อิทธิพลของสภาวะในการใช้เอนไซม์ต่อการผลิตเจลาตินจากหนังสัตว์ใหญ่

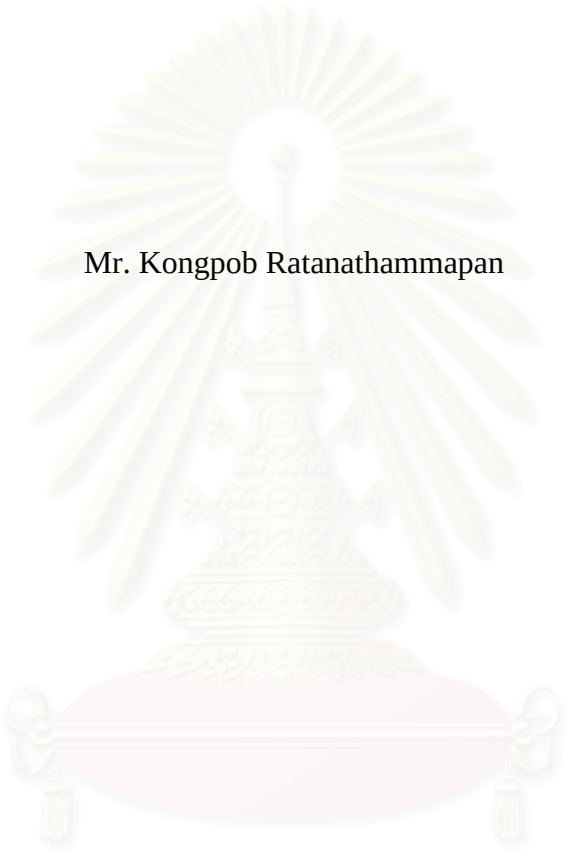
นายก้องภพ รัตนธรรมพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 947-17-6481-2

EFFECTS OF ENZYMATIC CONDITIONS ON GELATIN PRODUCTION FROM LARGE ANIMAL RAW HIDE

Mr. Kongpob Ratanathammapan



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2004

ISBN 947-17-6481-2

Thesis Title	EFFECTS OF ENZYMATIC CONDITIONS ON GELATIN PRODUCTION FROM LARGE ANIMAL RAW HIDE
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Field of study	Chemical Engineering
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ก้องภพ รัตนธรรมพันธ์ : อิทธิพลของสภาวะในการใช้เอนไซม์ต่อการผลิตเจลาตินจากหนังสัตว์ใหญ่ (EFFECTS OF ENZYMATIC CONDITIONS ON GELATIN PRODUCTION FROM LARGE ANIMAL RAW HIDE). อ.ที่ปรึกษา: รศ.ดร. ศิริพร ดำรงค์ศักดิ์กุล, อ.ที่ปรึกษาร่วม: ดร. กิตตินันท์ โกมลภิส, 93 หน้า. ISBN 947-17-6481-2

