limed split, provided by World Pet International Co., Ltd. The dried split was ground into an average size of not exceed 1.18 mm (mesh number 16). The pH of raw hide was used in the adjustment of pH of hide slurry by HCl and buffer solution.

4. Copper II sulphate (anhydrous) was supplied by Unilab.
5. Sodium carbonate (anhydrous) , ammonium sulfate and potassium sodium tartrate were supplied by Univar.
6. NaOH, sodium phosphate monobasic monohydrate, trichloroacetic acid (TCA) and Tris-HCl were supplied by Merck.
7. Bovine serum albumin (BSA), sodium phosphate dibasic heptahydrate and, azo-casein were purchased from Fluka.
8. HCl conc. 37 %, chloroform, methanol and toluene were purchased from Lab-Scan.
9. Folin-ciocalteu's phenol reagent was supplied by Carlo Erba.
10. Ethyl alcohol absolute anhydrous was supplied by Mallinckrodt
11. Acetic acid and sodium dodecyl sulphate (SDS) were supplied by HBD Chemical.
12. Glycerol, glycine, coomassie brilliant blue R 250, bromophenol blue, acrylamide, bis-acrylamide, ammonuim persulfate, and tetramethyl ethylenediamine (TEMED) were supplied by Sigma-Aldrich.
13. Standard marker low molecular weight was supplied by Amersham.
14. Gelatin (laboratory grade) was purchased from Khurusapha.

4.2 Equipments

1. pH meter (PP-50) from Sartorius
2. Centrifuge (Kubota 7820) from Kubota Corporation
3. Centrifuge (Kubota 5100) from Kubota Corporation
4. Centrifuge (Avanti J-30I) from Beckman Coulter
5. Lyophilizer (PowerDry LL3000) from Heto
6. Nitrogen analyzer set from Buchi
7. Reactor set (1 L reactor with semi-circle stirrer and water bath)
8. Vacuum filtration set (1L sucksion flash with buchner funnel and vacuum pump)
9. UV-VIS spectrophotometer (6405 UV/VIS) from Jenway
10. Oven (Binder)
11. Furnace (F48000) from Barnstead International
12. Texture analyzer (TA-XT2i) from Stable micro systems
13. Rheometer (HAAKE RS 600)
14. Electrophoresis kit from Bio-RAD
15. Power supply from Bio-RAD

4.3 Experiments

The experiments are divided into two parts: crude extraction of proteolytic enzymes from papaya latex, and enzymatic hydrolysis of raw hide. The crude extraction is subdivided into seven parts: (i) Papaya latex characterization, (ii) Protein determination, (iii) Activity assay, (iv) Crude extraction by solvent, (v) Precipitation, (vi) Lyophilization, and (vii) Molecular weight determination. Experimental procedures of this part are summarized in Figure 4.1.

The enzymatic hydrolysis is subdivided into nine parts: (i) Raw hide characterization, (ii) pH adjustment, (iii) Hydrolysis reaction, (iv) Gelatin separation, (v) Protein determination, (vi) Gel strength determination (vii) Viscosity determination, (viii) Molecular weight determination, and (ix) Ash determination. All experiments in this section are shown in the diagram in Figure 4.2.

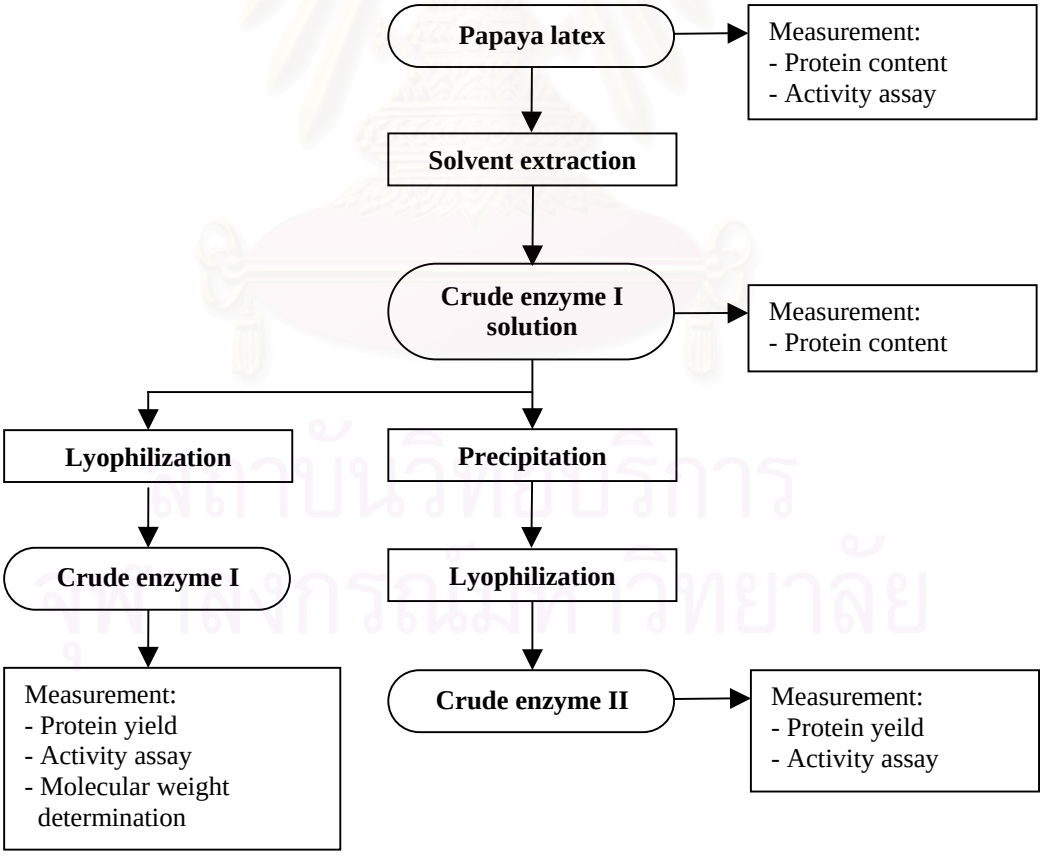

Figure 4.1 The diagram of crude extraction of proteolytic enzymes

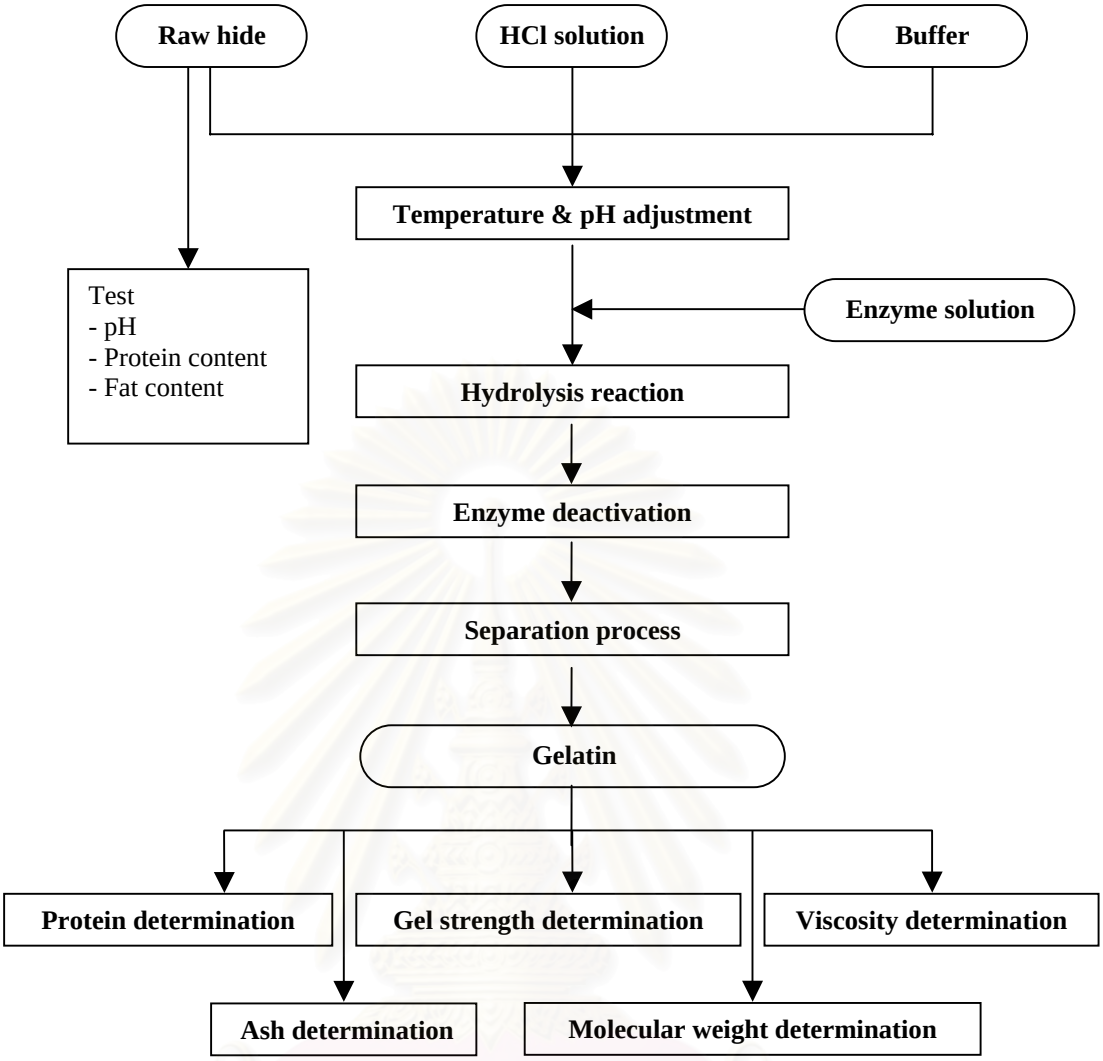

Figure 4.2 The diagram of enzymatic hydrolysis of raw hide

4.3.1 Crude extraction of proteolytic enzymes

4.3.1.1 Papaya latex characterization

Water content

The water content of papaya latex was determined by lyophilization. The fresh latex (100 g) was contained in a plasticware and frozen at -20^{o}C for 18 hours. Water was removed from latex at frozen state in the chamber of lyophilizer at -40^{o}C under vacuum for 12 hours. The weight of dried latex was measured. The water content of latex was calculated from the following equation.

$$W = \frac{m_0 - m_1}{m_0} \times 100$$

where

W is the percentage of the water content.

m_0 is the initial weight of the papaya latex.

m_1 is the weight of the dried papaya latex.

Soluble solid, fiber and gum content

The fresh latex (100 g) was centrifuged at 3,000 rpm (1,700g) for 20 minutes to separate the solid (fiber and gum) and liquid (water and soluble solid) phases. The liquid phase was put in a plasticware and frozen at -20^{o}C for 18 hours before lyophilizing at -40^{o}C for 12 hours. The weight of soluble solid was measured. The content of fiber and gum in latex was determined from the following equation.

$$F = \frac{m_1 - s}{m_0} \times 100$$

where

F is the percentage of fiber and gum.

m_0 is the initial weight of the papaya latex.

m_1 is the weight of the dried papaya latex.

s is the weight of the soluble solid.

4.3.1.2 Protein determination

In this study, Lowry method was used to determine the percentage of protein content in papaya latex, extracted enzyme solution, and gelatin solution.

Stock reagents

1. Reagent A as Copper sulphate solution (1% w/v)
2. Reagent B as Sodium potassium tartrate (2% w/v)
3. Reagent C as Sodium hydroxide solution (0.2 M)
4. Reagent D as Sodium carbornate solution (4% w/v)
5. Reagent F as Folin-Ciocalteau reagent solution (50% v/v)

Test procedure

Solution E was prepared by mixing 49 ml, 49ml, 1ml, and 1ml of stock reagents D, C, B, and A, respectively. Because of the precipitation of copper, solution E must be freshly prepared for each test. The 0.25 ml of papaya latex was diluted to 100 ml with distilled water. The 0.5 ml of diluted papaya latex solution was mixed with 2.5 ml of solution E in a test tube and shaken by a vortex mixer. After 10 minute, 0.25 ml of solution F was added and then shaken. Reaction was occurred at room temperature for 30 minute. After that, the absorbance of the sample at the wavelength of 750 nm was measured using a UV-VIS spectrophotometer. A blank test was performed using distilled water instead of diluted papaya latex. The standard curve was prepared by using bovine serum albumin, BSA, at various concentrations in the range of 0-300 μ g/ml. The reported protein content in gelatin solution was determined from the average of 3-repeated test values.

4.3.1.3 Enzyme activity assay (http://secure.megazyme.com/downloads/en/data/S-AZCAS.pdf**)**

Reagents

1. Azo-casein solution (0.2% w/v)
   One gram of azo-casein, 2 ml of absolute ethanol and 1 ml of toluene were mixed and adjusted to the volume of 500 ml by distilled water.
2. Trichloroacetic acid solution (10% w/v)

3. 0.1 M phosphate buffer

Solution A: 0.2 M stock solution of sodium phosphate monobasic was prepared by mixing 27.8 g of sodium phosphate monobasic monohydrate with distilled water and adjusted to 1,000 ml.

Solution B: 0.2 M stock solution of sodium phosphate dibasic was prepared by mixing 53.6 g of sodium phosphate dibasic heptahydrate with distilled water and adjusted to 1,000 ml.

The 0.1 M phosphate buffer at desired pH was prepared by mixing x ml of solution A and y ml of solution B and adjusted the volume to be 200 ml with deionized water as shown in Table 4.1.

Table 4.1 The mixing amount (ml) of solution A and solution B in 0.1 M phosphate buffer

x (ml)	y (ml)	pH	x (ml)	y (ml)	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	89.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

Test procedure

The activity assay of enzyme was preformed at various conditions, i.e. various temperature and pH. The 0.9 ml of 0.1 M phosphate buffer (at the desired pH) and 0.1 ml of papaya latex were mixed in a test tube and shaken by a vortex mixer. The 1 ml of azo-casein solution was added to the mixing solution and further shaken. The

solution was incubated in water bath at the desired temperature for 20 minutes. After that, the 2 ml of TCA solution was added to the solution for precipitation of non-hydrolyzed substrate, and then the solution was put in a ice bath at about 4^{o}C to stop the hydrolysis reaction. Finally, the solution was centrifuged at 3,000 rpm (1,660g) for 20 minutes to separate the solid and liquid phases. The absorbance of the sample at the wavelength of 440 nm was measured using a UV-VIS spectrophotometer. Blank test was prepared by adding the TCA solution to the substrate before azo-casein solution was added. The yield of activity of crude enzyme was determined from the following equation.

$$Y = \frac{S}{L} \times 100$$

where

Y is the yield of activity of crude enzyme.

S is the total activity of supernatant.

L is the total activity of papaya latex.

4.3.1.4 Solvent extraction

The solvents used to extract proteolytic enzymes from papaya latex in this work were distilled water and phosphate buffer pH6. Papaya latex and solvent were mixed at various volumetric ratios of latex to solvent from 1:0.5 to 1:4 as presented in Table 4.2. The mixture was stirred at 4^{o}C for 10 minutes and centrifuged at 4^{o}C 3,000 rpm (1,700g) for 30 minutes. The supernatant was separated from residue by a vacuum filtration kit with Whatman filtration paper grade 4 (20-25 μm). A part of extracted enzyme solution was separated and lyophilized prior to characterization, i.e. protein determination and enzyme activity assay. The other part of extracted enzyme solution was proceeded to the step of precipitation using ammonium sulfate.

Table 4.2 The volumetric ratios of papaya latex to solvent

Ratio between papaya latex and solvent	1:0.5	1:1	1:2	1:4
Papaya latex (ml)	100	75	50	30
Solvent (ml)	50	75	100	120

4.3.1.5 Precipitation with ammonium sulfate

The filtrate from solvent extraction step was precipitated in 45wt% of saturated ammonium sulfate solution at 4^{o}C. The 45 ml of saturated ammonium sulfate solution and 55 ml of filtrate were mixed, and gently stirred at 4^{o}C for 2 hours. After that, the precipitate was separated by centrifugation at 4^{o}C, 3,000 rpm (1,700g) for 20 minutes prior to lyophilization.

4.3.1.6 Lyophilization

To dry the extracted enzyme solution using lyophilization technique, the extracted enzyme solution from solvent extraction step and precipitation step were frozen at -50^{o}C for 12 hours. The frozen samples were lyophilized at the temperature of -40^{o}C and the pressure under 0.5 mmHg. The dry samples were kept in a refrigerator (4^{o}C) before use.

4.3.1.7 Molecular weight determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of proteolytic enzyme.

Reagents

1. 30% acrylamide, 0.8% bis-acrylamide
   The 29.2 g of acryamide and 0.8 g of bis-acrylamide were dissolved with deionized water, adjusted the volume to be 100 ml, and filtrated by Whatman filtration paper grade 4.
2. 1.5 M Tris-HCl pH 8.8
   The 18.17 g Tris-HCl was dissolved in deionized water. The pH was adjusted to 8.8 with 1 M HCl, and the volume was adjusted to 100 ml.
3. 1.5 M Tris-HCl pH 6.8
   The 6.06 g Tris-HCl was dissolved in deionized water. The pH was adjusted to 6.8 with 1 M HCl, and the volume was adjusted to 100 ml.

4. 1% SDS solutoion

   1 g of SDS was dissolved with 100ml of distilled water.

5. 10% of ammonium persulfate

   0.1 g of ammonium persulfate was dissolved with 1 ml of deionized water. This solution should be freshly prepared before casting gel.

6. Tetramethyl ethylenediamine (TEMED)

   TEMED was used as catalytic reagent for polymerization of polyacrylamide gel.

7. 5X Sample buffer

   The 0.005 g of bromophenol blue was dissolved in 12.5 ml of 0.5 M TCA, 10 ml of glycerol, 30 ml of 10% SDS and diluted to 100 ml with distilled water.

8. Electrophoresis buffer

   The 3.03 g of Tris-HCl, 14.40 g of glycine and 1.0 g of SDS were dissolved in distilled water (1,000 ml).

9. Staining solution

   Stain solution was prepared by mixing 0.04 g of Coomassie Brilliant Blue R in 27 ml 95% ethanol, 0.5 g of copper sulfate in 63 ml of distilled water, 10 ml of acetic acid, and filtrated by Whatman filtration paper grade 4.

10. First destaining solution

    First destaining solution was prepared by dissolving 5 g of copper sulfate with 120 ml of 95% ethanol, 70 ml of acetic acid and 810 ml of distilled water, then filtrated by Whatman filtration paper grade 4.

11. Second destaining solution

    Second destaining solution was prepared as same as first destaining solution, but without copper sulfate.

Test procedure

Two glass plates were assembled with two-side spacer, clamp, and grease as presented in Figure 4.3. The separating gel was prepared at 12.5% polyacrylamide as presented in Table 4.3. The gel solution was degassed under vacuum for 5 minutes before TEMED was added. This solution was poured into glass plate assembly carefully, and then overlaid with distilled water to ensure a flat surface and to exclude

air. The distilled water was removed after the separating gel was set. The stacking gel was prepared at 3% polyacrylamide (as shown in Table 4.3) and degassed before TEMED was added. A stacking gel is large pore gel, overlaid on top of separating gel, and inserted with a comb. A comb was removed after a stacking gel had been set. The gel was left into chamber of electrophoresis kit, then electrophoresis buffer was filled. The pH gradient was adjusted overall gel during prerunning with current of 16 mA per gel for 30 minutes. The 0.05 g of extracted enzyme was dissolved with 10 ml of distilled water. Sample solution was mixed with the sample buffer in the volumetric ratio of 4:1 and boiled for 10 minutes in water bath to ensure that polypeptide chains were unfolded and stretched. The 20 μl of a boiled solution was filled into each well of a stacking gel. The 8 μl of standard protein and 12 μl of 20% 5X sample buffer were mixed and filled into a well of gel as a protein marker. The other wells were filled with 12 μl of 20% 5X sample buffer. The gel was applied with the current of 16 mA per gel until the marker was reached the bottom of the gel. The gel was removed from the spacers, and covered with a staining solution for 12 hours. The gel was destained until background was clear and dried on a glass plate. Standard protein was low molecular weight group: phophorylase b (MW 97,000), albumin (MW 66,000), ovabumin (MW 45,000), carbonic anhydrase (MW 30,000). trypsin inhibitor (MW 20,100), and α -lactalbumin (MW 14,000).