งานวิจัยนี้ทำการศึกษาผลของสภาวะได้แก่ ความเป็นกรด-ด่าง อุณหภูมิ และเวลา ที่ใช้ในการผลิตเจลาตินจากหนังสัตว์ใหญ่ที่มีต่อผลได้ของเจลาติน และสมบัติของเจลาตินที่ผลิตได้ ได้แก่ ความแข็งของเจล และ ความหนืด โดยผลได้ของเจลาตินจะวัดจากความเข้มข้นของสารละลายเจลาตินที่ได้ด้วยวิธีการของเลารี (Lowry) ส่วนสมบัติของเจลาตินจะวัดจากสารละลายเจลาตินที่ความเข้มข้น 12.5% โดยน้ำหนัก เอนไซม์ที่ใช้ในงานวิจัยนี้มีสองชนิด คือ ปาเปน และ นิวเตรส ในงานวิจัยนี้หนังชั้นในของหนังสัตว์ใหญ่จะถูกไฮโดรไลซิสโดยเอนไซม์ที่สภาวะต่าง ๆ กัน โดย อุณหภูมิที่ใช้อยู่ในช่วง 40-70°C และความเป็นกรด-ด่างอยู่ในช่วง 6-8 จากการศึกษาพบว่า ในช่วงต้นของปฏิกิริยาประมาณ 10 นาทีแรกจะได้ปริมาณเจลาตินที่สกัดได้ต่อช่วงเวลาสูงที่สุดหลังจากนั้นปริมาณเจลาตินที่สกัดได้ก็จะเพิ่มขึ้นเล็กน้อยตามเวลาการทำปฏิกิริยาที่เพิ่มขึ้น โดยจากการทดลองพบว่าตัวแปรสำคัญที่ควบคุมอัตราการเกิดปฏิกิริยาและปริมาณผลได้ของเจลาตินคือปริมาณของสารตั้งต้น อัตราส่วนที่เหมาะสมของเอนไซม์ปาเปนต่อสารตั้งต้นในการสกัดเจลาตินที่ทำให้ได้ผลได้ของเจลาตินดีที่สุดคือ 0.3:200 สภาวะที่เหมาะสมในการทำงานของเอนไซม์ คือ ที่อุณหภูมิ 70°C และความเป็นกรด-ด่างที่ 6-7 สำหรับนิวเตรส สภาวะที่เหมาะสมในการทำงานของเอนไซม์คือ ที่อุณหภูมิ 40-50°C และความเป็นกรด-ด่างที่ 6-7 โดยปริมาณผลได้ของเจลาตินจากเอนไซม์ทั้งสองชนิดนี้มีค่าโดยประมาณ 70-80% ของปริมาณโปรตีนในสารตั้งต้น ผลการศึกษาสมบัติของเจลาตินที่สกัดได้จากการใช้เอนไซม์ทั้งสองชนิดนี้พบว่า เจลาตินที่ได้จากการใช้เอนไซม์ปาเปนนั้นมีความแข็งของเจลและความหนืดค่อนข้างต่ำ (ประมาณ 2.9-5.6 เซนติพอยส์) เมื่อเทียบกับเจลาตินเกรดการค้าประเภท เอ (Type A) ที่ใช้ในการทำอาหารและเกรดที่ใช้ในห้องปฏิบัติการ ส่วนเจลาตินที่ได้จากการใช้เอนไซม์นิวเตรสนั้นไม่สามารถเกิดเจลได้และมีความหนืดต่ำมาก (ประมาณ 1.5-3 เซนติพอยส์) ดังนั้นเอนไซม์ปาเปน จึงน่าจะเหมาะสมสำหรับใช้ในการผลิตเจลาตินที่มีความแข็งของเจลและความหนืดต่ำ ส่วนเอนไซม์นิวเตรสนั้นเหมาะสมในการใช้ในการผลิตโปรตีนไฮโดรไลเสทหรือคอลลาเจนไฮโดรไลเสท (protein hydrolysate/collagen hydrolysate)

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต.....
สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2547	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4470213321 : MAJOR CHEMICAL ENGINEERING

KEY WORD: GELATIN/ PAPAIN/ NEUTRASE/ HYDROLYSIS/ GEL STRENGTH/ VISCOSITY/ PROTEIN HYDROLYSATE

KONGPOB RATANATHAMMAPAN: EFFECTS OF ENZYMATIC CONDITIONS ON GELATIN PRODUCTION FROM RAW HIDE. THESIS ADVISOR: ASSOCIATE PROFESSOR SIRIPORN DAMRONGSAKKUL, Ph.D., THESIS CO-ADVISOR: KITTINAN KOMOLPIS, Ph.D, 93 pp. ISBN 947-17-6481-2

This research aims to study the effects of enzymatic conditions (temperature, pH and time) on gelatin production on the yield of gelatin and gelatin properties such as gel strength and viscosity. The yield of gelatin is determined by Lowry method and the gelatin properties are determined from gelatin solution 12.5% wt. Two types of commercial enzymes were used in this work: papain and neutrase. In this study, raw hide was hydrolyzed by enzyme at various conditions. The temperature was varied in the range of 40-70^{o}C and pH 6-8. From the results of gelatin recovery, it was found that at the beginning of the hydrolysis reaction, the percentage of gelatin recovery was sharply increased up to about 10 minute, then started to slightly increase. This was due to the low concentration of raw hide substrate which was the main factor controlling the reaction rate and the percentage gelatin recovery. The optimal ratio of papain per raw hide for highest gelatin recovery is 0.3:200. The optimum working conditions of papain are at 70^{o}C and pH 6-7. For neutrase, the optimum working conditions are 40-50^{o}C and pH 6-7. The percentage of recovered protein at optimal conditions is around 70-80%. From the results of gelatin properties, it was found that the gel strength and viscosity of gelatin from papain hydrolysis is relatively low, around 2.9-5.6 cP, comparing with Type A gelatin (food and laboratory grade). The protein recovered from neutrase hydrolysis can not from gel and the viscosity is very low (1.5-3 cP). So that papain is suggested to be used for the production of low gel strength gelatin while neutrase is suitable to be used for protein hydrolysate or collagen hydrolysate production.

Department	Chemical Engineering	Student's signature……..
Field of study	Chemical Engineering	Advisor's signature……..
Academic year	2004	Co-Advisor's signature…….