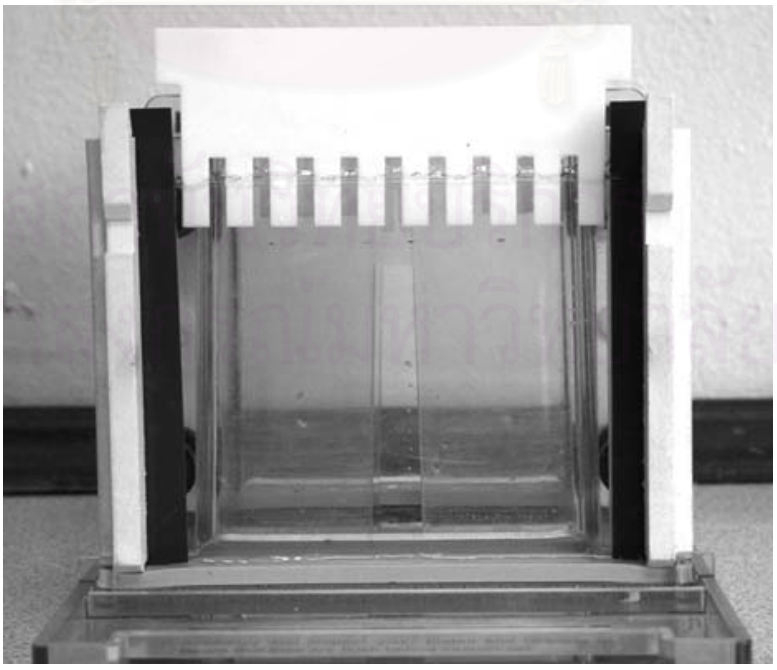

Figure 4.3 Front view of gel plate assembly.

Table 4.3 Reagents of polyacrylamide gel electrophoresis.

Reagent	Stacking gel 3% polyacrylamide	Separating gel 10% polyacrylamide	Separating gel 12.5% polyacrylamide
- 30% acrylamide + 0.8% bis-acrylamide	0.5 ml	3.325 ml	4.175 ml
- 1.5 M Tris-HCl pH 8.8	-	2.50 ml	2.50 ml
- 0.5 M Tris-HCl pH 6.8	1.25 ml	-	-
- 1% SDS	50 µl	100 µl	100 µl
- Distilled water	3.175 ml	4.025 ml	3.175 ml
- 10% ammonium persulfate	25 µl	50 µl	50 µl
- TEMED	5 µl	5 µl	5 µl

4.3.2 Enzymatic hydrolysis of raw hide

4.3.2.1 Raw hide characterization

Protein determination

Kjeldahl method was used to determine the protein content of raw hide by the Nitrogen analyzer set. The 0.1 g of raw hide was digested with 15 ml of concentrated H_2SO_4 solution and 0.5 g of selenium oxide was added as a catalyst in order to increase the rate of organic break-down during the acid digestion and reduce the reaction time to 90 minute. The clear acid digested mixture was left to cool down at room temperature, and then back titrated with NaOH (50% w/w), boric acid (2% w/w) and concentrated H_2SO_4 using the Nitrogen analyzer set. The percentage of nitrogen content that calculated from back titration was multiplied by a specific protein factor (6.38) to convert to the percentage of protein content in raw hide.

Fat determination

The method of Folch, fat extraction, was used to determine fat content in raw hide. The raw hide was mixed with chloroform/methanol solvent (2/1 v/v). The weight of raw hide sample to chloroform/methanol volume ratio was 1:20. The mixture was stirred for 15-20 minute at room temperature using a magnetic stirrer and then filtrated to separate the liquid phase and solid phase. 4 ml of water was added to

the solution to separate methanol from chloroform. After mixing for a few seconds, the solution was centrifuged at 2,000 rpm (755g) for 15 minute to separate the two liquid phases. The upper phase (water/methanol) was removed by a dropper, and the lower phase (chloroform/fat) was filled into a foil dish and then left in an oven at 40^{o}C for 4 hours to evaporate chloroform. The dried fat was kept in a desiccator at room temperature.

4.3.2.2 pH adjustment

Since as-received raw hide was limed, to prepare the raw hide slurry at the desired pH, the raw hide slurry was neutralized with HCl solution, prepared by diluting 37% HCl conc. with deionized water to make up volume of 200 ml. The volume of 37% HCl conc. used depended on the desired pH of the hydrolysis reaction, as presented in Table 4.4. After that, 0.1 M phosphate buffer was used to maintain the pH of the raw hide slurry during hydrolysis reaction.

Table 4.4 The volume of 37% HCl conc. in solution and pH of raw hide slurry

37% HCl (ml)	pH of slurry
6	8
4	7
2	6

4.3.2.3 Hydrolysis reaction

The hydrolysis reaction was carried out in a 1 L reactor. The ratio of raw hide to phosphate buffer solution (including HCl) was 1:4 (200g:800ml). Firstly, The hydrolysis reaction was performed by mixing 200 g of raw hide with 200 ml of HCl solution. The slurry was well mixed using an over-head stirrer at the speed of 75 rpm, then 500 ml of phosphate buffer was added. The raw hide slurry and the 100 ml of phosphate buffer were heated to the desired temperature by a water bath. When the desired temperature (hydrolysis temperature) of hide slurry was reached, the enzyme was dissolved into the remaining 100 ml of preheated phosphate buffer solution, and then added into the raw hide slurry. The amount of each enzyme used in the reaction was first varied in order to determine the optimum enzyme to substrate ratio (see

results in Section 5.2.2). After that the optimum amount of each enzyme used was fixed. The hydrolysis reaction was considered to start at this point and sample solutions were collected from the reactor at 6, 10, 20, 40, 60 and 90 minutes, periodically. The collected samples were kept in a freezer (-20^{o}C) to inhibit the hydrolysis reaction. Finally the samples were heated to 90^{o}C for 15 minutes to deactivate enzyme.

4.3.2.4 Gelatin separation

Centrifugation and filtration techniques were used to separate gelatin solution from hide residue that might be in the collected sample solution. The solution was centrifuged with the speed of 12,000 rpm (21,600g) for 30 minutes at 25^{o}C. After that, the liquid phase was separated from residue by a vacuum filtration kit with Whatman filtration paper grade 2 (8 μm pore size). The 0.25 ml of filtered gelatin solution was brought to determine the protein concentration by Lowry method as previously described in Section 4.3.1.2. The percentage of gelatin recovery was calculated based on the following equation.

$$P = \frac{G}{K\left(\frac{H}{V}\right)} \times 100$$

where

P is the percentage of gelatin recovery; wt%.

G is the protein concentration of gelatin solution; g/ml.

K is the protein fraction of raw hide.

H is the weight of raw hide; g.

V is the volume of buffer solution (including HCl); ml.

The remaining gelatin solution was preheated in a microwave oven at 100 W for 15 minutes, and then dried in a conventional oven at 60^{o}C. The dried gelatin was kept in a desiccator, prior to the determination of the gel strength, viscosity, molecular weight, and ash.

4.3.2.5 Gel strength determination

Gel preparation

The measurement of gel strength was modified from the British Standard (BS EN ISO 9665:200). The 12.5% (w/w) gelatin solution was prepared by dissolving the 5 g $\pm$ 0.01 g of gelatin in 35 ml of water in a test bottle (a cylinder glass bottle with an inside diameter of 39 mm). The mixture was stirred to completely wet the dried gelatin then placed in the oven at 50^{o}C for 15 minutes. The mixture was continuously stirred at 50^{o}C until homogeneous gelatin solution was obtained. To prevent gel cracking, the bottle was left to cool down for 15 minute at room temperature and then placed in a refrigerator at 4^{o}C for 16-18 hour.

Gel strength determination

The gel strength of gelatin at 4^{o}C was determined using texture analyzer, equipped with 1.27 cm diameter hemisphere head cylindrical, cross-head speed of 0.8 mm/s. The gel strength of the gelatin sample was determined by measuring the force required to depress the center of gel surface vertically to a depth of 4 mm $\pm$ 0.01 mm. The measured gel strength was in the double bloom standard. The gel strength was reported as the averaged value of the three measurements.

4.3.2.6 Viscosity determination

The determination of viscosity was modified from The British Standard (BS EN ISO 9665:200). The 12.5% (w/w) gelatin solution was prepared, same as for gel strength measurement. To measure the viscosity of gelatin solution, 1 ml of gelatin solution was placed between a plate-and-plate (35 mm diameter) geometric head of HAAKE RS 600. The gelatin solution was held for 3 minutes at the constant temperature of 60^{o}C. One hundred data points of the viscosity of gelatin solution were recorded at the constant shear rate of 400 s^{-1} for 5 minutes.

4.3.2.7 Molecular weight determination

1 g of dried gelatin was used to characterize the molecular weight, using 10% polyacrylamide gel, following the procedure described in Section 4.3.1.7.

4.3.2.8 Ash determination

Determination of ash was modified from The British Standard (BS EN ISO 9665:200). A clean crucible was prepared by heating at 550°C ± 5°C for 2 hours and cooled in a desiccator at room temperature. After that, the weight of crucible was measured. The 0.5 g ± 0.001 g of dried gelatin was weighed into the crucible. The dried gelatin was heated in a furnace (F48000) at 550°C ± 5°C for 6 hours. The dried ash in the crucible was cooled in a desiccator at room temperature. The weight of ash was measured using a 4-digit balance. The percentage of the ash was determined from the following equation.

$$A = \frac{m_1}{m_0} \times 100$$

where

A is the percentage of the ash.

m_0 is the initial weight of the dried gelatin.

m_1 is the weight of the ash.

CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Crude extraction of proteolytic enzymes from papaya latex

5.1.1 Crude extraction of proteolytic enzymes

5.1.1.1 Papaya latex

Compositions of latex

The compositions of papaya latex were determined and presented in Table 5.1. It was seen that main component in papaya latex was water (81.7wt%). The rest was dry mater (18.3wt%) including protein, fiber and gum, and other soluble solid. The results on the latex compositions corresponded with the work of Azarkan *et al*. (2003), studying the purification of enzymes stored in latex of papaya. It was reported that main composition of latex was water (80wt%). Papaya latex contained about 15-20wt% of dry matter and 40wt% of this dry matter was enzymes, mainly cysteine endopeptidases which was more than 80wt% of total enzyme fraction.

Table 5.1 The compositions of papaya latex

Composition of papaya latex	Content (wt%)
Water	81.7
Protein	11.5
Fiber and gum	5.6
Other soluble solid	1.2

Activity of proteolytic enzymes in papaya latex

At 70^{o}C and pH6, the enzyme activity of as-received papaya latex from various lots reached in the range of 1.7-1.8 unit/ml. The activity of papaya latex from this variety, Khag Dam, was not different as reported by the work of Mahaphon *et al*

(1987). They have studied on the activity of latex from 3 varieties of papaya: Go-Goa, Khag Nuan, and Wat Plange. From this report, it was found that average activity of papaya latex from same variety was similar, but there was a difference in average activity in 3 varieties.

As known that proteolytic enzymes, which are the most important component in papaya latex, were heat sensitive, as-received papaya latex was stored at -20^{o}C. The activity of proteolytic enzymes in papaya latex was determined every two weeks in order to identify the stability of enzyme activity in fresh latex. The conditions for activity assay were set at 70^{o}C and pH 6.

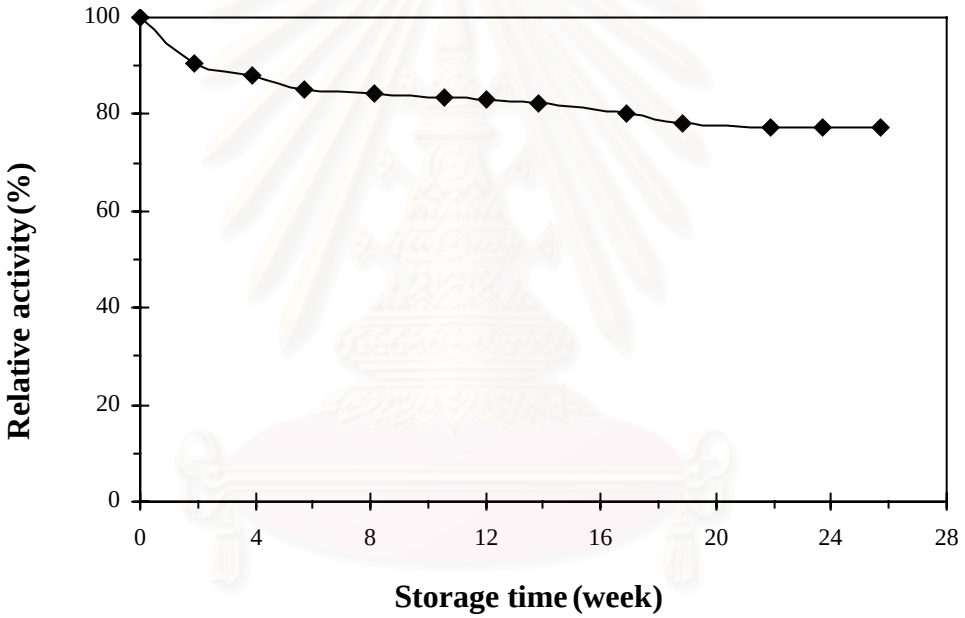

Figure 5.1 Relative activity of papaya latex stored at -20^{o}C for 26 weeks.

Figure 5.1 presented the relative activity of papaya latex stored at -20^{o}C. It was noticed that the relative activity of stored papaya latex decreased rapidly (about 13%) in the first 4 weeks. After that, the relative activity slowly decreased. After latex was stored for 22 weeks, the relative activity was consistent at around 77% of the initial activity. In this work, papaya latex received was used in solvent extraction process during the storage time of 6-8 weeks. This could possibly limit the variation of activity decreasing in papaya latex.

5.1.1.2 Crude enzyme from papaya latex by solvent extraction

Crude enzyme was extracted from papaya latex at 4^{o}C by two solvents; water and 0.1 M phosphate buffer pH6. They were inexpensive and commonly used for extraction of enzyme from plants. The volumetric ratios of papaya latex to solvent were varied from 1:0.5 to 1:4. The extracted samples were collected at 5, 10, 30 and 60 minutes. Figure 5.2 and 5.3 presented the activity of crude extracted enzyme and protein yield of extraction, respectively. It was found that the activity of crude extracted enzyme and yield of extraction did not depend on time. This was because mass transfer rate of enzyme was very high. The activity of crude enzyme extracted from both solvents was similar, about 1.4-1.5 unit/ml. The yield of water extraction was higher than the one of buffer extraction for each ratio. Therefore the optimal extraction time was assigned at 10 minutes.

The yields of extraction of papaya latex at the fixed extraction time of 10 minutes and various ratios of latex to solvents ratios from 1:0.5 to 1:4 were presented in Figure 5.4. The yield of enzyme extraction by water were higher than buffer for all ratios. As volumetric ratios of papaya latex to water were increased from 1:1 to 1:4, the yield of water extraction was increased slightly from 77.8% to 85.7%. However, the use of high volumetric ratio, i.e. 1:4, would result in low protein concentration, which was difficult to lyophilize or precipitate. Therefore the extraction ratio of latex to water was fixed at 1:1. As volumetric ratios of papaya latex to buffer solution were increased from 1:1 to 1:4, the yield of buffer extraction was decreased from 64.9% to 37.7%. It was found that the optimal ratio of buffer extraction was 1:1. It would be the result of pH of supernatant from water extraction, buffer extraction, and pI of enzyme (pH5, pH6, and pI8.75, respectively). In general, enzyme precipitated in solution at its pI where the net charges of enzyme equal to zero. At all ratios of latex to solvent, protein concentration from water extraction was higher than that from buffer extraction because pH of supernatant from water extraction was lower than that from buffer extraction. At low volumetric fraction of solvent, the protein yield of enzyme extraction using solvent was limited by volume of solvent. On the other hand, at high volumetric fraction of solvent, the protein yield of enzyme extraction was limited by the difference between pH of solution and pI of enzyme. This result was the same as that reported by Apichaikijdamrong *et al*. (1990). They studied the papain extraction

from waste papaya peel at 4°C using phosphate buffer solution as a solvent and suggested that optimal ratio of papaya peel to phosphate buffer solution was 1:1.

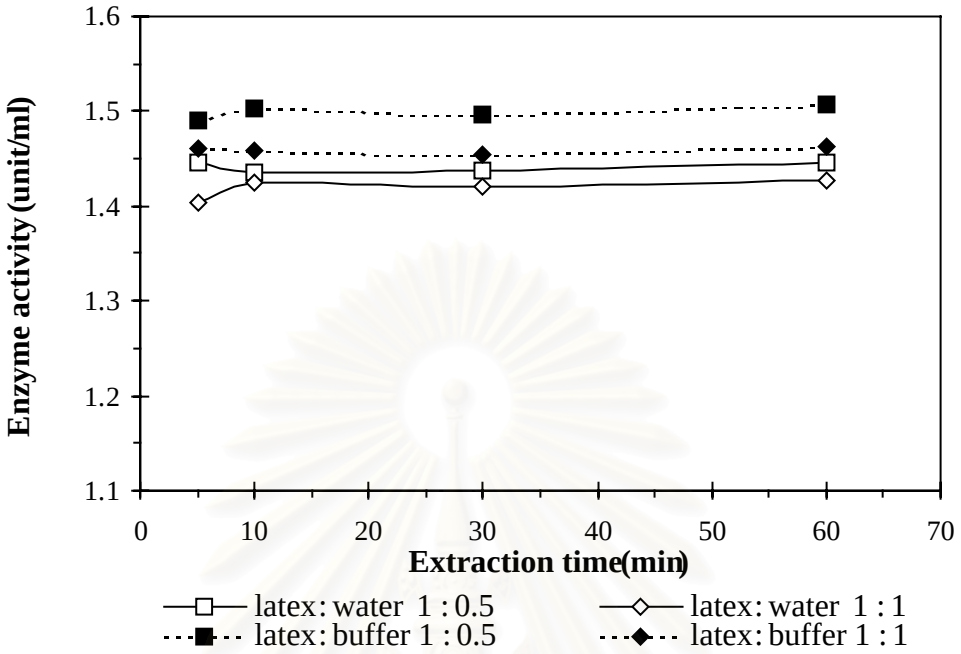

Figure 5.2 Activity at 70°C and pH 6 of crude enzyme extracted from the ratios of latex:solvent = 1:0.5 and 1:1

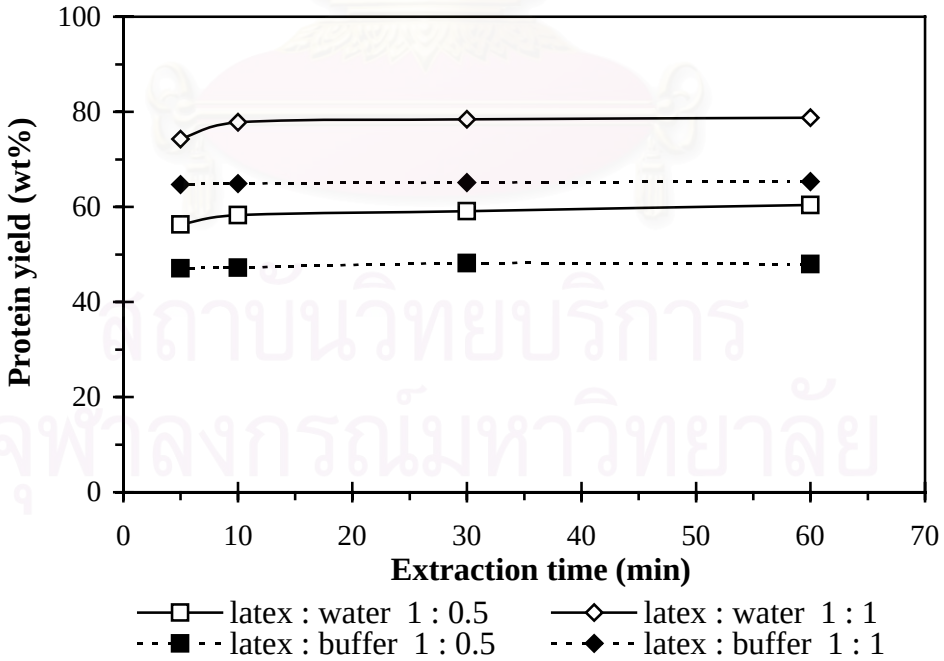

Figure 5.3 The yield of enzyme extraction from the ratios of latex:solvent = 1:0.5 and 1:1

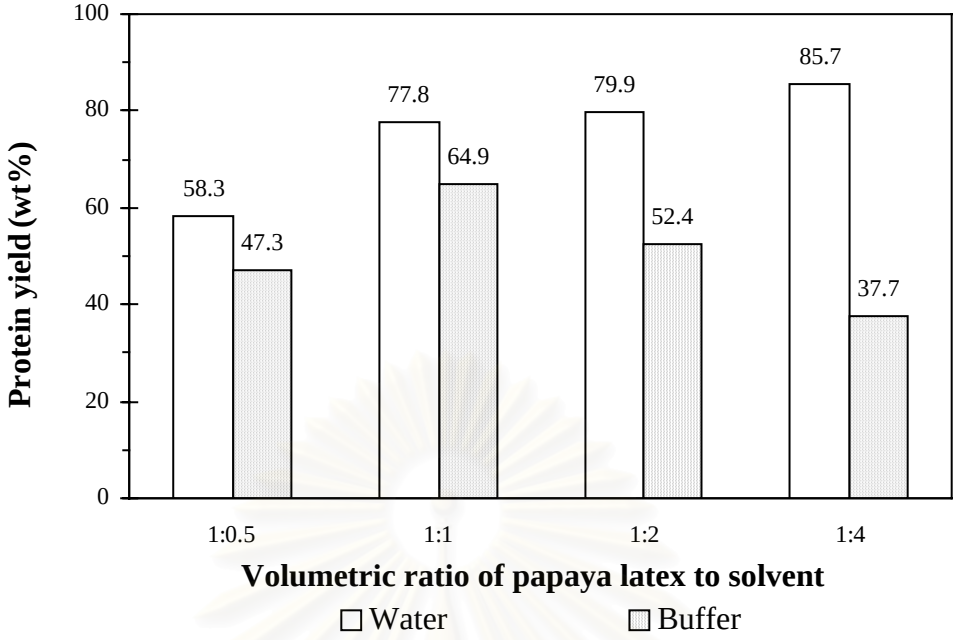

Figure 5.4 Protein yield of enzyme extraction at 10 minutes by two solvents at various ratios.