ACKNOWLEDGEMENTS

The author would like to express his sincere gratitude to Associate Professor Dr. Siriporn Damrongsakkul, who supervised, suggested and encouraged him throughout this work, to Dr. Kittinan Komolpis for his guidance and invaluable suggestions. The author is also grateful to Associate professor Dr. Tawatchai Charinpanitkul, Assistant professor Dr. Seeroong Prichanont and Mr. Prechar Sangterapitikul for serving as the chairman and the members of the thesis committee, respectively, whose comments were constructively and especially helpful.

The author would like to thank Ms. Parichat Lobyaem for her help in molecular weight determination (SDS-PAGE).

The author would like to thank the staffs of Analytical Instrument Center and Laboratory for their helps with experiments. He would like to extend his grateful thanks to all members of polymer group at the Department of Chemical Engineering, Chulalongkorn University as well.

Finally, his greatest thank should go to his family especially his parents, who always support, undoubtfully believe and understand him.

CONTENTS

CONTENTS (continued)

PAGE

CONTENTS (continued)

PAGE

CHAPTER I

INTRODUCTION

In present, gelatin is used in many industries such as food industry, pharmaceutical industry, photographic industry and cosmetic industry. For food industry, gelatin is often used in frozen food and daily product. In photographic industry, gelatin is used in coating of photographic film and paper. The type of gelatin applications is mostly classified by the gel strength of gelatin which can be very different depending on protein sources and manufacturing methods.

Gelatin is a large and water-soluble protein that obtained by partial hydrolysis of collagen, the main protein component in skin, bones, hides, and white connective tissues of animal bodies. In gelatin manufacturing industry, the major source of raw material is from tannery industry. In each step of tanning processing, a large amount of solid wastes is generated such as horn, hoof, bones, and small pieces of hide. In order to increase the market value and reduce waste problem, the solid residual is used as the raw material for gelatin production. The typical method of gelatin production can be classified into two methods: the acid process and the liming process. The acid process uses acid solution to hydrolyze collagen and the gelatin obtained from this method is called type A gelatin. Raw materials for this method are pigskins and the processing duration is around 10-45 hours. On the contrary, alkaline or lime is used to hydrolyze protein in the liming process and the gelatin obtained from this method is called type B gelatin. Bones and hides are typical raw materials in liming process that usually takes around 30-100 days. These two typical gelatin production processes have several disadvantages, especially large amount of wastes generated and the limitation of raw material.

In early of 1900s, the first method that applied enzyme to hydrolyze collagen was developed by American and Japanese workers and the gelatin obtained from this method was not classified as type A or B gelatin (Ward *et al*., 1977). Nowadays, the enzymatic process for gelatin production is more attractive. Comparing with the liming and acid processes, the enzymatic process has several advantages such as the reduction of processing time and wastes. In addition, it can be applied with all sources of raw material such as fish scale, horn, hoof, leather waste, and animal bone. Many works have reported the studies of enzymatic hydrolysis used in the process of protein waste treatment (Guerard *et al*., 2001 and 2002; Bajza *et al*., 2001 and Taylor *et al*., 1989 etc.). The study of the effects of enzymatic conditions on the gelatin production has not received much attention. Therefore this work aims to investigate the effects of enzymatic conditions on the yield and physical properties of recovered gelatin from the hydrolysis reaction of raw hide. The proteolytic enzyme used in this study is papain and neutrase.

1.1 Objectives

1. To investigate the effect of enzymatic conditions on the yield and the physical properties of recovered gelatin from the hydrolysis reaction of raw hide.

2. To compare the effect of enzyme on the yield and the physical properties of recovered gelatin from the hydrolysis reaction of raw hide.

1.2 Scope of work

1. Investigate the effect of enzymatic conditions on the reaction rate and the yield of recovered gelatin from the hydrolysis reaction of raw hide. The interested enzymatic conditions were:
    1.1 Hydrolysis temperature from 50-70^{o}C for papain and 40-50^{o}C for neutrase.
    1.2 The pH of raw hide slurry from 6-8.
    1.3 Reaction time from 0-90 minute.

2. Determine the physical properties of recovered gelatin such as:
    2.1 Gel strength
    2.2 Viscosity

3. Investigate the effect of enzyme (papain and neutrase) on the reaction rate, yield and physical properties of recovered gelatin from the hydrolysis reaction of raw hide.

CHAPTER II

THEORY

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

Animal skin is composed 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. About 90% of solid matter in skin is protein and in fresh skin the protein content is around 35% of total weight.

One of the main functions of the skin is to keep the temperature of the body constant. The temperature can be controlled by the sweat glands (permitting loss of heat) and the fat glands (retaining body heat). Skin is one of the principal organs of the body that protects against bacterial invasion or mechanical damage. Another function of skin is the color filters that protect underlying tissues from the ultraviolet rays of the sun. Animal skin that received by the tanner can be divided into three distinct layers: flesh, derma or corium and epidermis. These three general layers are described below. Flesh is not a part of skin, it composing with adipose tissue, yellow connective tissue, blood vessel nerves and voluntary muscle. Before tanning process, flesh must be removed from skin in fleshing step. Derma, corium or true skin can be divided into two portions: an upper portion and a lower portion. The upper portion contains glands, muscles and hair follicles. The lower portion contains collagen fibers. Epidermis is similar to derma but the major composition is keratin.

Raw hide is the treated skin of large animal (after fleshing) such as cow and buffalo. It is composed with derma and epidermis, of which the composition is showed in Table 2.1. Protein in raw hide can be classified into two types: structural and non-structural protein, as showed in Table 2.2.

Table 2.1 The composition of raw hide (Sharphouse, 1980)

Composition of raw hide	content (%)
Water	64
Protein	33
Fats	2
Mineral salts	0.5
Other substances	0.5

Table 2.2 The composition of protein in raw hide (Sharphouse, 1980)

Composition of protein in raw hide (%)	
Structural protein	
Collagen	29
Keratin	2
Elastin	0.3
Non-structural protein	
Albumins, Globulins	1
Mucins, Mucoids, etc.	0.7

2.2 Collagen (www.greatvistachemicals.com)

Collagen is a major structural protein, forming molecular cables that strengthen the tendons and vast, resilient sheets that support the skin and internal organs. Bones and teeth are collagen that composed with mineral crystals. About one half of the total body collagen is in the skin and about 70% of the material other than water in dermis of skin and tendon is collagen. (Lee *et al*., 2001) Collagen provides structure to our bodies, protecting and supporting the softer tissues and connecting them with the skeleton. It has great tensile strength and responsible for skin elasticity, the degradation of collagen leads to wrinkles that accompany aging. Collagen is composed of a lot of amino acids such as glycine, proline, hydroxyproline and hydroxylysine. The regular arrangement of amino acids in collagen is generally follows the pattern Gly-X-Y, where Gly is glycine, X is proline, and Y is proline or hydroxyproline. There are very few other proteins with such regularity. The resulting structure forms a collagen fiber or collagen helix, composing of three polypeptide

chains (tropocollagen or α-chain), wound together in a tight triple helix to compose the complete molecule of collagen structure (see Figure 2.1).

The fundamental structural unit is a tropocollagen, a molecular rod about 2600 Å in length and 15 Å in diameter with a molecular weight of 300,000 daltons. The polypeptide chains of the two amino acids, alternating with one another and punctuated by the presence of certain other amino acids, are coiled in a left-handed helix. The different chains are bonded to each other by H-bridges as described: 1. NH from the glycines and CO from residues of the other chain. These H-O bonds are perpendicular on the axis of the collagen chains. 2. H-bridges of hydroxyl-groups of hydroxy prolines. 3. H-bridges with water molecules. All these bonds stabilize the triple-helical structure of collagen. (http://www.science.uva.nl/) The systematic packing of the triple helix caused strength and resilience to the collagen fibers. Additional mechanical and chemical stability was from intra- and intermolecular crosslink (see Figure 2.1). Intramolecular crosslink was formed between two α-chains in the non helical section of the same molecule by aldol condensation of two aldehydes. Intermolecular crosslink was occurred between the telopeptide region of one collagen molecule and the adjacent molecule by aldimine formation (covalent bond). (Friess, 1998)

In the fibrils, the tropocollagen is cross-linked by covalent bonds primarily located at the molecule ends, so-called telopeptides. Native tropocollagen is highly resistant to alkaline, acid and enzymatic hydrolysis, owing to its densely-packed in the helix structure. However, the telopeptides do not have such a high resistance, since they are not integrated in the helix structure but may be regarded as randomized or globular regions. When collagen molecule in tissue is heat-denatured, the covalent cross-links between the molecules were broken up. However, such heating not only breaks up the cross-links, but also causes random hydrolysis of the bonds in the tropocollagen (intramolecular crosslink), resulting in gelatin of poor quality. (U.S patent 5,877,287)