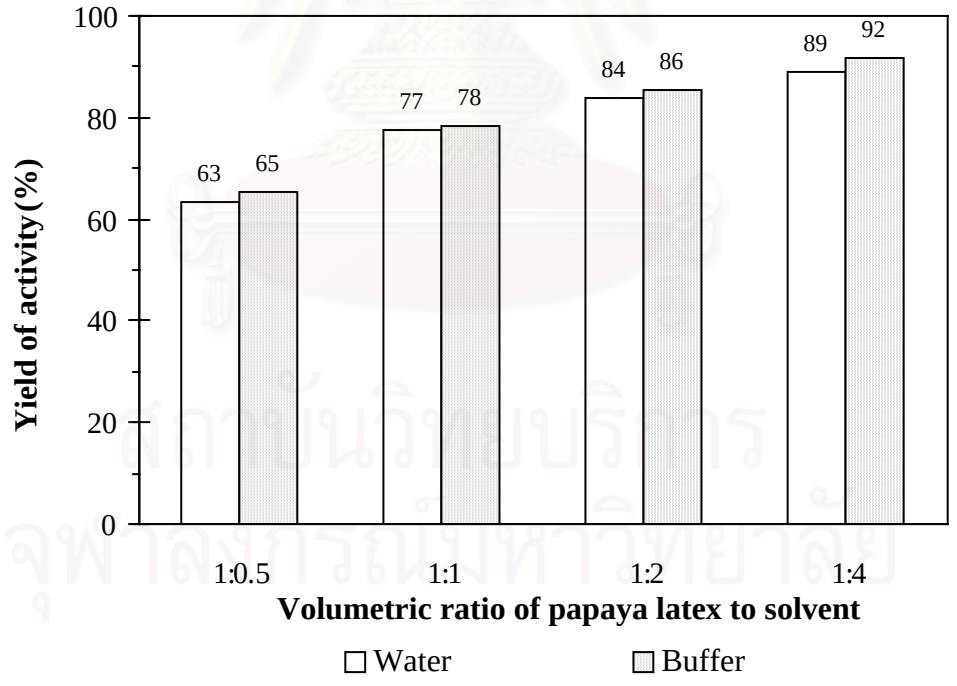

Figure 5.5 Yield of activity of enzyme extraction at 10 minutes by two solvents at various ratios.

Figure 5.5 presented the yield of activity of crude extracted enzyme. It was found that the yields of activity from water and buffer extraction were similar, the protein yield from buffer extraction was less than that from water extraction, though. It might be because phosphate buffer pH6 could preserve activity of enzyme better than water.

At the high volumetric solvent fraction, the yield of activity from extraction was high but the enzyme concentration was too low to proceed to the next step. So crude enzymes from solvent extraction at the ratio of latex to solvent = 1:1 were selected. Therefore a part of crude enzymes were taken to lyophilize, and the other part was further proceeded to the step of purification by precipitation with ammonium sulfate.

After lyophilization, the activity of dried crude enzymes extracted from the ratio of latex to solvent 1:1 was assayed as a function of temperature and pH6. Figure 5.6 presented the activity of 1% w/v crude enzyme solution from solvent extraction at various temperatures and pH 6. Activities of crude enzymes were increased as the temperature increased from 55^{o}C to 75^{o}C, but decreased when the temperature was higher than 75^{o}C for the case of water extraction. For the use of buffer solvent, the activity was rather consistent when increasing the temperature from 75^{o}C to 85^{o}C. The highest activity of crude enzyme was found to be at around 75^{o}C. The highest activity of crude enzyme extracted by water was slightly higher than that by buffer.

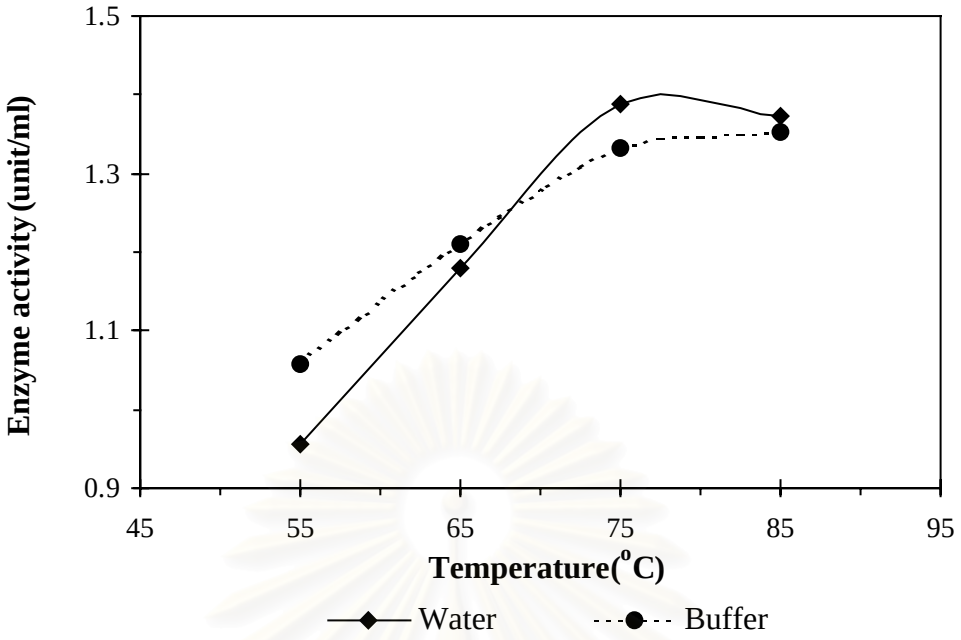

Figure 5.6 Activity of 1% w/v crude enzyme from extraction ratio of latex to solvent 1:1 at various temperatures, pH6.

5.1.1.3 Precipitation by ammonium sulfate

The activity yield of extracted enzyme before and after precipitation using ammonium sulfate was shown in Figure 5.7. After precipitation step using ammonium sulfate, it was found that the yield of activity of extracted enzyme was decreased by 15% and 20% for the case of water and buffer extraction, respectively. This corresponded to the fact that the yield of activity would be lower when it was purified. The activity of extracted enzyme using water as the solvent, presented in Figure 5.8, showed that, after precipitation by ammonium sulfate, there was a decrease in the activity of proteolytic enzymes by 15–68% over the temperature range of 55-85°C. The results on the yield and activity of extracted enzyme before and after precipitation corresponded with the work of Wuttiwiangtham (1993). He has reported that the yield of activity and activity of extracted enzyme after precipitation with ammonium sulfate and sodium chloride were lower than before precipitation but specific activity was higher.

From the results on the yield of activity and activity of enzyme from solvent extraction and precipitation step, crude enzyme from water extraction at the ratio of

latex to solvent 1:1 (further referred as crude extracted enzyme) was selected to be used for raw hide hydrolysis compared to the use of commercial papain.

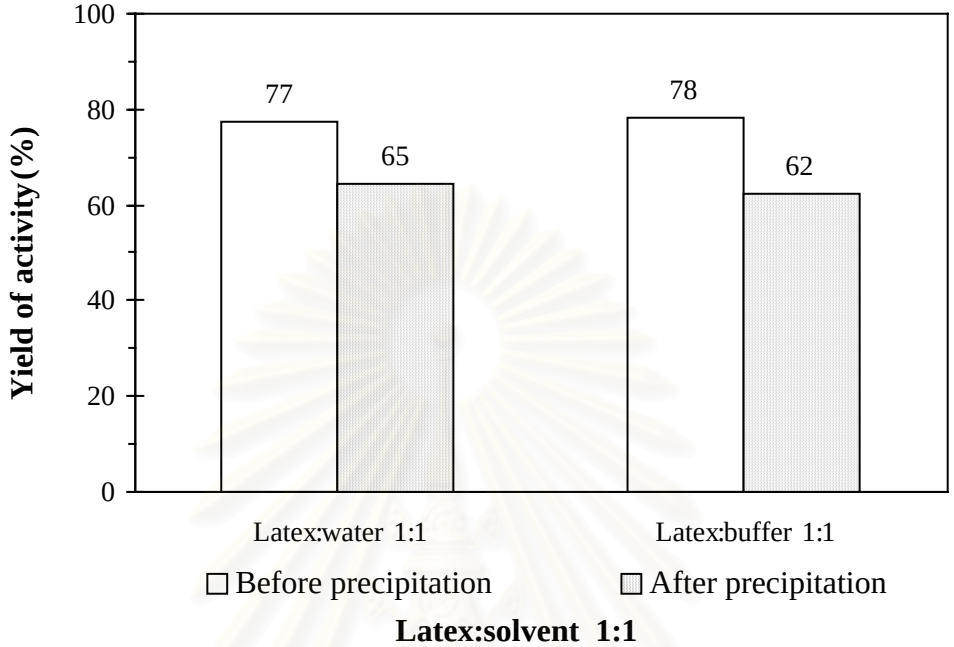

Figure 5.7 Yield of activity of extracted enzyme before and after ammonium sulfate precipitation

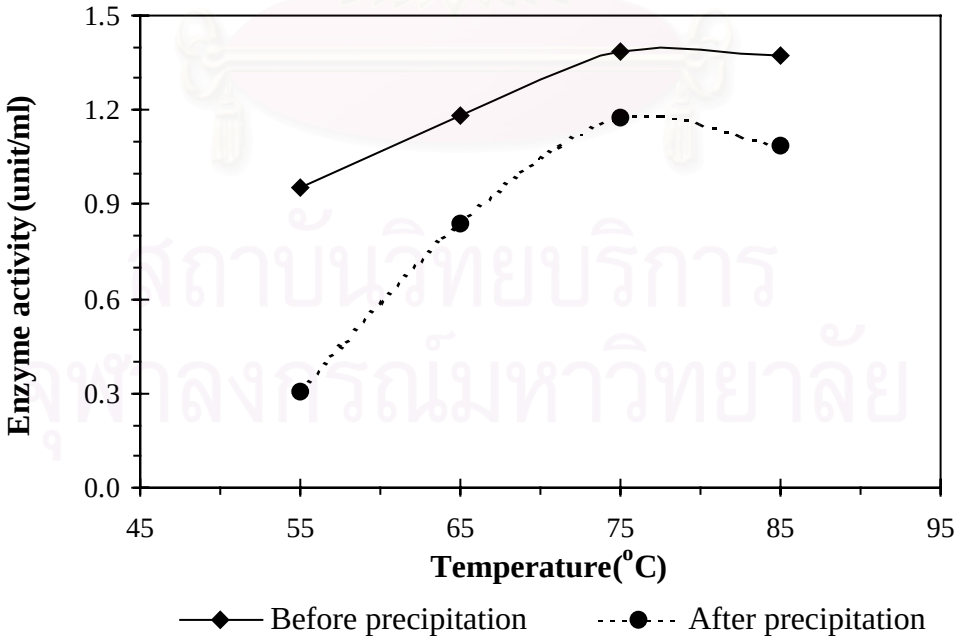

Figure 5.8 Activity of 1% w/v crude enzyme solution from water extraction at ratio 1:1 before and after precipitation at various temperatures at pH 6

5.1.2 Comparison of crude extracted enzyme and commercial papain

In this section, crude extracted enzyme and commercial papain were characterized and compared, in terms of protein content, activity, stability of enzyme activity, and molecular weight of enzyme.

5.1.2.1 Protein content

Protein contents of crude extracted enzyme from papaya latex and commercial papain were reported in Table 5.2. It was noticed that the protein content of crude extracted enzyme was more than that of commercial papain. This could be due to the reason that extracted enzyme contained several types of proteolytic enzymes. The commercial papain might also have additives and stabilizers for commercial uses.

Table 5.2 Protein content of crude extracted enzyme and commercial papain

Enzyme source	Protein content (%wt)
Crude extracted enzyme	74.32
Commercial papain	8.11

5.1.2.2 Activity

Figure 5.9 and 5.10 presented the activity of crude extracted enzyme and commercial papain, respectively. The trend of the activity of both enzymes was similar. It was increased as the temperature increased from 55^{o}C to 75^{o}C, then started to be decreased as the temperature was higher than 75^{o}C. This could be due to the reason that enzymes were denatured at high temperature. At low temperature, the activity of crude extracted enzyme at pH 6 was the lowest, and it was sharply increased up to the highest activity when the temperature reached 75^{o}C. At higher pH (pH 7-8), the activity of crude extracted enzyme was less sensitive to the temperature. The optimal temperature and pH for the highest activity of both enzymes were at pH 6-7 and 75^{o}C. For commercial papain, the activity was very sensitive to the temperature. The optimum condition for the highest activity of commercial papain was found to be the same as crude extracted enzyme. The highest activity of crude extracted enzyme was slightly lower than that of commercial papain, i.e. the highest

activity of crude extracted enzyme and commercial papain were 1.4 and 1.6 unit/ml, respectively.

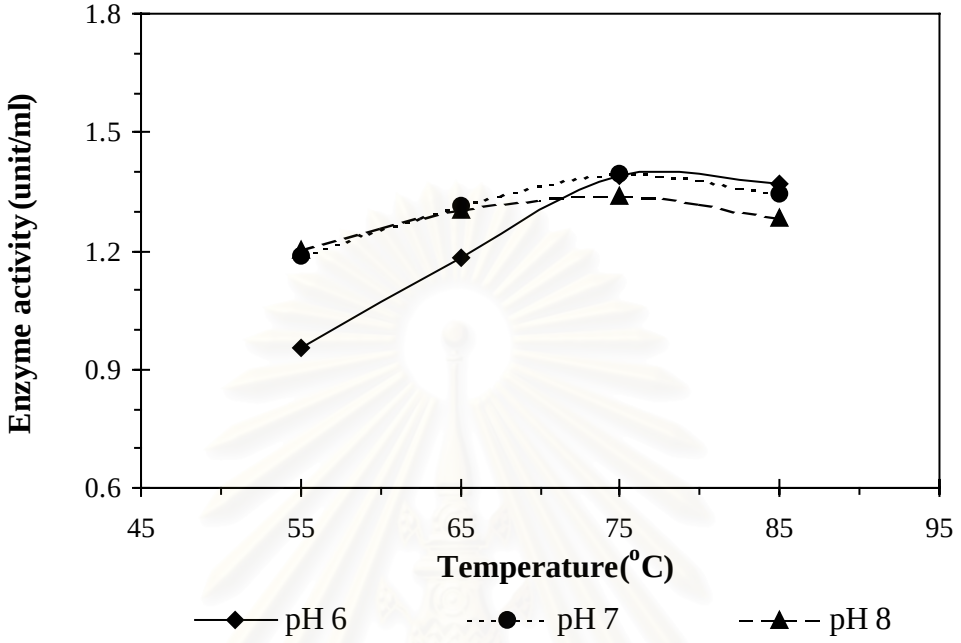

Figure 5.9 Activity of 1% w/v crude extracted enzyme solution (latex:water ratio 1:1) at various temperatures and pH

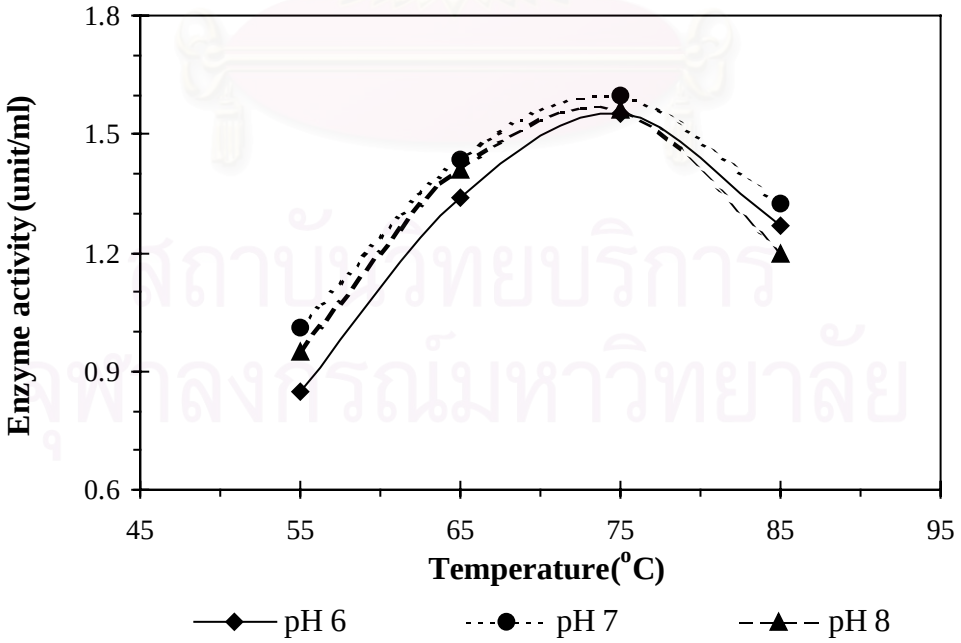

Figure 5.10 Activity of 1% w/v commercial papain solution at various temperatures and pH

5.1.2.3 Stability of enzyme activity

To use the enzyme in the hydrolysis of raw hide, it was necessary to know the stability of enzyme while hydrolysis reaction proceeded. The stability of both enzymes during hydrolysis reaction were investigated using a blank hydrolysis reaction batch (without raw hide) at 75-85^{o}C and pH 7. The sample solutions were collected from the reactor at different hydrolysis times from 1 to 50 minutes for enzyme activity assay. The relative activity of both enzymes was shown in Figure 5.10. At 75^{o}C, the results indicated that commercial papain was more stable than crude enzyme. The relative activity of commercial papain decreased by 3% and 8% within 40 and 50 minutes of hydrolysis reaction, respectively. The activity of crude enzyme decreased by 3%, 6%, and 10% within 30, 40, and 50 minutes of hydrolysis reaction, respectively. This demonstrated that both enzymes could be used in hydrolysis reaction at 75^{o}C and pH 7 for 30-40 minutes. When hydrolysis reaction proceeded longer than 40 minutes, the activity of both enzymes started to significantly drop. At 85^{o}C, stability of crude extracted enzyme was better than commercial papain and both of them were less stable than those at 75^{o}C. It was implied that both enzymes were heat sensitive and easier to deactivate at higher temperature.