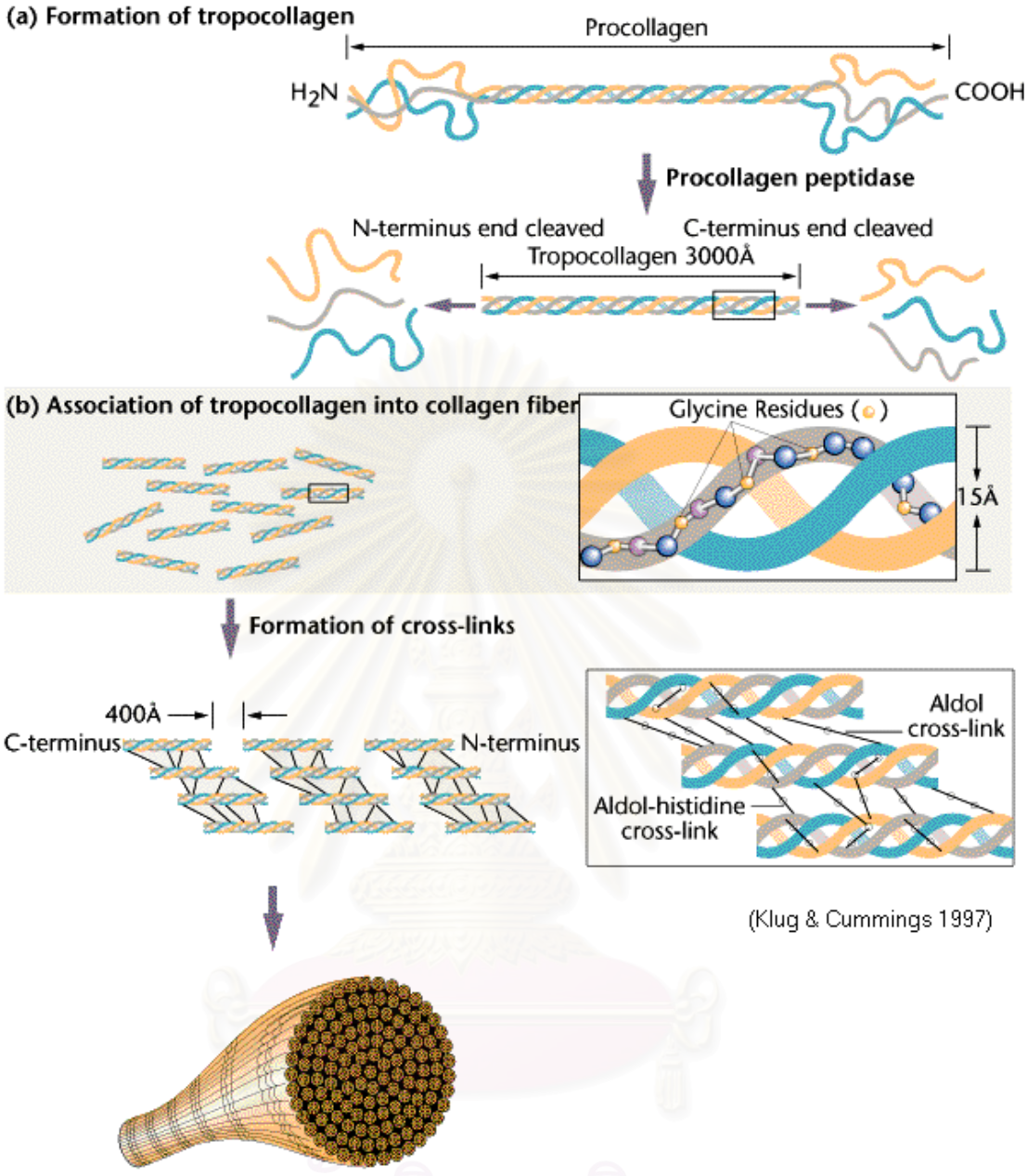


Figure 2.1 Model of triple helix structure of collagen. (www.mun.ca/biology/scarr/Collagen_structure.gif)

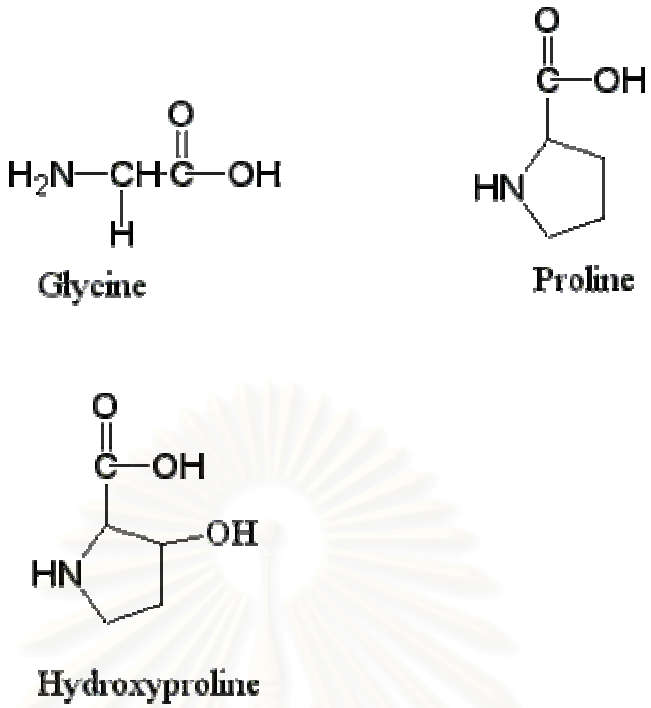


Figure 2.2 The regular amino acids in collagen

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

Gelatin is a protein obtained by partial hydrolysis of collagen, the chief protein component in skin, bones, hides and white connective tissues of the animal body (see Figure 2.3). Type A gelatin is produced by acid processing of collagenous raw material and type B gelatin is produced by alkaline or lime processing. Because it 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 and can be considered as containing lower molecular-weight fractions of gelatin.

Use of animal glues was first recorded ca 4000 BC in ancient Egypt. In the past centuries, glue and crude gelatin is extracts by boiling bone and hide pieces and allowing the solution to cool and gel. But in seventeenth century, the first commercial gelatin manufacturing began. At the beginning of the nineteenth century, commercial production methods were improved to achieve the manufacture of high molecular weight collagen extracts with good quality that form characteristic gelatin gels.

The type of gelatin application are classified by 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.

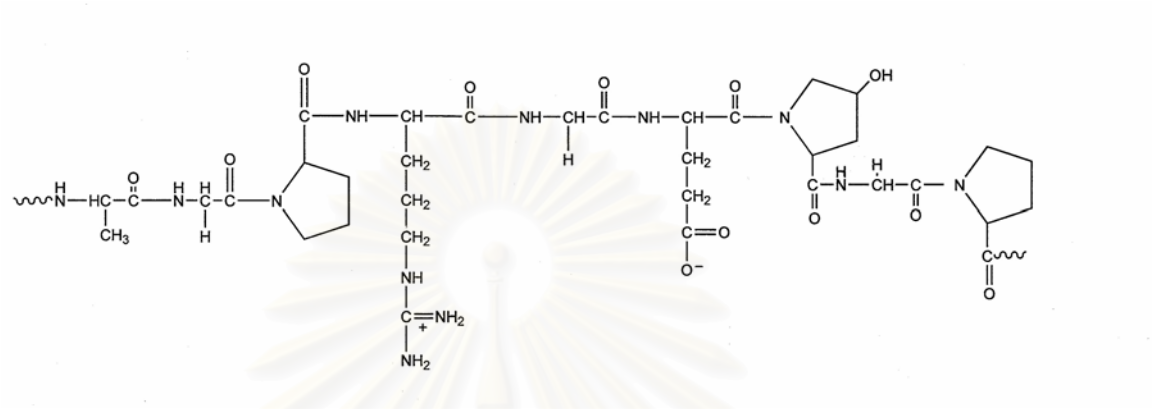


Figure 2.3 A typical chemical formulation of gelatin. (www.sbu.ac.uk)

2.3.1 Chemical composition and structure

Gelatin is not a single 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. Molecular weight distribution is measured by fractional precipitation with ethanol or 2-propanal and by complexing with anionic detergent molecules. The coacervates are isolated and recovered as gelatin fractions.

From the analysis, gelatin is composing with amino acids from 0.2% tyrosine to 30.5% glycine and 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 of gelatin behaves in solution 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 is depends on the temperature during the polymer-polymer and polymer-solvent interaction leading to bond formation. The rigidity of the gel is approximately proportional to the square of the gelatin concentration. Crystallites can be detected by x-ray diffraction pattern, and believed to be at the junctions of the polypeptide chains.

Dry gelatin can be stored in airtight containers at room temperature for many years. However, it decomposes above 100^{o}C but for complete combustion, temperatures above 500^{o}C are required. 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 decrease with increasing temperature because of hydrolysis.

2.3.2 Manufacturing process

In the past, bones and ossein (decalcified bone) 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 bones 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 (type A gelatin) and liming process (type B gelatin).

Most type A gelatin in made from pork skins and has grease as a marketable by-product. The process includes macerating of skins, washing to remove extraneous matter, and swelling for 10-30 hour in 1-5% hydrochloric, phosphoric, or sulfuric acid. Then four to five extractions are made at temperatures increasing from 55-65^{o}C for the first extract and 95-100^{o}C for the last extract. Each extraction is about 4-8 hour. Then grease is removed, the gelatin solution filtered and deionized for most applications. The solution is concentrated to 20-40% solids by continuous vacuum evaporation in several stages. The viscous solution is chilled before extruded into noodles and then dried at 30-60^{o}C on a continuous wire-mesh belt. 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 dry gelatin is ground and blended to specification.