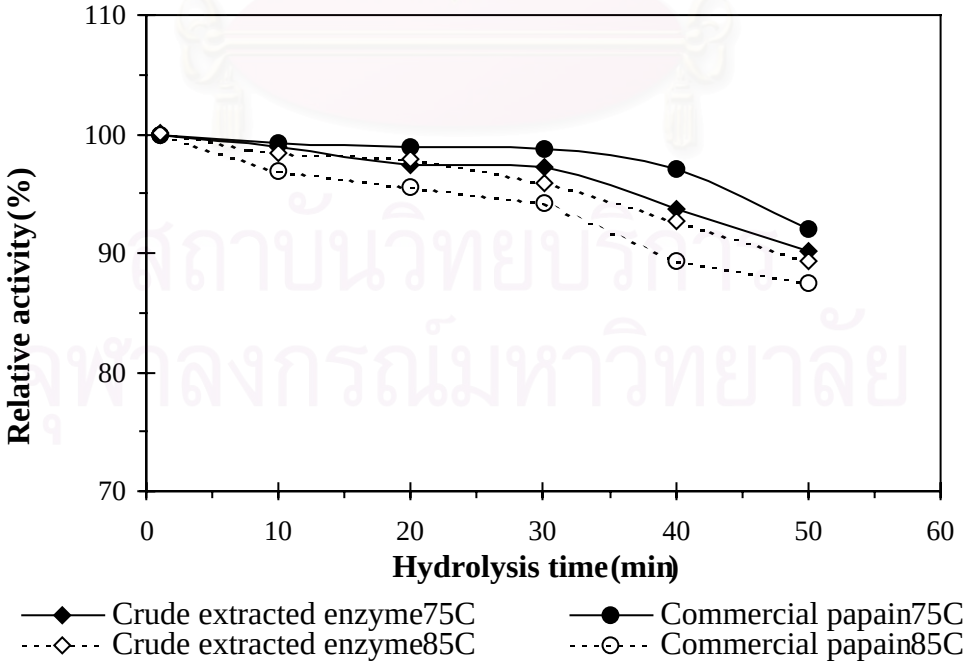

Figure 5.11 Relative activity of crude extracted enzyme and commercial papain at 75 and 85^{o}C, pH 7

5.1.2.4 Molecular weight of enzyme

Figure 5.12 presented the molecular weight of low molecular weight standard marker, crude extracted enzyme, and commercial papain in lane a, b, and c respectively. It could be noticed that for crude extracted enzyme, there were at least 3 main molecular weight bands; 35kDa, 21kDa, and 14kDa. The lowest molecular weight band, 14kDa, was dye marker from the 5X sample buffer. The approximate molecular weight of 35kDa and 21kDa were identified as chymopapain and papain, respectively. In case of commercial papain (lane c), at least 2 protein bands; 21kDa and 14kDa appeared on SDS-PAGE results (Monti *et al*, 2000). Chymopapain was found in crude extracted enzyme because it was the main proteolytic enzyme in papaya latex. Comparing the band of papain at 21kDa in lane b and c, it could be observed that the quantity of papain in crude extracted enzyme was less than that in commercial papain. As known that, papain was the most active proteolytic enzymes in papaya latex, this could result in the lower activity of crude extracted enzyme comparing to that of commercial papain. This corresponded to the results on highest activity of both enzymes reported in Section 5.1.2.2.

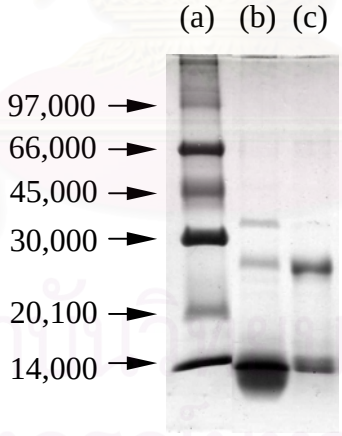

Figure 5.12 SDS-PAGE analysis of: (a) low molecular weight standard marker, (b) crude extracted enzyme, and (c) commercial papain

5.2 Enzymatic hydrolysis of raw hide

5.2.1 Raw hide

Raw hide used in this work was limed split from tanning factory. Usually, liming reagents were used to preserve rawhide from decomposing by microorganism. The source of raw hide, amount of liming reagent and liming time in the tanning treatment were the major factors that caused the difference in residual mineral, protein and fat contents in raw hide. Enzymatic hydrolysis could be affected by protein and fat contents in raw hide. Therefore protein and fat contents of the raw hide sample were determined and presented in Table 5.3. It was observed that protein and fat content in raw hide were 63.62wt% and 15.04wt%, respectively. The remaining was mineral salt and other substances.

Table 5.3 The compositions of raw hide

Composition of raw hide	Content (wt%)
Protein	63.62
Fat	15.04
Others	21.34

5.2.2 Optimum ratio of enzyme to raw hide

The objective of this section was to find the optimum ratio of enzyme to substrate (raw hide) for the hydrolysis batch of 200 g of raw hide. The amount of enzyme used was varied while the recovery of gelatin was determined as a function of hydrolysis time.

Crude extracted enzyme

The hydrolysis of raw hide was carried out at the condition for the highest activity of crude extracted enzyme (75^{o}C, pH 7). The gelatin recovery at various ratios of crude extracted enzyme to raw hide from 0:100 to 0.040:100 (from 0-0.08 g of crude enzyme to 200 g of raw hide) were presented in Figure 5.13.

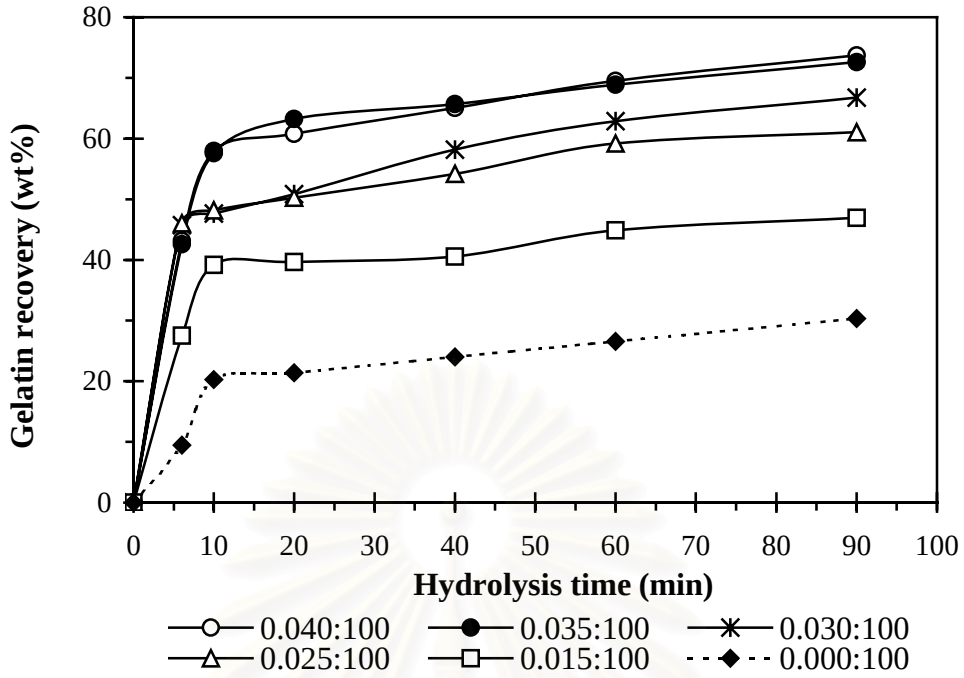

Figure 5.13 Gelatin recovery at various ratios of crude extracted enzyme to raw hide at 75°C, pH7

From Figure 5.13, as crude extracted enzyme to raw hide ratio was increased, the gelatin recovery was increased until the ratio reached 0.350:100. The average percentage of gelatin recovery was increased about 8.7% as the crude enzyme to raw hide was increased by 16.67% (from 0.030:100 to 0.035:100). But the average percentage of gelatin recovery was consistent as the crude enzyme to raw hide ratio increased from 0.035:100 to 0.040:100. It could be stated that the optimum ratio of crude enzyme to raw hide was 0.035:100. Consequently the ratio of crude enzyme to raw hide was assigned at 0.035:100 (0.07 g of crude extracted enzyme to 200 g of raw hide) for further investigation in this work.

Commercial papain

The same investigation was carried out for the case of using commercial papain. The gelatin recovery at various ratios of commercial papain to raw hide from 0:100 to 0.2:100 (from 0-0.4 g of commercial papain to 200 g of raw hide) was shown in Figure 5.14.

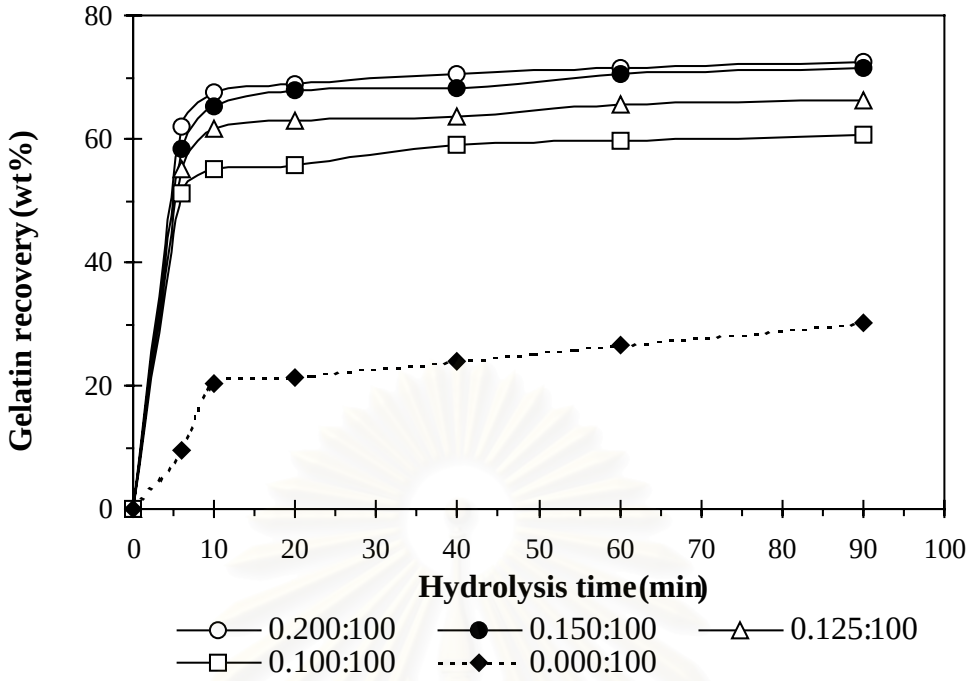

Figure 5.14 Gelatin recovery at various ratios of commercial papain to raw hide at 75^{o}C, pH7

It was noticed that the gelatin recovery was increased as commercial papain to raw hide ratio was increased until commercial papain to raw hide ratio was at 0.15:100. The average percentage of gelatin recovery increased 8 % when the commercial papain to raw hide ratio was increased 20% (from 0.125:100 to 0.150:100) and the average percentage of gelatin recovery did not change when further increasing the commercial papain to raw hide ratio. The optimum commercial papain to raw hide ratio was found to be 0.15:100. Therefore the ratio of commercial papain to raw hide used in further investigation was assigned at 0.15:100 (0.3 g of commercial papain to 200 g of raw hide).

5.2.3 Effects of hydrolysis conditions on gelatin recovery

5.2.3.1 Effects of temperature on gelatin recovery

To investigate the effects of various hydrolysis temperatures at a fixed pH on gelatin recovery, the raw hide hydrolysis reactions were carried out at various temperatures from 65°C to 85°C, pH 7.

Crude extracted enzyme

The percentage of gelatin recovery from crude extracted enzyme hydrolysis reaction was depicted in Figure 5.15. It could be noticed that each initial rate of gelatin recovery was greatly increased (at first 10 minutes of hydrolysis time), then the rate of gelatin recovery was slowly increased when the hydrolysis reaction was continued. The reason for decreasing in recovery rate of gelatin recovery might be due to the deactivation of enzyme by heat, decrement of protein substrate, and product inhibition. Ratanathammapan (2005) reported that the decrease of protein substrate was the main factor controlling the hydrolysis rate.

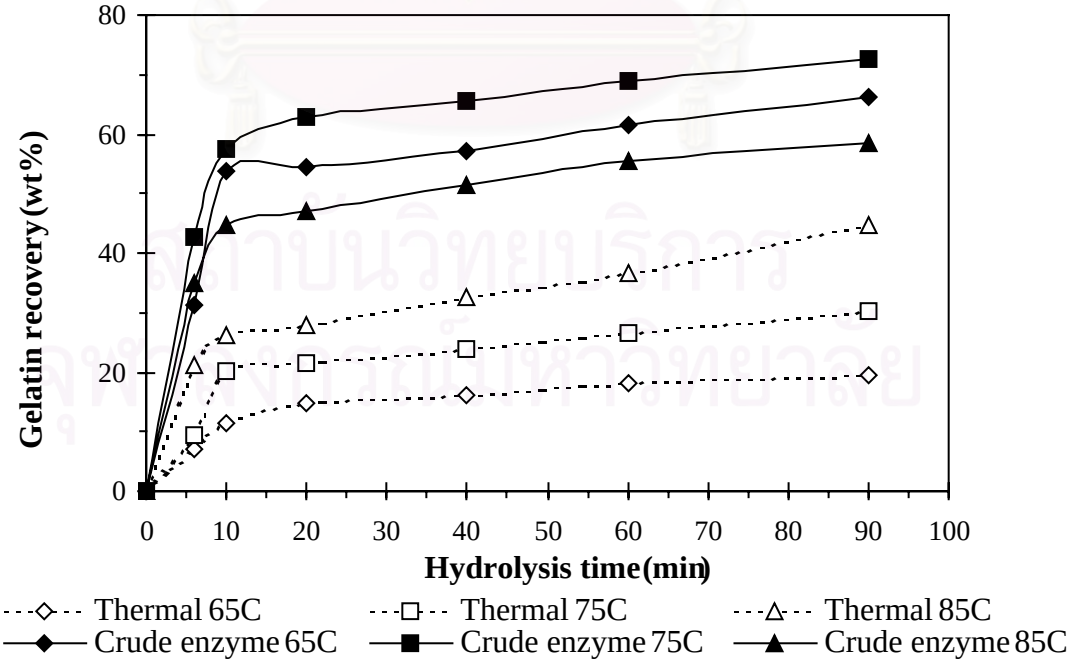

Figure 5.15 Gelatin recovery from enzymatic hydrolysis and thermal hydrolysis at pH 7 and various temperatures

When the temperatures of crude extracted enzyme hydrolysis at pH 7 were varied from 65^{o}C to 85^{o}C, it was found that the percentage of gelatin recovery at 65^{o}C, 75^{o}C and 85^{o}C were in the same pattern as previously described. However, the percentages of gelatin recovery at three temperatures were different, i.e. the percentage of gelatin recovery at 75^{o}C was the highest and following by the one at 65^{o}C and 85^{o}C. When the hydrolysis reaction continued to 90 minutes, the percentage of gelatin recovery at 75^{o}C, 65^{o}C, and 85^{o}C was 72wt%, 66wt%, and 58wt%, respectively. The highest gelatin recovery achieved at 75^{o}C was the result from the highest activity of crude extracted enzyme.

In this work the enzymatic hydrolysis reaction was carried out at high temperature (from 65^{o}C to 85^{o}C) at which collagen in raw hide could be thermally hydrolyzed. Therefore the gelatin recovery in Figure 5.15 were the combination of both thermal and enzymatic hydrolysis reaction. The gelatin recovery of thermal hydrolysis reaction at high temperature was very important so the thermal hydrolysis of raw hide at each constant temperature was determined. Results shown in Figure 5.14 indicated that the percentages of gelatin recovery from thermal hydrolysis at pH7 were significantly different depending on the temperature. The highest temperature resulted in highest percentage of gelatin recovery. At the hydrolysis time of 90 minutes, the gelatin recovery of thermal hydrolysis at 65^{o}C, 75^{o}C and 85^{o}C was about 19wt%, 30wt% and 45wt%, respectively. This was because the triple helix structure of collagen molecules in raw hide was stable with hydrogen and a number of covalent bonds. When temperature was above 40^{o}C, theses bonds were cleaved and collagen was converted to gelatin (Gomez-Guillen *et al.*, 2005). The result on thermal hydrolysis corresponds with the study of Muyonga *et al* (2004). When the temperature of the thermal hydrolysis was increased from 50^{o}C to 95^{o}C, the percentage of total protein recovered from skin and bone of Nile perch was increased.

To avoid the effects of thermal hydrolysis, the actual gelatin recovery from only crude extracted enzyme hydrolysis was achieved by subtracting the gelatin recovery from thermal hydrolysis from that of enzymatic hydrolysis, as illustrated in Figure 5.16.

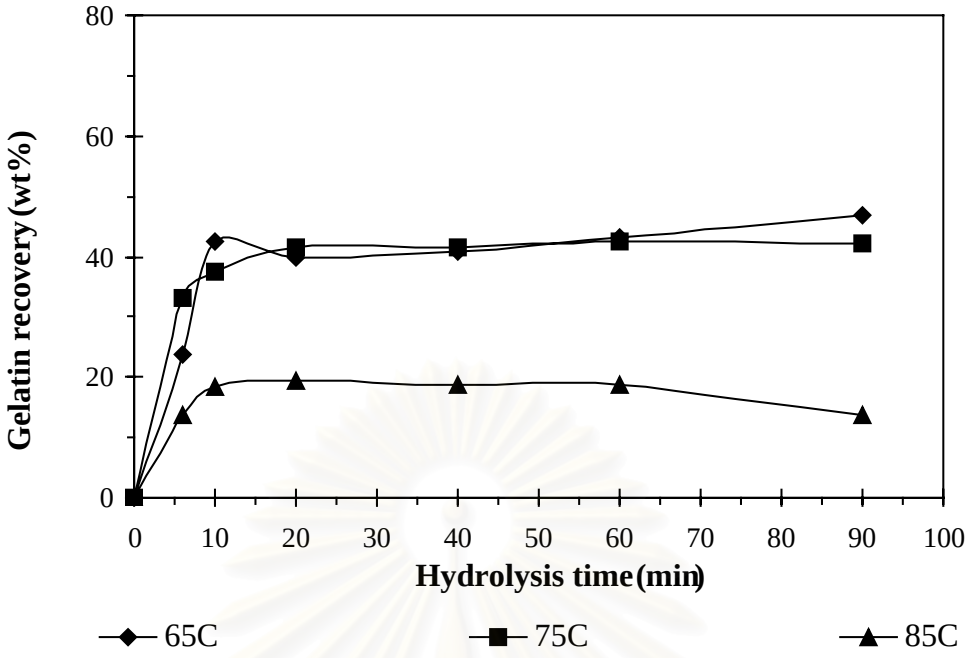

Figure 5.16 Gelatin recovery from only enzymatic hydrolysis using crude enzyme at pH 7 and various temperatures

From Figure 5.16, the actual gelatin recovery of crude extracted enzyme hydrolysis at 65°C and 75°C were similar but that at 85°C was lowest. It might be because of the competition between thermal and enzymatic hydrolysis. The gelatin recovery from thermal hydrolysis at 75°C was more than that at 65°C. At 75°C, the substrate concentration for enzymatic hydrolysis was not as high as at 65°C. As a result, the enzymatic hydrolysis at 75°C was limited. Therefore, the actual gelatin recovery at 65°C and 75°C were similar. At 85°C, the gelatin recovery from thermal hydrolysis was highest. It was because the substrate concentration for enzymatic hydrolysis was very low composing to at lower temperature and, moreover, enzyme activity was lowest. So, the actual gelatin recovery at 85°C was lowest.