Type B gelatin is usually made from bones, but also from bovine hides and pork skins. Firstly, the bones for type B gelatin must be crush and degreased. Secondly, 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 to remove the mineral content. An important by product, dibasic calcium phosphate, is precipitated and recovered from the spent liquor. The demineralized bones, i.e., 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 is the primary process that results in low isoelectric ranges for type B gelatin. After washing for 15-30 hours to remove the lime, the ossein is acidified to pH 5-7 with an appropriate acid. Then the extraction processing for type A gelatin is followed. Throughout the manufacturing process, cleanliness is important to avoid contamination by bacteria or proteolytic enzymes.

Bovine hides and skins are the main sources of raw material for type B gelatin and are supplied in the form of splits, trimmings of dehaired hide, raw hide pieces, or salted hide pieces. Like pork skins, the hides are cut to smaller pieces before being processed. Sometimes the term calfskin gelatin is used to call hide gelatin. The liming of hides usually takes a little longer than the liming of ossein.

2.3.3 Physical and chemical properties

Commercial gelatin is produced in mesh sizes ranging from coarse granules to fine powder. In Europe, gelatin is also produced in thin sheets for used in cooking. It is a vitreous, brittle solid, faintly yellow in color. Dry 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.

Gelation

Perhaps 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 forms a gel. 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 gel strength, the test procedure for its determination is great importance. Commercially, gelatin gel strength 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 after the inventor of the lead shot device. In the early 1990s, the testing equipment utilizing sensitive load cells for the measurement are commonly used. The conversion temperature for gelatin is determined as a setting point, i.e., 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, trifluoroethanol and formamide. Gelatin is practically insoluble in less polar organic solvents such as acetone, carbon tetrachloride, ethanol, ether, benzene, dimethyformamide and most other nonpolar organic solvents. Many water soluble organic solvents are compatible with gelatin, but interfere with gelling properties. Dry 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 means is used extensively in photographic products and this so-called hardening permanently 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) 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 present. The isoionic point of Type A gelatin is varied in pH range from 7-10, and the isoionic point of type B gelatin is varied in pH range from 5.2-4.8 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. Addition of salts decreases the viscosity of gelatin solutions. This effect is most evident for concentrated gelatin solutions. 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. Conventionally, 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 effective protective colloid that can prevent crystal, or particle, aggregation, thereby stabilizing a heterogeneous suspension. 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. 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.

Coacevation

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. The effect of pH and electrolyte content on swelling property of gelatin can be explained by the simple Donnan equilibrium theory. This explains why gelatin exhibits the lowest swelling at its isoelectric point. 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. The rate of swelling follows approximately a second-order equation. 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 the humidity or moisture content changes.

2.3.4 Application 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. For example, a 250 Bloom gelatin may be utilized at concentrations ranging from 0.25% in frozen pies to 0.5% in ice cream. In sour cream and cottage cheese, gelatin inhibits water separation. Marshmallows containing as much as 1.5% gelatin to restrain the crystallization of sugar, thereby keeping the marshmallows soft and plastic and gelatin also increases viscosity and stabilizes the foam in the manufacturing process. Many lozenges, wafers and candy coatings contain up to 1% gelatin to decreases the dissolution rate.

In meat products, such as canned hams, various luncheon meats, corned beef, chicken rolls, 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 significantly. Gelatin has also found new uses as an emulsifier and extender in the production of reduced-fat margarine products. The largest use of edible gelatin in the United States is in the preparation of gelatin desserts (1.5-2.5% concentrations). 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 the pharmaceutical industry for the manufacture of soft and hard capsules. The formulations are made with water or aqueous polyhydric alcohols. Capsules are usually preferred over tablets in administering medicine. 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. Methods have been developed to encapsulate dry powders and water-soluble materials which may first be mixed with oil. 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. Coated or cross-linked gelatin is used for enteric capsules. Gelatin is used as a carrier or binder in tablets, pastilles and troches. For arresting hemorrhage during surgery, a special sterile gelatin sponge known as absorbable gelatin sponge of gelfoam is used. The gelatin is partially insolubilized by a cross-linking process. When moistened with a thrombin or sterile salt solution, the gelatin sponge is slowly dissolved by tissue enzymes. Special fractionated and prepared type B gelatin can be used as a plasma expander.

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. A new gelatin product may require 6-12 months of testing, including extensive field testing prior to commercialization. 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. The quality and uniformity standards are highest for emulsion gelatin because it controls silver halide nucleation, crystal growth, chemical sensitization, latent image stability and numerous other factors affecting the total photographic response.