Commercial papain

The percentage of gelatin recovery from commercial papain hydrolysis reaction at each constant temperature and pH7 were presented in Figure 5.17. The patterns of gelatin recovery from commercial papain hydrolysis were similar to patterns from crude extracted enzyme hydrolysis, but the initial rates of commercial papain hydrolysis were higher. At the end of hydrolysis reaction, the percentage of

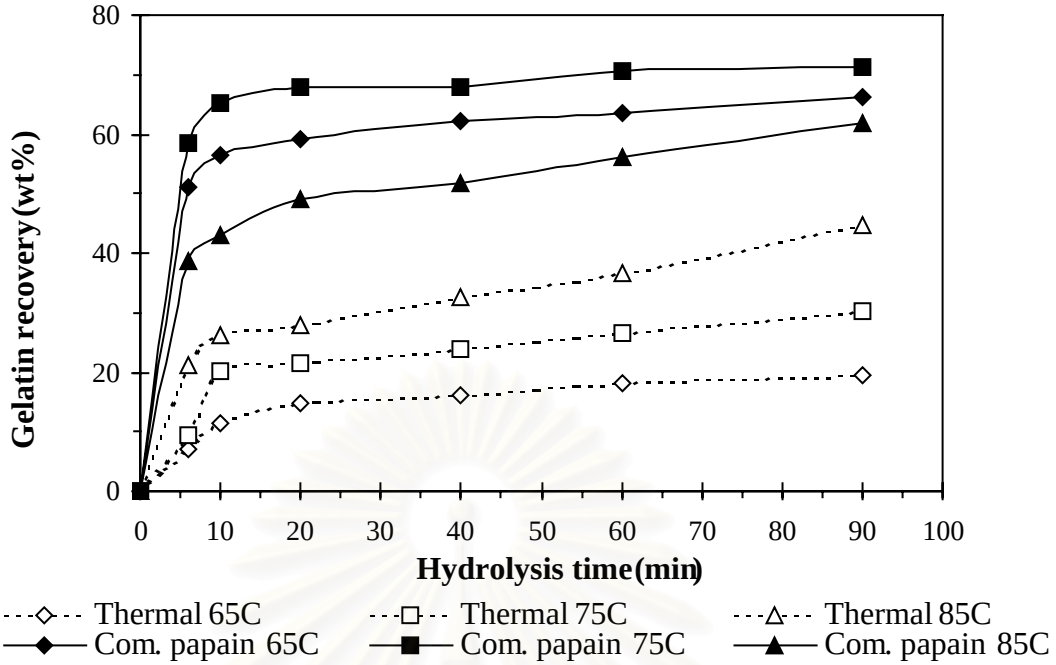

Figure 5.17 Gelatin recovery from enzymatic hydrolysis and thermal hydrolysis at pH 7 and various temperatures

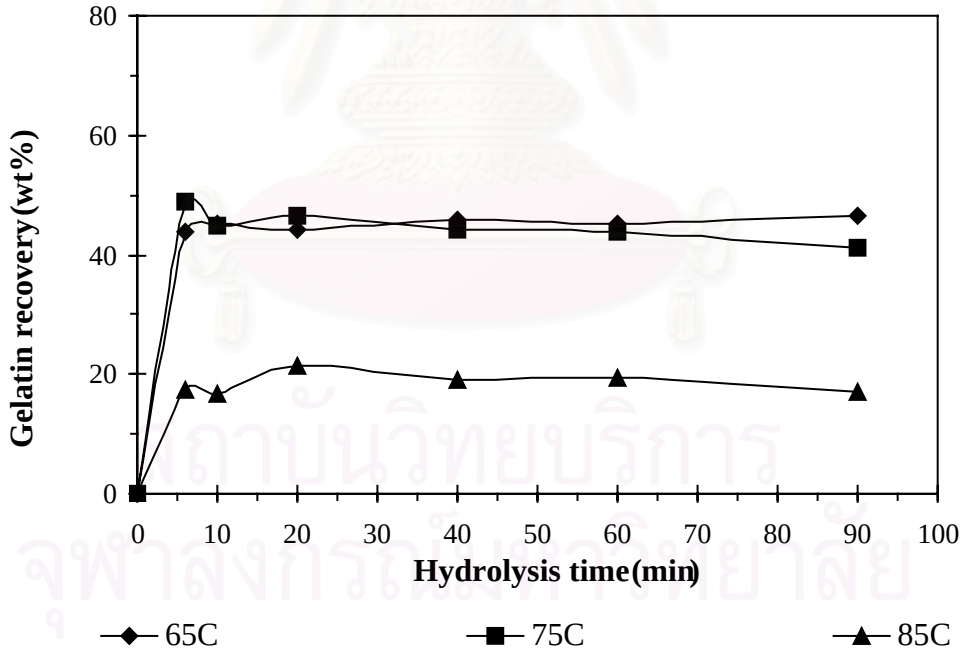

Figure 5.18 Gelatin recovery from only enzymatic hydrolysis using commercial papain at pH 7 and various temperatures

gelatin recovery at 65^{o}C, 75^{o}C, and 85^{o}C was 66wt%, 71wt%, and 61wt%, respectively. This might be because the stability and papain purity of the commercial papain was higher than those of the crude extracted enzyme

Figure 5.18 presented the actual gelatin recovery after subtracting the gelatin recovery of thermal hydrolysis from that of enzymatic hydrolysis. It was noticed that the patterns of actual gelatin recovery from only commercial papain hydrolysis at three temperatures (65^{o}C, 75^{o}C and 85^{o}C) were the same as the case of crude extracted enzyme. The results on the gelatin recovery and the actual gelatin recovery from commercial papain hydrolysis corresponded with the work of Ratanathammapan *et al*. (2005). It was reported that, the highest gelatin recovery was found at 75^{o}C and pH7. At pH 7, the actual gelatin recoveries from 65^{o}C and 75^{o}C at were similar.

5.2.3.2 Effects of pH on gelatin recovery

From section 5.2.3.1, the results on the effects of enzymatic hydrolysis temperature on the gelatin recovery showed that the optimum hydrolysis temperature for the highest gelatin recovery at pH 7 was 75^{o}C. The hydrolysis temperature was then fixed at 75^{o}C when investigating the effect of pH (from 6 to 8) on gelatin recovery from raw hide.

Crude extracted enzyme

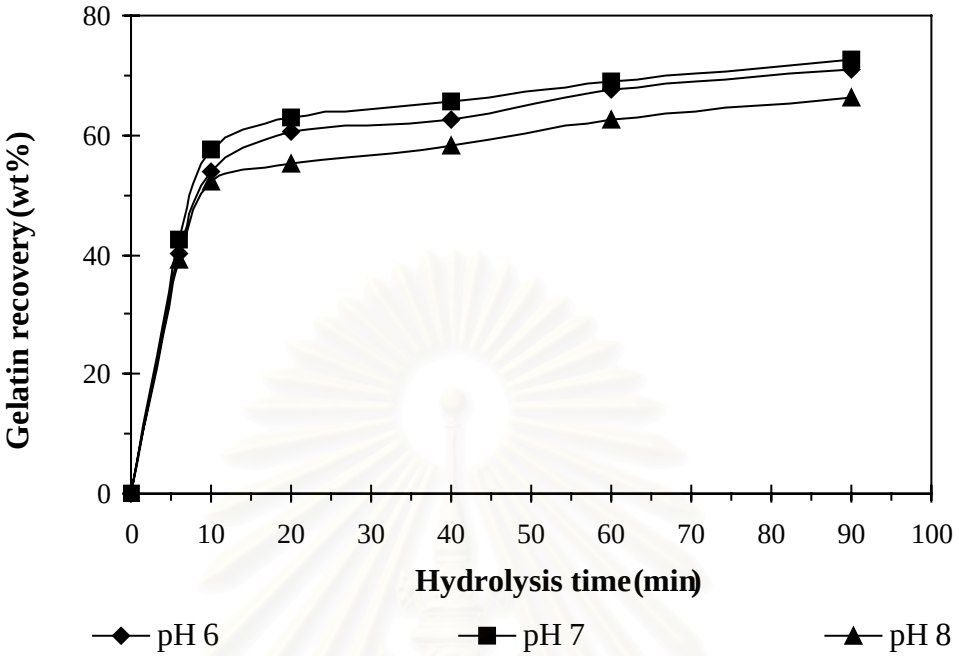

Figure 5.19 Gelatin recovery from enzymatic hydrolysis at constant temperature of 75°C and various pH

The percentages of gelatin recovery from crude extracted enzyme hydrolysis reaction at 75°C and various pH were presented in Figure 5.19. It was observed that the initial rates of gelatin recovery (6 minutes) at three different pH were very similar. After 6 minutes of hydrolysis time, the percentages of gelatin recovery at pH 6 to 8 were increased slightly with the same pattern until the end of hydrolysis reaction at the time of 90 minutes. The percentages of gelatin recovery from crude extracted enzyme hydrolysis at pH 6 and 7 were similar and higher than the one at pH 8. This was because the activity of crude extracted enzyme at pH 6 and pH 7 was similar and slightly higher than that at pH 8. The percentages of gelatin recovery from crude extracted enzyme hydrolysis at pH 6, 7 and 8 reached 70wt%, 72wt% and 66wt%, respectively.

Commercial papain

Figure 5.20 showed the percentages of gelatin recovery from commercial papain hydrolysis reaction at 75°C and various pH from 6 to 8.

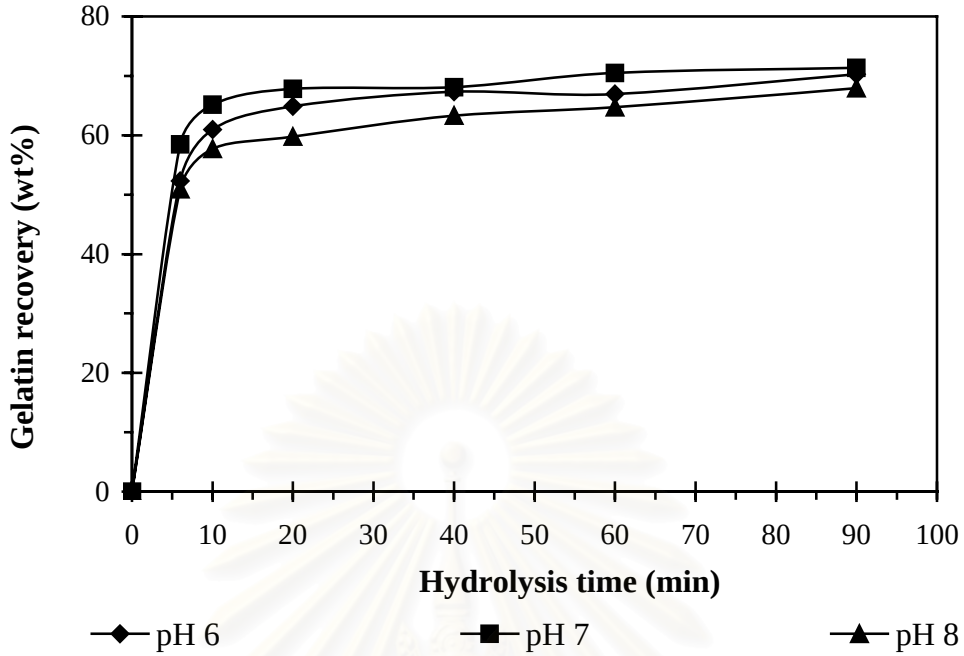

Figure 5.20 Gelatin recovery from enzymatic hydrolysis at constant temperature of 75^{o}C and various pH

It was found that the trend of gelatin recovery from the commercial papain hydrolysis at 75^{o}C and various pH was the same as the trend of the crude extracted enzyme hydrolysis. It was also due to the activity of commercial papain of which the trend was similar to that of crude extracted enzyme. When the hydrolysis time reached 90 minutes, the percentages of gelatin recovery at pH 7, pH 6, and pH 8 were 71wt%, 70wt% and 68wt%, respectively.

From the result of gelatin recovery, it could be observed that effects of temperature on gelatin recovery were stronger than the pH. The crude extracted enzyme and commercial papain hydrolysis reactions gave the highest gelatin recovery at 75^{o}C, pH7 and the lowest at 85^{o}C, pH7. The gelatin recovery of crude extracted enzyme and commercial papain were the result from their activities. At the beginning of hydrolysis reaction, the percentage of gelatin recovery from hydrolysis reaction using crude extracted enzyme was increased at a lower rate comparing to the one using commercial papain. At the end of reaction, the percentages of gelatin recovery from crude extracted enzyme and commercial papain were similar.

5.2.4 Effects of hydrolysis conditions on gelatin properties

5.2.4.1 Effects of temperature on gelatin properties

Crude extracted enzyme

The gel strength and viscosity of gelatin solution from crude extracted enzyme hydrolysis reaction of raw hide at various temperatures and pH 7 were illustrated in Figure 5.21 and 5.22, respectively.

From Figure 5.21, it was found that the gel strength of gelatin solution obtained at 90 minutes was higher than the one at 10 minutes for each temperature. At 10 and 90 minutes of reaction, the gel strength of gelatin solution at 85^{o}C was highest, following by the one at 65^{o}C and 75^{o}C. At 90 minutes, the highest gel strength obtained from the hydrolysis reaction at 85^{o}C, pH 7 was 187.6 g Bloom, and the lowest obtained from the one at 75^{o}C, pH 7 was 58.4 g Bloom. The lowest gel strength was observed at temperature of 75^{o}C corresponding to the condition for the highest enzyme activity. As the enzyme activity was high, the enzyme shortened the peptide chains rapidly to the low molecular weight or LMW peptide chains. The reason for the low gel strength of gelatin solution might be due to the low molecular weight fraction of gelatin solution. At 85^{o}C, enzyme was deactivated by heat. The molecular weight of gelatin obtained from this condition might be higher than that obtained from 65^{o}C and 75^{o}C.

From Figure 5.22, it could be noticed that the viscosity of gelatin solution extracted at 10 and 90 minutes were similar. The viscosity of gelatin solutions obtained from the hydrolysis reaction at 65^{o}C and 75^{o}C were similar (2.6-3.1 cP) but slightly lower than the one at 85^{o}C (4.2-4.4 cP). The viscosity of gelatin solution obtained from the hydrolysis reaction at 85^{o}C, pH 7 and 90 minutes was highest (4.4 cP). At this hydrolysis condition, the highest gel strength of gelatin was also observed. This would be the result of long chain gelatin or high molecular weight gelatin.

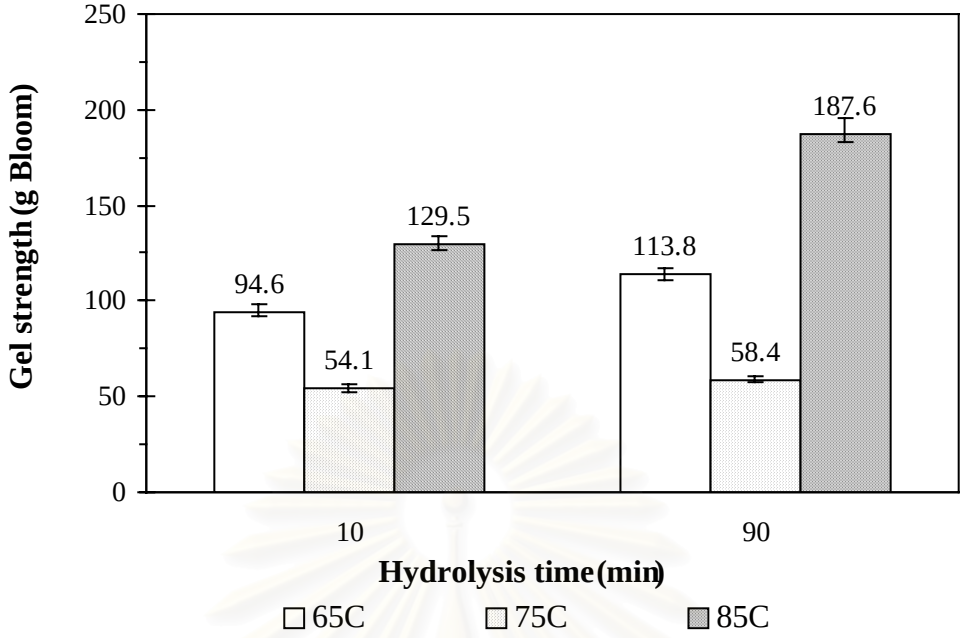

Figure 5.21 Gel strength of gelatin from hydrolysis reaction using crude extracted enzyme at various temperatures, pH 7

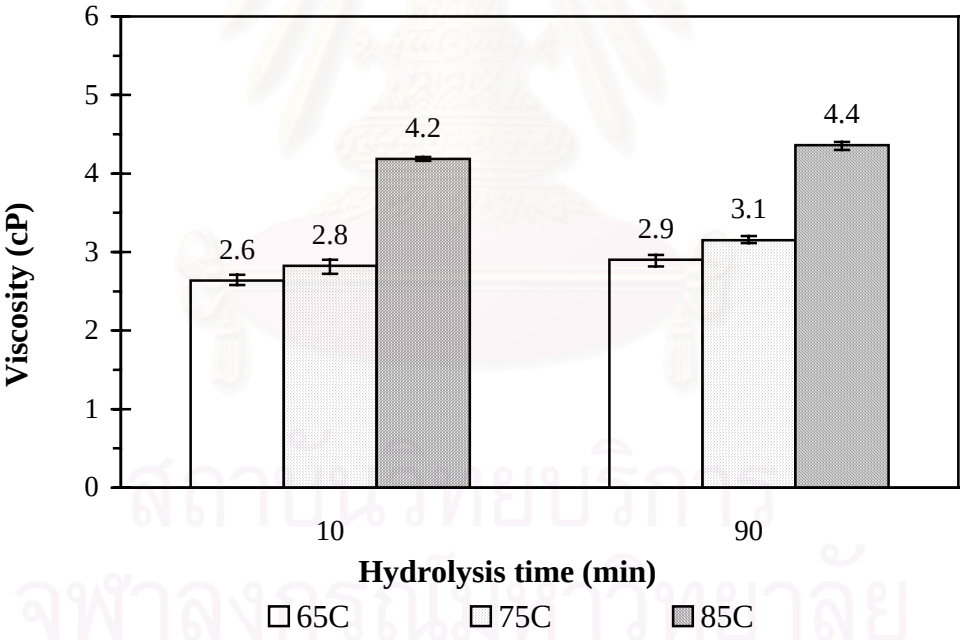

Figure 5.22 Viscosity of gelatin solution from hydrolysis reaction using crude extracted enzyme at various temperatures, pH 7

Commercial papain

Figure 5.23 and 5.24 showed the gel strength and viscosity of gelatin solution from commercial papain hydrolysis reaction of raw hide at various temperatures (65^{o}C to 85^{o}C), and pH 7, respectively.

From Figure 5.23, it was found that the gel strength of gelatin solution from commercial papain hydrolysis was similar to that from crude extracted enzyme hydrolysis, shown in Figure 5.21. At 90 minutes, the gel strength of gelatin solution at pH 6, pH 7, and pH 8 was 104.3 g Bloom, 55.8 g Bloom, and 166.4 g Bloom, respectively.

When the temperature of hydrolysis reaction at pH7 varied from 65^{o}C to 85^{o}C, it was seen that the viscosity of gelatin solution obtained tended to be gradually increased. The viscosity of gelatin solution obtained at 10 and 90 minutes of hydrolysis similar.

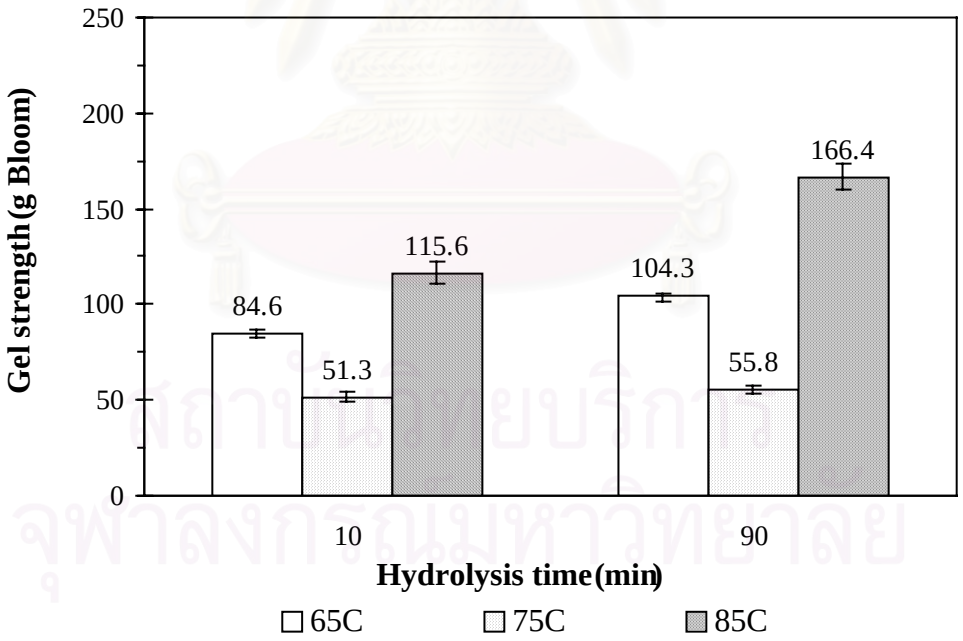

Figure 5.23 Gel strength of gelatin from hydrolysis reaction using commercial papain at various temperatures, pH 7

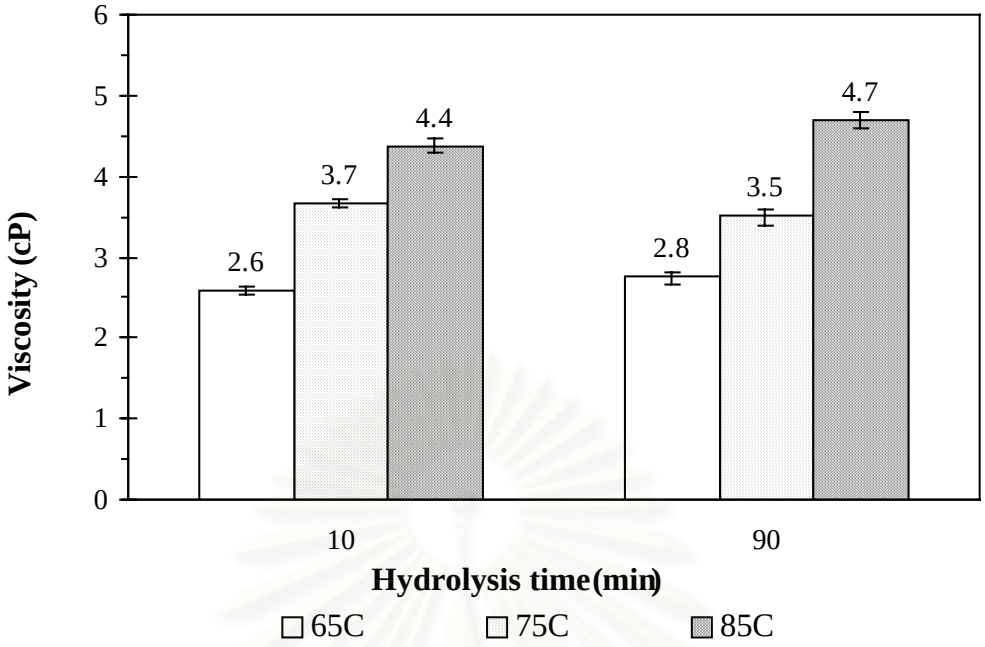

Figure 5.24 Viscosity of gelatin solution from hydrolysis reaction using commercial papain at various temperatures, pH 7

5.2.4.2 Effects of pH on gelatin properties

Crude extracted enzyme

Figure 5.25 and 5.26 demonstrated the gel strength and viscosity of gelatin solution from crude extracted enzyme hydrolysis reaction of raw hide at various pH and 75^{o}C, respectively.

It was noticed that when the hydrolysis reaction at 75^{o}C continued from 10 to 90 minutes, the gel strength of gelatin obtained at pH 6 and pH 7 were similar but the one from pH 8 was significantly increased. This was because the optimum hydrolysis condition of crude extracted enzyme at 75^{o}C was at pH 6 and pH 7. The short peptide chains of gelatin were then obtained at these conditions. For pH 8, the gel strength of gelatin was highest because the activity of enzyme was lower than the one of pH 6 and pH 7. This might be because of the high molecular weight gelatin at low enzyme activity condition (pH 8). When the hydrolysis reaction continued to 90 minutes, the gel strength of gelatin at pH 6, pH 7, and pH 8 was 51.7g Bloom, 58.4 g Bloom, and 162.1 g Bloom, respectively.

From Figure 5.26, it could be observed that the viscosity of gelatin solution from hydrolysis reaction at 75°C and various pH was similar. When each hydrolysis reaction at fixed pH was continued from 10 to 90 minutes, the viscosity did not change. They were in the range of 2.8-3.8 cP.

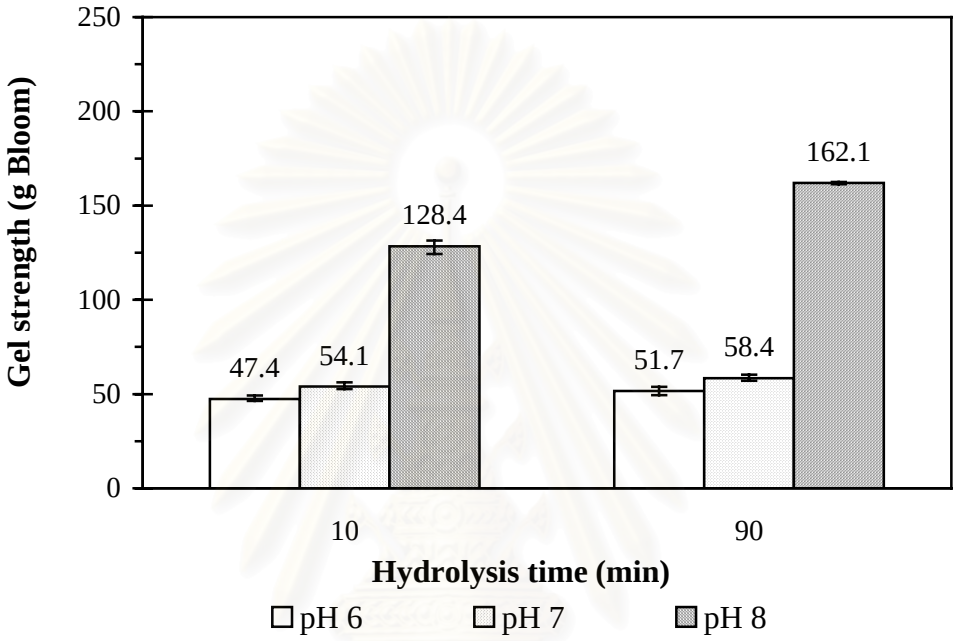

Figure 5.25 Gel strength of gelatin from hydrolysis reaction using crude extracted enzyme at various pH, 75°C

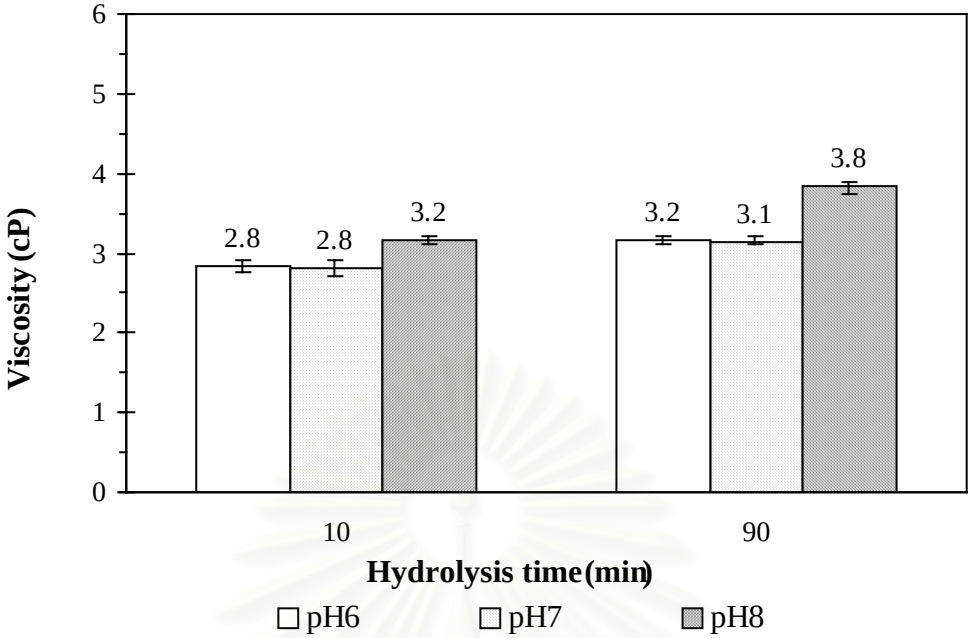

Figure 5.26 Viscosity of gelatin solution from hydrolysis reaction using crude extracted enzyme at various pH, 75°C

Commercial papain

The gel strength and viscosity of gelatin solution from the commercial papain hydrolysis at 75°C and various pH were showed in Figure 5.27 and 5.28, respectively.

It was observed that the gel strength and viscosity of gelatin solution obtained at pH 6, pH 7 and pH 8 were in the same pattern as those of gelatin obtained from crude extracted enzyme hydrolysis. The gel strength of gelatin at pH 8 was the highest and following by the one at pH 7 and pH 6.

The viscosity of gelatin solution from commercial papain hydrolysis at 75°C was higher than that from crude extracted enzyme hydrolysis (especially at pH 8). It was because the activity of commercial papain sharply dropped more than that of crude extracted enzyme at pH 8. Hence the high molecular weight and viscosity of gelatin solution from commercial papain hydrolysis were higher than that from crude extracted enzyme hydrolysis. They were varied in the range of 2.9-4.6 cP.

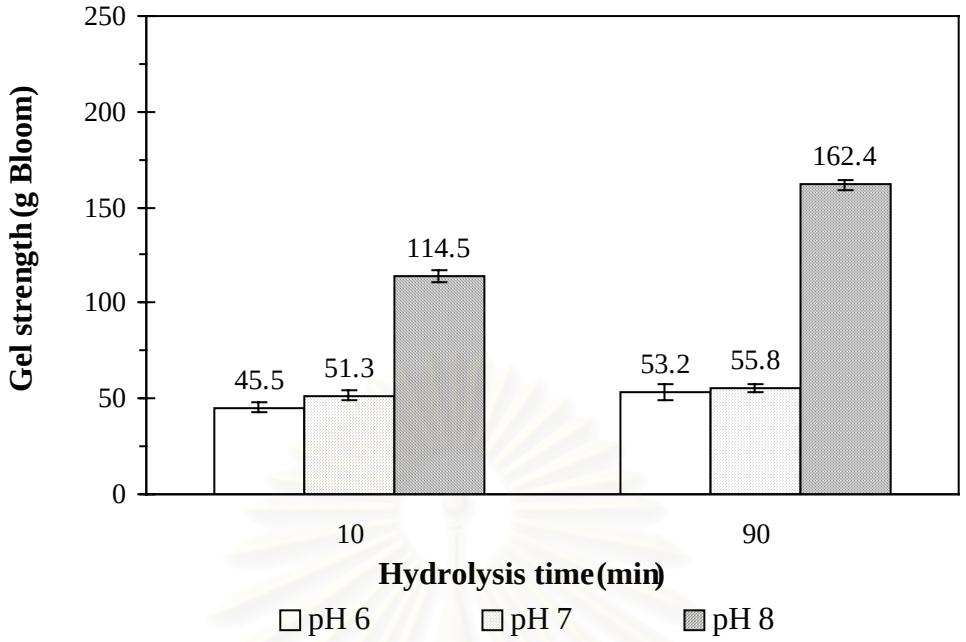

Figure 5.27 Gel strength of gelatin from hydrolysis reaction using commercial papain at various pH, 75°C

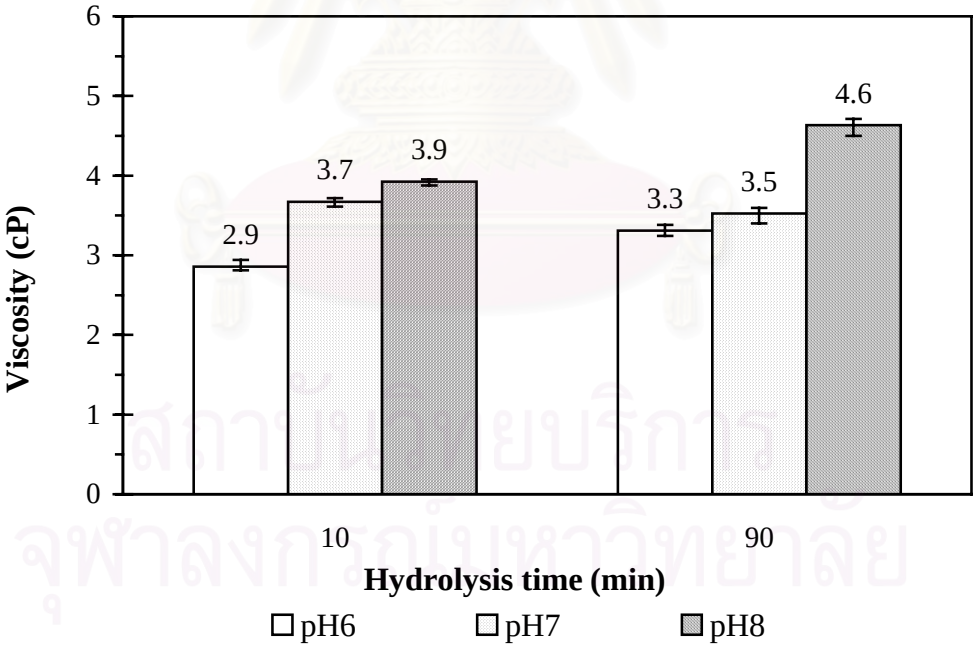

Figure 5.28 Viscosity of gelatin from hydrolysis reaction using commercial papain at various pH, 75°C

5.2.4.3 Molecular weight of recovered gelatin

The molecular weight of dialyzed gelatin recovered from crude extracted enzyme and commercial papain hydrolysis at pH 7 was determined with SDS-PAGE as presented in Figure 5.29.

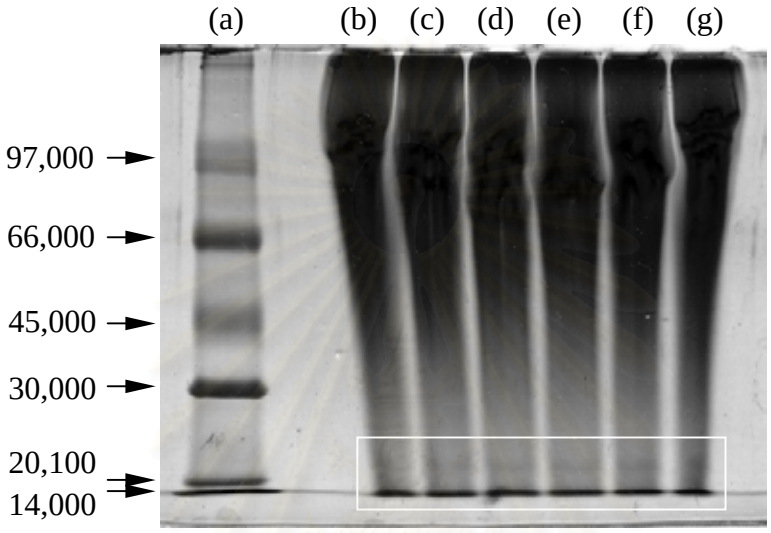

Figure 5.29 SDS-PAGE analysis of gelatins:

(a) low molecular weight standard marker
(b) crude extracted enzyme hydrolysis at 75^{o}C and 10 minutes
(c) crude extracted enzyme hydrolysis at 75^{o}C and 90 minutes
(d) crude extracted enzyme hydrolysis at 85^{o}C and 90 minutes
(e) commercial papain hydrolysis at 75^{o}C and 10 minutes
(f) commercial papain hydrolysis at 75^{o}C and 90 minutes
(g) commercial papain hydrolysis at 85^{o}C and 90 minutes

It was found that for all gelatin samples, there were at least 2 molecular weight bands; 14kDa and 21kDa on SDS-PAGE results. The band of protein at 21kDa might be papain used in hydrolysis reaction. It was noticed that smear of protein appeared on all gelatin samples. The molecular weight of gelatin could not be determined by SDSPAGE technique. It might be because of the impurities in the solution and the denaturation of gelatin.

5.2.4.4 Ash of recovered gelatin

Ash content of gelatin obtained from enzymatic hydrolysis reaction was presented in Table 5.4.

Table 5.4 Ash content of gelatin obtained from hydrolysis reaction using crude extracted enzyme and commercial papain

Hydrolysis condition of gelatin sample		Ash content (wt%)
Crude extracted enzyme	75^{o}C, pH 7, 90min	16.2
E:S = 0.035:100	85^{o}C, pH 7, 90min	16.9
Commercial papain	75^{o}C, pH 7, 90 min	16.3
E:S = 0.15:100	85^{o}C, pH 7, 90 min	16.2

It was found that average ash contents of gelatin samples obtained from various hydrolysis reactions were similar (about 16-17 wt%). According to ISO, the ash content of food and parametrical gelatin is not allowed to be greater than 0.18wt%. The ash content of extracted gelatins in this work were higher than the ash content of gelatin in ISO. It was because there were impurities in gelatin obtained from this work such as mineral salt and fat. If these gelatins were purified, the ash content might be decreased.

5.2.5 Comparison of crude enzyme and commercial papain hydrolysis

Table 5.5 and 5.6 summarized the percentage of gelatin recovery, gel strength, and viscosity of gelatin obtained from crude extracted enzyme and commercial papain hydrolysis, respectively.

Table 5.5 The relationship of percentages of gelatin recovery and the gelatin properties from crude extracted enzyme hydrolysis at 90 minutes and pH 7

Temperature (^{o}C)	Gelatin recovery (wt%)	Gel Strength (g Bloom)	Viscosity (cP)
65	66.2	113.8	2.9
75	72.6	58.4	3.1
85	58.4	187.6	4.4

Table 5.6 The relationship of percentages of gelatin recovery and the gelatin properties from commercial papain hydrolysis at 90 minutes and pH 7

Temperature (^{o}C)	Gelatin recovery (wt%)	Gel Strength (g Bloom)	Viscosity (cP)
65	66.1	104.3	2.8
75	71.4	55.8	3.5
85	61.8	166.4	4.7

Comparing at each hydrolysis reaction, it could be observed that gelatin recovery from both types of hydrolysis was very similar but the properties of gelatin obtained were slightly different. The gel strength of gelatin from crude extracted enzyme hydrolysis was slightly higher than the one from commercial papain. The viscosity of gelatin solution from crude extracted enzyme hydrolysis was not different from that from commercial papain hydrolysis. It might be because the gelatin chain was short. This confirmed that the crude extracted enzyme from papaya latex could be effectively used in the hydrolysis of raw hide, same as the use of commercial papain.

When considering the amount and total activity of crude extracted and commercial papain used in the hydrolysis reaction at optimum condition for highest gelatin recovery (75^{o}C, pH7), were compared in Table 5.7, it could be observed that

the amount of crude extracted enzyme used was much less than that of commercial papain but the total activity of them were similar. That was the reason for the same gelatin recovery and properties of gelatin from crude extracted enzyme and commercial papain hydrolysis.

Table 5.7 Comparison of the activity and cost of crude extracted enzyme and commercial papain used in hydrolysis reaction at 75^{o}C and pH 7

Enzyme	Amount (g)	Total activity (unit)	Enzyme cost (baht/100 g enzyme)
Crude extracted enzyme	0.07	46	300
Commercial papain	0.3	47	1600

Comparing the cost of enzymes, the cost of commercial papain was quoted as the available lab grade enzyme which the cost of crude extracted enzyme from papaya latex was calculated based on the investment of papaya tree planting, latex collecting, and crude extraction of enzyme using water as solvent. It was shown that the cost of crude extracted enzyme was around five times lower than that of commercial papain. This ensured that crude enzyme by water extraction of papaya latex could be employed in the enzymatic hydrolysis of raw hide with a considerably cheaper cost comparing to commercial papain. Quality of gelatin obtained from both types of enzymatic hydrolysis was similar, i.e. low gel strength gelatin was obtained. This suggested that crude extracted enzyme could be used instead of commercial papain for the gelatin production from raw hide source.

5.2.6 Effects of enzyme to raw hide ratio on gelatin recovery and properties

From previously mentioned results, one can notice that at each condition of both crude extract enzyme and commercial papain hydrolysis, the percentage of gelatin recovery or gelatin yield could be related to the properties of gelatin. At the optimum condition for the highest activity of enzyme (75^{o}C, pH7), highest gelatin recovery from both enzymatic hydrolysis reactions was achieved but the gel strength of gelatin obtained was lowest. On the contrary, at the condition for relatively low activity of enzymes (such as 85^{o}C, pH7), the gelatin recovery from both enzymatic hydrolysis reactions was quite low while the relatively high gel strength of gelatin

was obtained. These were based on the hydrolysis reaction of which the optimum ratio of enzyme to raw hide substrate was fixed, i.e. 0.035:100 for crude extracted enzyme hydrolysis and 0.15:100 for commercial papain.

At this part, it was interesting to further examine the influence of enzyme to raw hide ratios on the gelatin recovery and gelatin property at each hydrolysis condition. At the optimum condition for highest enzyme activity, the enzyme to raw hide ration would be decreased while at the condition for relatively low enzyme activity, the enzyme to substrate ratio would be increased. From the previous result, the viscosity of gelatin solution was low (2.5 cP to 4.5 cP). It was because of the low molecular weight of gelatin. In this part, the viscosity of gelatin solution did not determined.

Crude extracted enzyme

At the condition for the highest activity of enzyme (75^{o}C, pH7), the ratio of crude extracted enzyme to raw hide was decreased from 0.035:100 to 0.:100. The percentage of gelatin recovery and gel the strength of gelatin were illustrated in Figure 5.30, and 5.31, respectively. (The percentage of gelatin recovery was reproduced from Figure 5.13.)

When the ratio of crude extracted enzyme to raw hide was increased, it was observed as expected that at hydrolysis time of 90 minutes, the percentage of gelatin recovery was decreased which the gel strength of gelatin obtained was increased. It could be noticed that the gel strength of gelatin from the hydrolysis at low enzyme to raw hide ratio (0.015:100) and that from thermal hydrolysis (0:100) were similar which the recovery of gelatin of the former one was about 20wt% than that of the later one. This implied that the addition of very small amount of crude enzyme resulted in an increase in gelatin recovery but had no effect on gel strength of gelatin obtained.

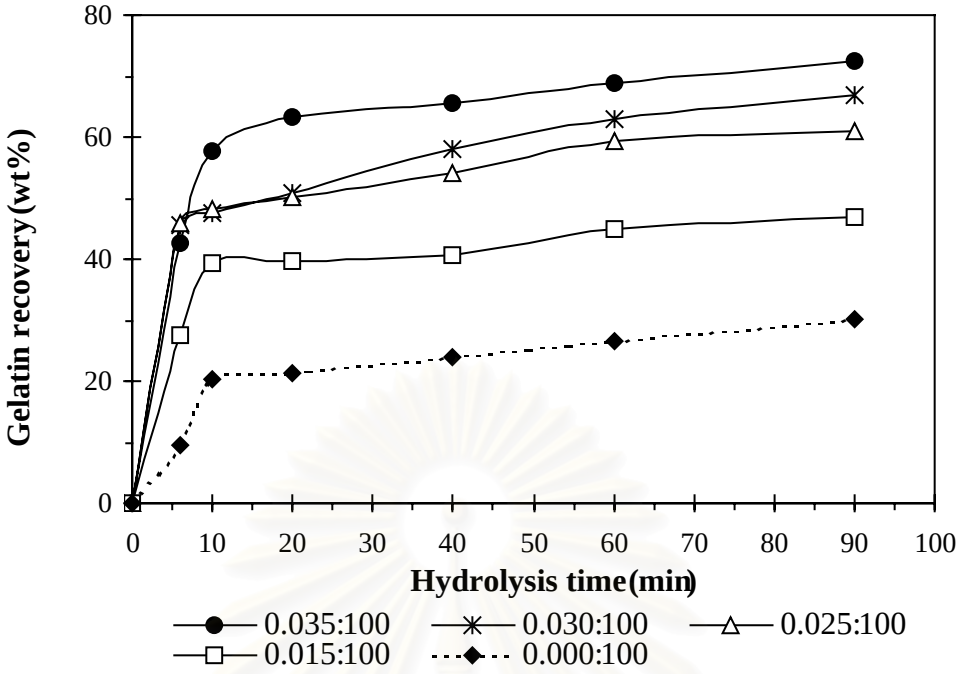

Figure 5.30 Gelatin recovery from crude extracted enzyme hydrolysis at various enzyme to raw hide ratios, 75°C and pH 7

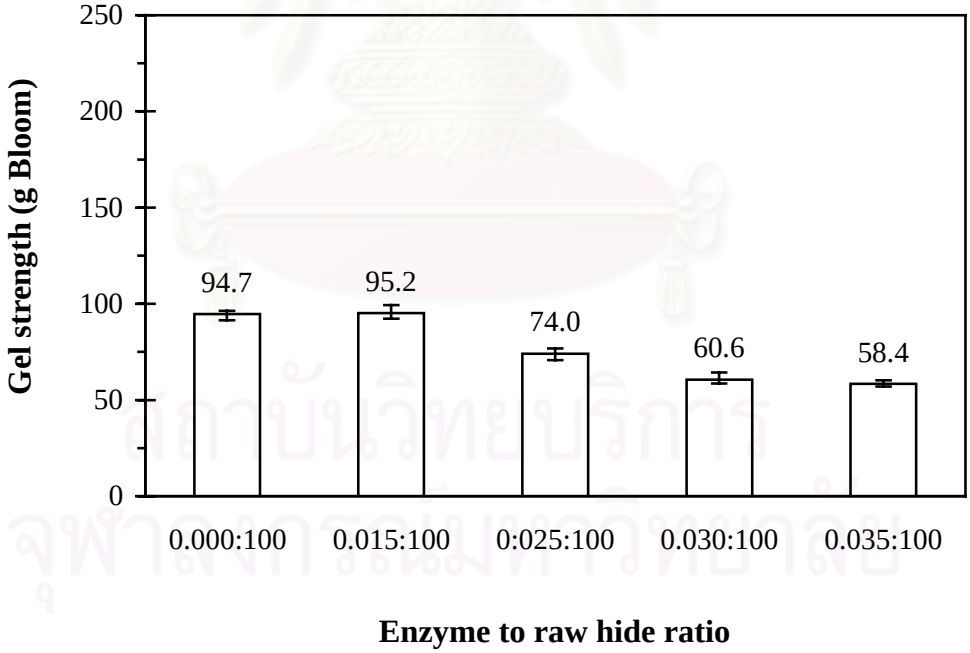

Figure 5.31 Gel strength of gelatin from crude extracted enzyme hydrolysis at various enzyme to raw hide ratios, 75°C, pH 7, and 90 minutes

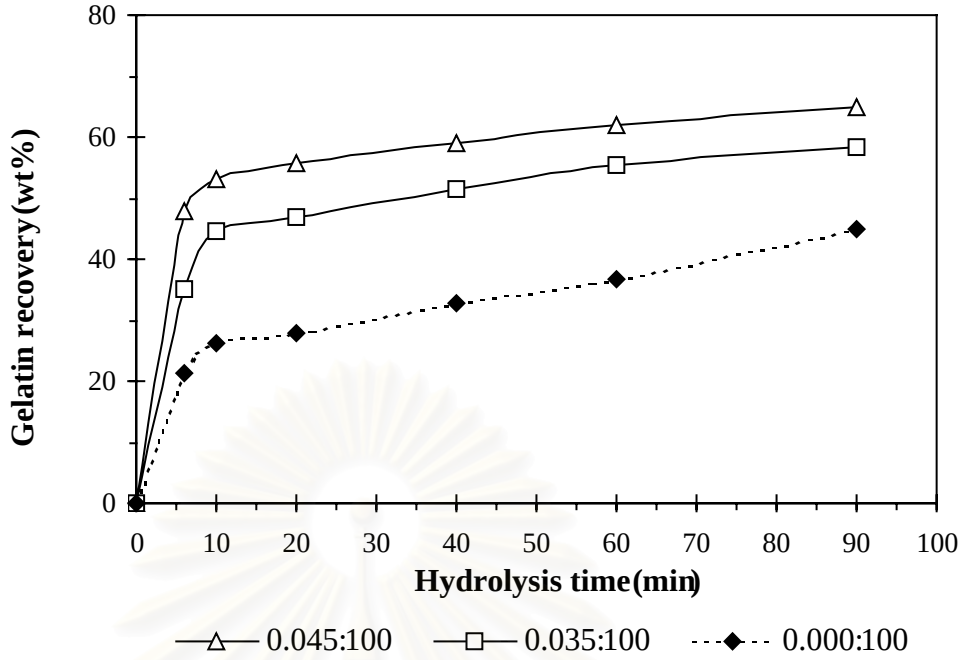

Figure 5.32 Gelatin recovery from crude extracted enzyme hydrolysis at various enzyme to raw hide ratios, 85°C and pH 7

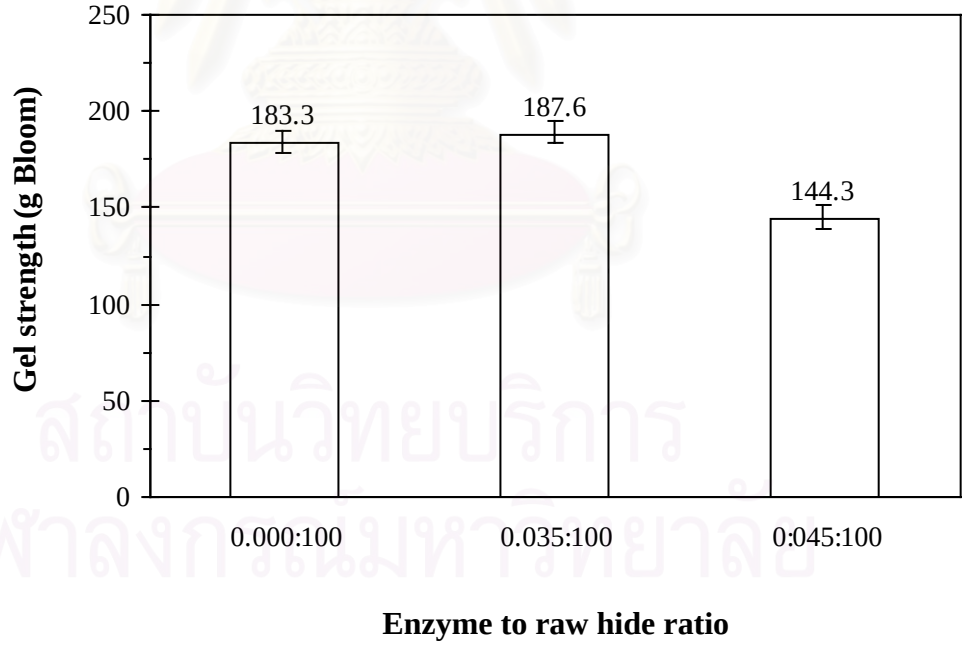

Figure 5.33 Gel strength of gelatin from crude extracted enzyme hydrolysis at various enzyme to raw hide ratios, 85°C, pH 7 and 90 minutes

At the relatively low activity of enzyme (85^{o}C, pH7), the ratio of crude extracted enzyme to raw hide was increased from 0.035:100 to 0:045:100. Figure 5.32, and 5.33 presented the percentage of gelatin recovery and gel strength of gelatin, respectively.

It was observed that percentage of gelatin recovery was increased and the gel strength of the gelatin was decreased when the enzyme to raw hide ratio was increased 0.035:100 to 0.045:100. It was because the activity of enzyme at the ratio of 0:045:100 was higher than that at the ratio of 0.035:100. However, the gel strength of gelatin obtained at the ratio of 0:100 (thermal hydrolysis) and 0:035 were similar.

The result suggested that crude extracted enzyme to raw hide ratio also had an pronounced influence on the percentage of gelatin recovery and the property of gelatin received. One could, therefore, desire the yield of the gelatin recovery and the property of gelatin by manipulating either enzyme to raw hide ratio or the hydrolysis conditions (temperature and pH of hydrolysis reaction).

Commercial papain

For the case of commercial papain hydrolysis at the highest enzyme activity (75^{o}C, pH7), the enzyme to raw hide ratio was decreased from 0.15:100 to 0:100. Figure 5.34, and 5.35 showed the percentage of gelatin recovery, and gel strength of gelatin, respectively.

It could be noticed that the percentage of gelatin recovery was increased but the gel strength of gelatin was decreased as the ratio of commercial papain to raw hide ratio decreased. The gel strength of gelatin obtained from commercial papain hydrolysis at various enzyme to raw hide ratios were similar and those were lower than the gel strength obtained from thermal hydrolysis (enzyme to rawhide ratio at 0:100). It might because the enzyme activity was very high, then the protein chains were shortened to low molecular weight gelatin rapidly.

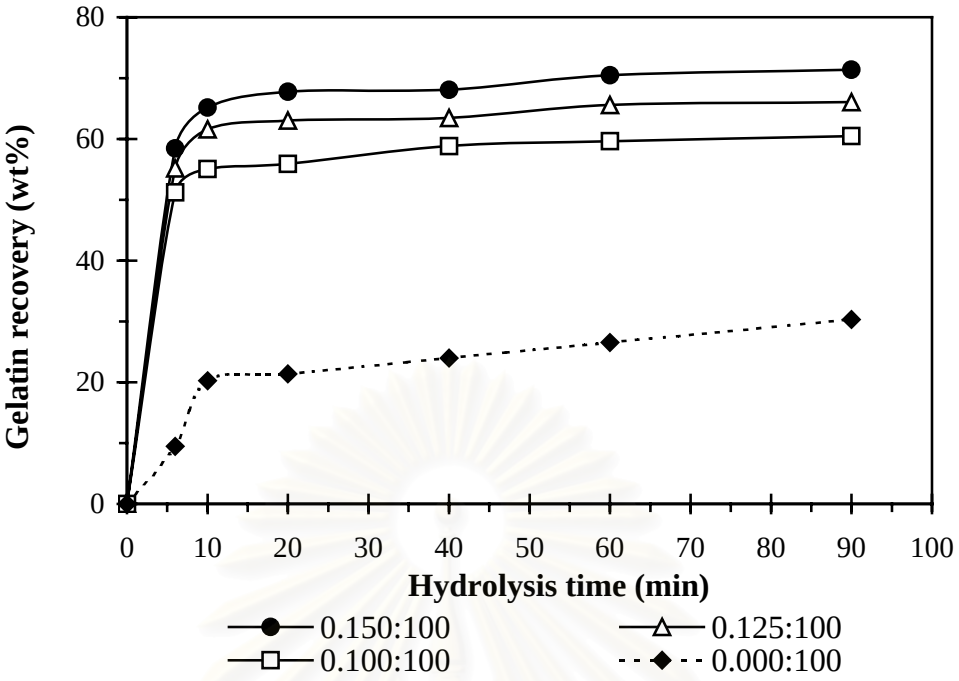

Figure 5.34 Gelatin recovery from commercial papain hydrolysis at various enzyme to raw hide ratios, 75^{o}C and pH 7

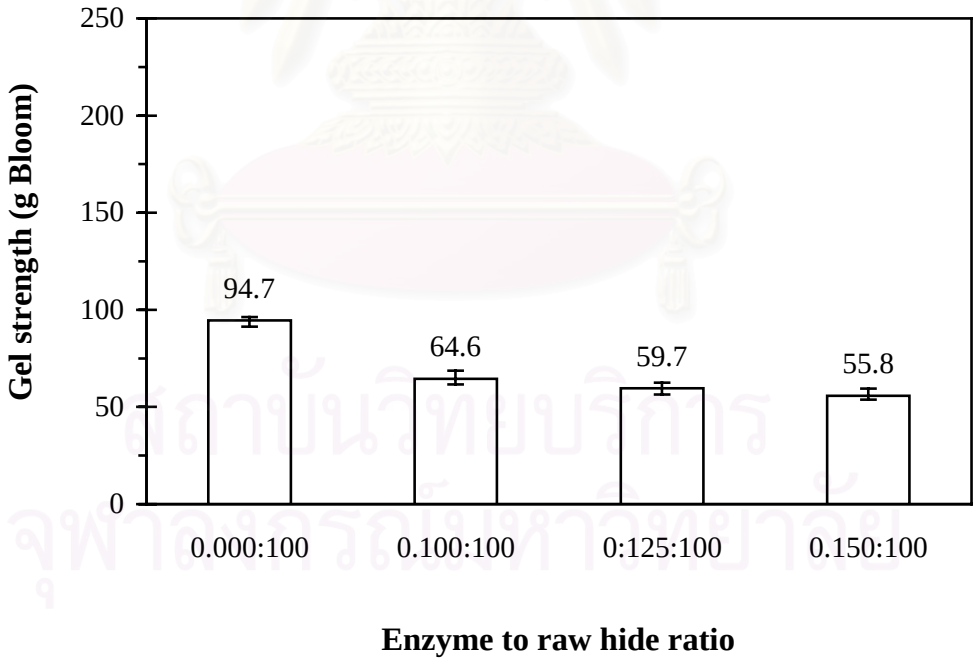

Figure 5.35 Gel strength of gelatin from commercial papain hydrolysis at various enzyme to raw hide ratios, 75^{o}C, pH 7, and 90 minutes

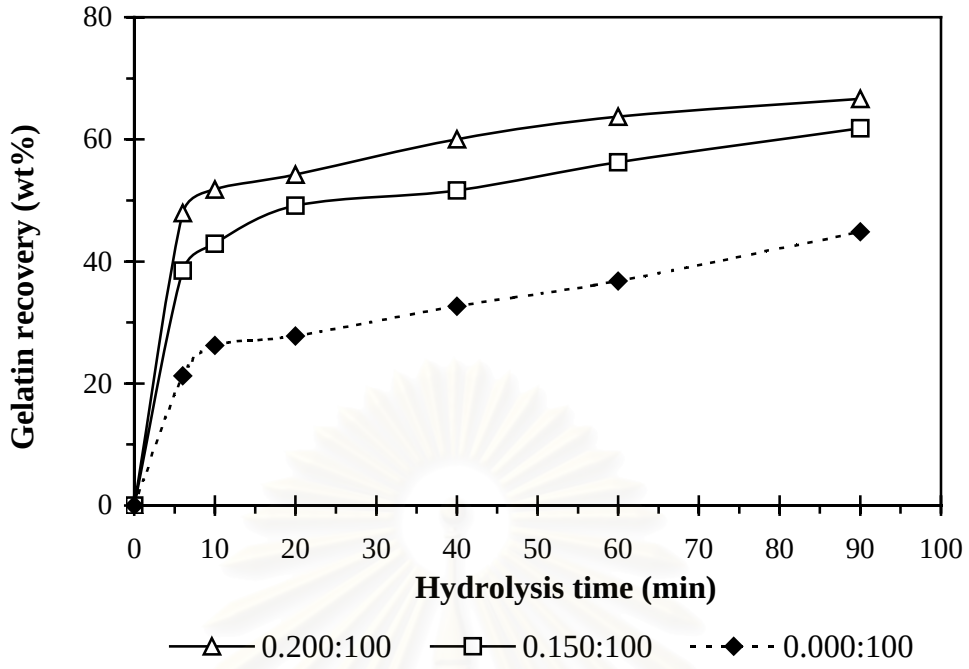

Figure 5.36 Gelatin recovery from commercial papain hydrolysis at various enzyme to raw hide ratios, 85^{o}C and pH 7

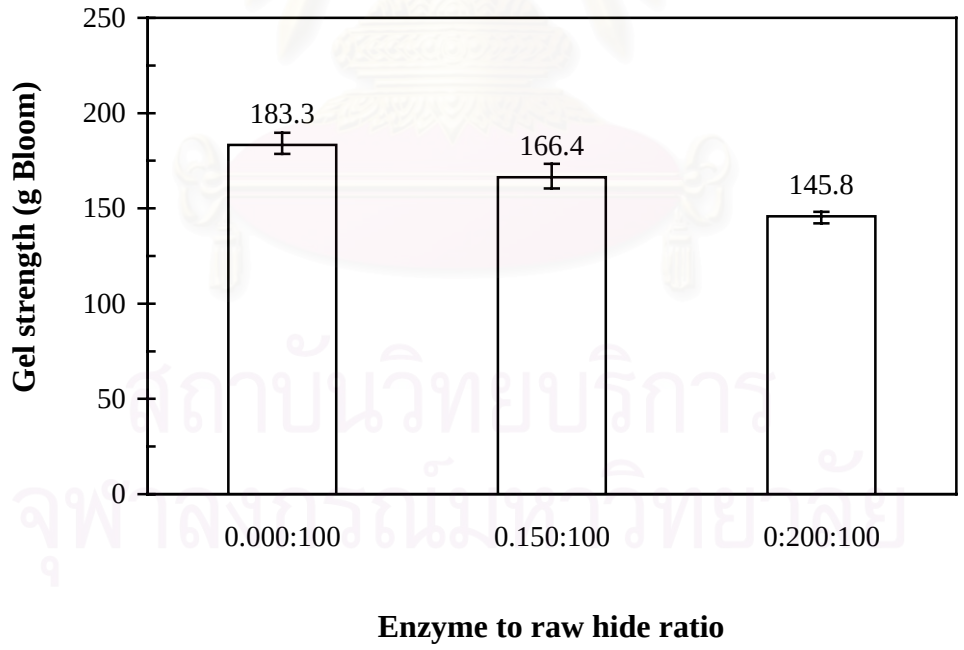

Figure 5.37 Gel strength of gelatin from commercial papain hydrolysis at various enzyme to raw hide ratios, 85^{o}C, pH 7, and 90 minutes

In the case of the relatively low activity condition, 85^{o}C and pH 7, the commercial papain to raw hide ratio was increased from 0.15:100 to 0.20:100. Figure 5.36, and 5.37 illustrated the percentage of gelatin recovery, and gel strength of gelatin, respectively.

It was found that the gelatin recovery was increased but the gel strength was decreased while increasing the ratio of enzyme to raw hide from 0.15:100 to 0.20:100. This was in the same trend as observed in the case of crude extracted enzyme hydrolysis.

In conclusion, the gelatin recovery and properties of gelatin depended on the ratio of enzyme (crude extracted enzyme and commercial papain) to raw hide substrate, temperature, and pH. When the enzyme to raw hide ratio was increased, the gel strength of gelatin was decreased and the percentage of gelatin recovery was increased until it was consistent. At 75^{o}C and pH7, the percentage of gelatin from enzymatic hydrolysis was highest but the gel strength was lowest. The gelatin recovery and properties of gelatin could be controlled by the enzyme to raw hide ratio and hydrolysis condition. From the results of enzymatic hydrolysis, the gelatin recovery and properties of gelatin from crude extracted enzyme and commercial papain were comparable while the amount of crude extracted enzyme used was less than that of commercial papain. In addition, the cost of crude extracted enzyme was much cheaper than commercial papain. So that gelatin production from crude extracted enzyme hydrolysis could be performed with an equivalent efficiency but much less enzyme cost comparing to that from commercial papain hydrolysis.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This work aims to utilize proteolytic enzymes from local papaya latex in gelatin production from raw hide. The first part of the work is the crude extraction of proteolytic enzymes from papaya latex. Then, the second part is to use crude extracted enzyme in the hydrolysis of raw hide to produce gelatin. The conclusions on the yield of gelatin and the properties (gel strength and viscosity) of obtained gelatin using crude extracted enzyme and commercial papain can be summarized as follows.

Crude extraction of proteolytic enzymes from papaya latex

1. The yield and activity of extracted enzyme from solvent extraction did not depend on extraction time. But they depended on the solvent type and the ration of latex to solvent used

2. The optimum papaya latex to solvent ratio for the yield of extracted enzyme from buffer extractions was at 1:1. For water extraction, the extraction ratio for crude enzyme extraction was fixed at 1:1. The yield of extracted enzyme from water extraction was higher than the case of buffer extraction.

3. When enzyme from solvent extraction was precipitated with ammonium sulfate, the activity and yield of enzyme were decreased.

4. The highest activity of crude extracted enzyme from papaya latex and commercial papain at the working condition (75^{o}C, pH7) were 1.4 and 1.6 unit/ml, respectively

Enzymatic hydrolysis of raw hide

1. The optimum working condition of both crude extracted enzyme and commercial papain for highest gelatin recovery was at 75^{o}C and pH7.

2. The gelatin obtained from enzymatic hydrolysis reaction using either crude extracted enzyme or commercial papain had low gel strength and viscosity. The properties of gelatin did not depend on type of enzyme, but ratio of enzyme to raw hide and hydrolysis condition (temperature and pH). When the enzyme to raw hide ratio was increased, the percentage of gelatin recovery was increased but the gel strength of gelatin was decreased. The gelatin recovery was increased and the gel strength of gelatin was decreased when hydrolysis temperature was increased. At high temperature, the percentage of gelatin recovery was creased but the gel strength of gelatin was increased.

3. The percentage of gelatin recovery and properties of gelatin obtained from crude extracted enzyme and commercial papain hydrolysis were similar. But the amount and the cost of crude extracted enzyme used were much less than those of commercial papain. So that crude extracted enzyme from papaya latex could be used for gelatin production instead of the use of commercial papain.

6.2 Recommendations

1. Fat should be removed from limed split hide before raw hide has been ground. Because the efficiency of hydrolysis reaction using enzyme might be decreased by fat.

2. The effect of HCl solution in preparation step on gelatin recovery and gelatin properties should be explored.

3. Further study on the application of recovered gelatin with low gel strength should be explored.

4. The effect of purification process gelatin on gelatin properties should be further investigated.

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APPENDICES

A. Activity calculation

From method described in section 4.3.1.3, the activity of enzyme is calculated from the change of absorbance at the wavelength of 440 nm (the absorbance increase 0.1, the Unit of activity increases 1). For example, 0.1 ml of enzyme solution and controlled sample (no reaction from enzyme) have an absorbance 0.410 and 0.010, respectively. The activity of 0.1 ml of enzyme solution is 4 Unit. Therefore, the activity of 1 ml of enzyme solution is 40 Unit/ml. When comparing with the incubation time (20 minutes), the activity per time is 2 Unit/(ml·min). The calculation is showed as follows:

Activity of 0.1 ml of enzyme solution = (0.410-0.010)*10 = 4 Unit

Activity of 1 ml of enzyme solution = $\frac{1*4}{0.1} = 40$ Unit/ml

Activity per time = $\frac{40}{20} = 2$ Unit/ml

B. Yield of activity calculation

The yield of activity is calculated from dividing the total activity of extracted enzyme by total activity of papaya latex. For example, the activity of 30 ml of papaya latex is 1.355 unit/ml, total activity is 41 unit. After solvent extraction, the activity of 22.1 ml of supernatant is 1.424 unit/ml, total activity is 31 unit. So, the yield of activity is 77%. The calculation is showed as follows:

Total activity of 30 ml of papaya latex =1.355*30 = 40.65 Unit/ml

Total activity of 22.1 ml of supernatant =1.424*22.1 = 31.47 Unit/ml

Yield of activity $= \frac{31.47}{40.65} \times 100 = 77.42$ %

C. Total activity of enzyme used calculation

The total activity of enzyme used in hydrolysis of raw hide is calculated from the absorbance of the wavelength of 440 nm of 0.5w/v crude extracted enzyme and 0.2w/v commercial papain. The absorbance, activity, activity per gram, and total activity of crude extracted enzyme and commercial papain are presented in Table C1, C2, C3, and C4, respectively

Table C1 The absorbance of the wavelength of 440 nm of enzyme solutions

Type of enzyme solution	Crude extracted enzyme (0.5%w/v)	Commercial papain (0.2%w/v)
Absorbance (at 440 nm)	0.157	0.263

Table C2 The activity of enzyme solutions

Type of enzyme solution	Crude extracted enzyme (0.5%w/v)	Commercial papain (0.2%w/v)
Activity (unit/ml)	0.785	1.315

Table C3 The activity per gram of enzymes

Type of enzyme	Crude extracted enzyme (1g)	Commercial papain (1g)
Activity (unit/g)	657	157

Table C4 The total activity of enzyme used in hydrolysis of raw hide

Type of enzyme	Crude extracted enzyme (0.07g)	Commercial papain (0.3g)
Total activity (unit)	46	47

D. Protein content calculation (Lowry method)

The protein content is calculated from the absorbance of the protein solution at the wavelength of 750 nm. The absorbance value is converted to the amount of protein using the calibration curve. The calibration curves, Figure D1 and D2 were plotted using a BSA and a standard gelatin (lab grade, Khurusapha), respectively. Standard curve of BSA solution is used to calculate protein content of enzyme. In case of gelatin, standard curve of standard gelatin is used.

The concentration of protein is calculated based on the following equation.

$$C = \frac{A \times V}{F}$$

where

C is the concentration of protein;
μg/ml for enzyme and mg/ml for gelatin.

A is the absorbance value at the wavelength of 750 nm.

V is the volumetric ratio of water used to dilute to protein solution (400 ml/ml).

F is the conversion factor from calibration curve;
0.0025 ml/μg for enzyme and 1.2201 ml/mg for gelatin.

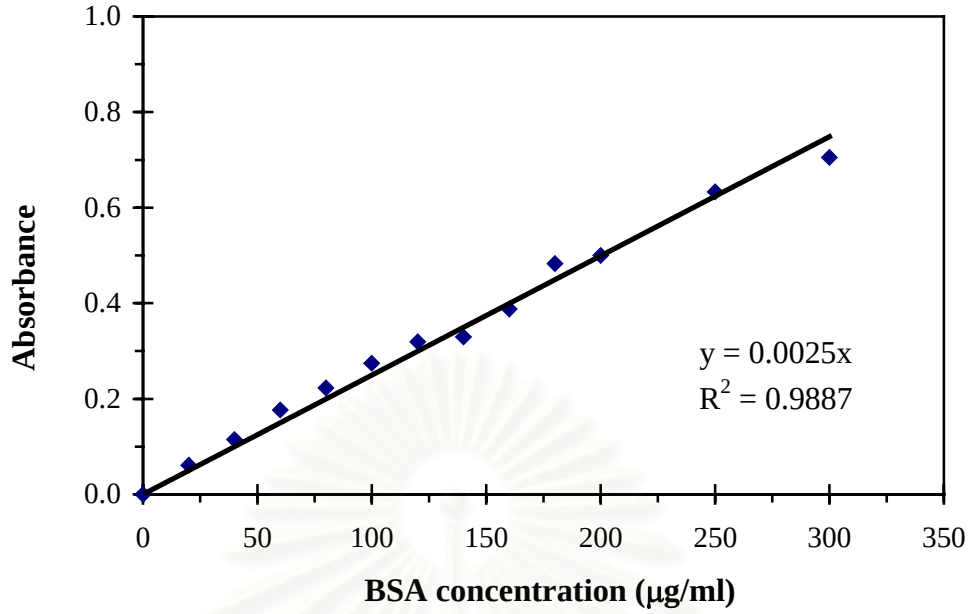

Figure D1 The calibration curve of BSA solution

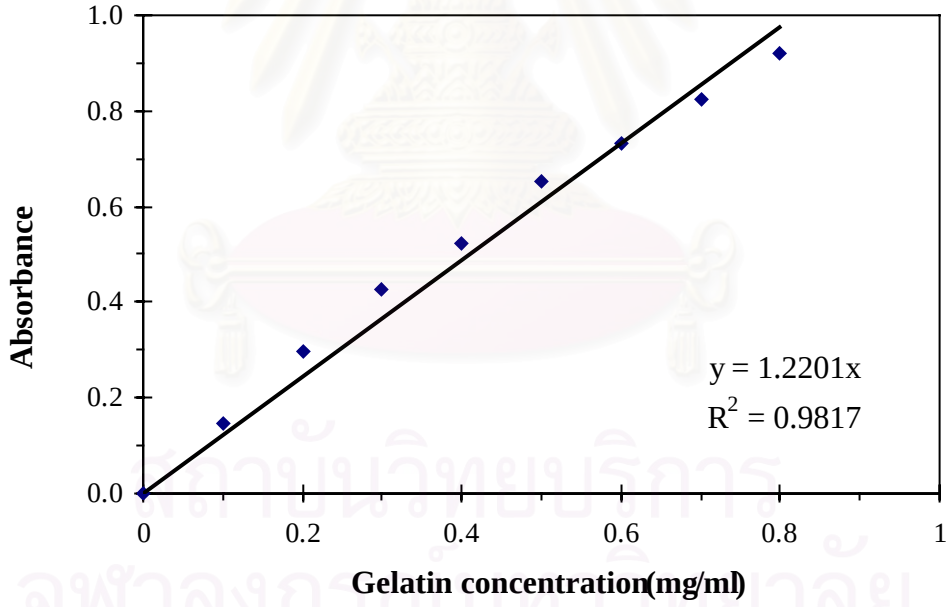

Figure D2 The calibration curve of standard gelatin solution

E. Protein molecular weight calculation (SDS-PAGE)

The Molecular weight of enzyme and gelatin were determined by SDS-PAGE. The relative mobility of protein band (Rm) is converted to the log function of molecular weight, log(MW), using the standard curve. After that, log(MW) is calculated to molecular weight of protein. The relative mobility is a mobility fraction between protein sample and the most mobile protein in each lane. The standard curve is constructed using a low molecular weight standard marker. Figure E1 and E2 show the calibration curve of low molecular weight standard maker for the case of enzyme and gelatin, respectively.

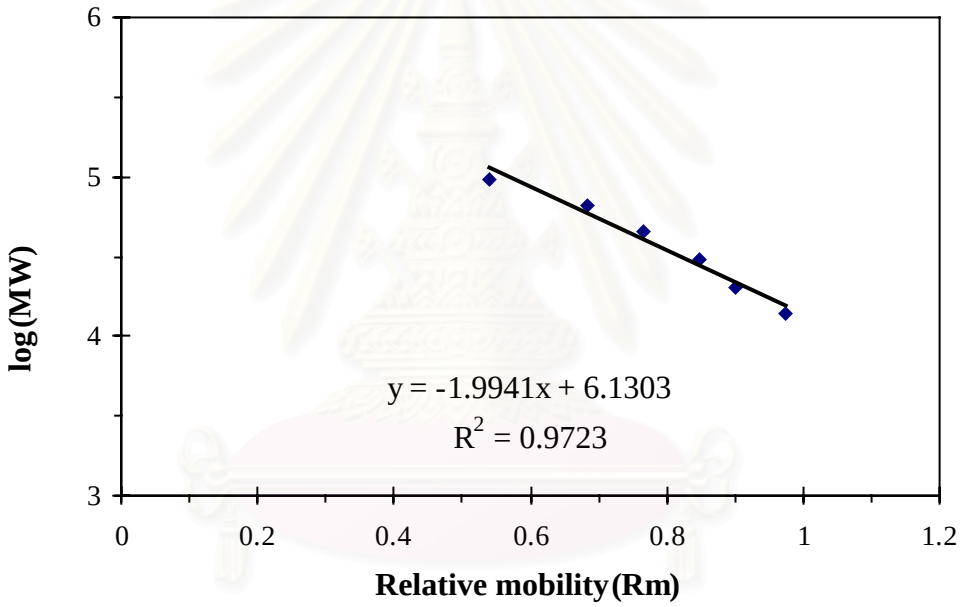

Figure E1 The calibration curve for proteolytic enzyme

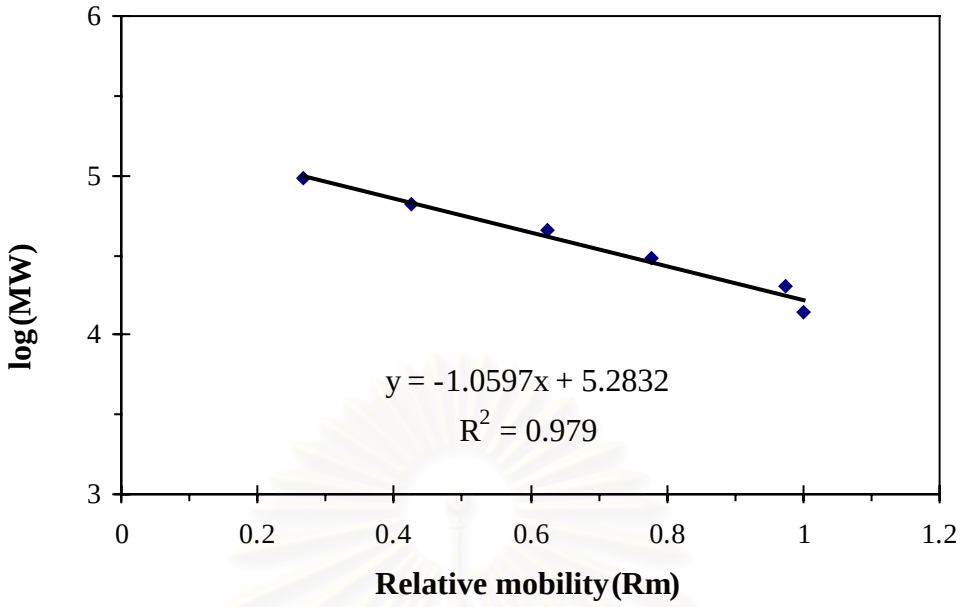

Figure E2 The calibration curve for the gelatin.

F. The relationship of percentage and gel strength of gelatin from enzymatic hydrolysis at various enzyme to raw hide ratios

The percentage of gelatin recovery and gel strength of gelatin obtained from enzymatic hydrolysis at various ratios were summarized in Table F1, F2, F3, and F4.

Table F1 The relationship of percentages of gelatin recovery and the gel strength of gelatin from crude extracted enzyme hydrolysis at various ratios, 90 minutes, 75°C and pH 7

Enzyme to raw hide ratio	0.000:100	0.015:100	0:025:100	0.030:100	0.035:100
Gelatin recovery (wt%)	30.3	47.0	61.1	66.8	72.6
Gel strength (g Bloom)	94.7	95.2	74.0	60.6	58.4

Table F2 The relationship of percentages of gelatin recovery and the gel strength of gelatin from crude extracted enzyme hydrolysis at various ratios, 90 minutes, 85°C and pH 7

Enzyme to raw hide ratio	0.000:100	0.035:100	0:045:100
Gelatin recovery (wt%)	44.8	58.4	64.8
Gel strength (g Bloom)	183.3	187.6	144.3

Table F3 The relationship of percentages of gelatin recovery and the gel strength of gelatin from commercial papain hydrolysis at various ratios, 90 minutes, 75°C and pH 7

Enzyme to raw hide ratio	0.000:100	0.100:100	0:125:100	0.150:100
Gelatin recovery (wt%)	30.3	60.5	66.1	71.4
Gel strength (g Bloom)	94.7	64.6	59.7	55.8

Table F4 The relationship of percentages of gelatin recovery and the gel strength of gelatin from commercial papain hydrolysis at various ratios, 90 minutes, 85°C and pH 7

Enzyme to raw hide ratio	0.000:100	0.150:100	0:200:100
Gelatin recovery (wt%)	44.8	61.8	66.6
Gel strength (g Bloom)	183.3	166.4	145.8

VITAE

Mr. Sittiruk Pitpreecha was born in Bangkok, Thailand on July 8, 1981. He graduated at high school level in 1999 from Chulalongkorn University Demonstration School in Bangkok. He received the Bachelor's Degree of Engineering with a major in Chemical Engineering from the Faculty of Engineering, Chulalonglorn University in 2002. After the graduation, he continued his study for a Master's Degree of Chemical Engineering at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University.

Some part of this work was selected for poster presentation in enzyme technology at BioThailand2005: Biotechnology Challenges in the 21st Century (BIOTEC, NSTDA, TSB, BOI), 2 - 5 November 2005, The Queen Sirikit National Convention Center, Bangkok, Thailand.