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 used to remove salts after silver halide precipitation in favor of precipitating, coagulating or derivatizing gelatin and washing the precipitate by decanting or utilizing ultrafiltration techniques; by new coating techniques that allow simultaneous coating of several layers at one time at speeds 10 times as fast as before; and by shot-time high temperature processing which may require new cross-linking agents 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. These materials with a low pH isoionic point form a coacervate at pH < 4.0. Precipitation in this case is accomplished by lowering the pH, washing at low pH and then increasing pH to above 6.5 to dissolve and redisperse the emulsion before

reconstituting it with gelatin. 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 and printing and processing equipment. Photographic technology offers a rapidly changing, highly sophisticated, very competitive market for photographic gelatin manufacturers.

In summary, the specifications of gelatin used in various industry applications are presented in Table 2.3, 2.4 and 2.5. (http://www.gelatin-gmia.com)

Table 2.3 A typical specification for edible gelatin.

Characteristic	Type A	Type B
pH	3.8 - 5.5	5.0 - 7.5
Isoelectric Point	7.0 - 9.0	4.7 -5.4
Gel Strength (Bloom)	50 - 300	50 - 300
Viscosity (mps)	15 - 75	20 - 75
Ash (%)	0.3 - 2.0	0.5 - 2.0

Table 2.4 A typical specification for pharmaceutical gelatin.

A typical hard capsule gelatin specification		
Characteristic	Type A	Type B
Gel Strength (Bloom)	240 - 300	200 - 250
Viscosity (mp)	44 - 55	45 - 60
pH	4.5 - 5.5	5.3 - 6.5
A typical soft capsule gelatin specification		
Characteristics	Type A	Type B
Gel Strength (Bloom)	150 - 200	125 - 175
Viscosity (mp)	25 - 35	30 - 45
pH	4.5 - 5.5	5.3 - 6.5
A typical tablet gelatin specification		
Characteristics	Type A	Type B
Gel Strength (Bloom)	75 - 150	75 - 150
Viscosity (mp)	17 - 35	20 - 35
pH	4.5 - 5.5	5.3 - 6.5

Table 2.5 A typical specification for photographic gelatin (Type B, bone)

Characteristic	Value
Moisture (%)	10.5 – 13.0
pH*	5.65 – 5.85
Viscosity (mp)*	74.0 – 95.0
Gel strength (Bloom)	250 – 310
Absorbance, 420nm*	0.0 – 0.158
Absorbance, 650nm*	0.0 – 0.032
Viscosity loss (%) **	< 5%

* Measured at 6.16% gelatin concentration and 40^{o}C.
** Measured at 10.0% gelatin concentration and 40^{o}C: gelatin held for 24 hours at 37^{o}C.

2.4 Enzyme (Garrett, 1995)

The use of enzyme in the food industry occurred long before the first discovery of enzymes. The oldest use of enzyme in food is the use of yeast in brewing and baking. The term ‘enzyme’ was first used by Kuhne in 1878 from the Greek language meaning ‘in yeast’. (Fox, 1991) The characteristic of enzymes can be divided into three distinctive features: catalytic power, specificity and regulation.

Catalytic power

Enzymes show enormous catalytic power, accelerating reaction rates as much as 10^{16} over uncatalyzed reactions, which is much greater than any synthetic catalysts can achieve, and enzymes accomplish these amazing feats in dilute aqueous solutions under mild conditions of temperature and pH. For example, the enzyme jack bean *urease* catalyzes the hydrolysis of urea. At 20^{o}C, the rate constant for the enzyme-catalyzed reaction is $3*10^{4}$/sec, the rate constant for uncatalyzed hydrolysis reaction of urea is $3*10^{-10}$/sec. Thus, 10^{14} is the ratio of the catalyzed rate to the uncatalyzed rate of reaction. Such a ratio is defined as the relative catalytic power of an enzyme.

Specificity

Enzyme is very selective, both in substances that it interacts and in the reaction that it catalyzes. The substances which an enzyme acts are traditionally called substrates. In an enzyme-catalyzed reaction, none of the substrate is converted into nonproductive side-reactions, so no wasteful by-products are produced. So the products from an enzyme reaction are also very specific. The selective qualities of an enzyme are collectively recognized as its specificity. The interaction between an enzyme and its substrates occurs through molecular recognition based on structural complementarity, such mutual recognition is the basis of specificity.

Regulation

Regulation of enzyme activity is proceed in various ways, ranging from controls over the amount of enzyme protein synthesized by the cell to more rapid modulation of activity through reversible interaction with metabolic inhibitors and activators. Because most enzymes are proteins, we can anticipate that the functional attributes of enzymes can be ascribed to the remarkable versatility found in protein structures.

2.4.1 Enzyme activity

In many situations, the actual molar amount of the enzyme is unknown. However, its amount can be measured in terms of the activity observed. The International Commission on Enzymes established by the International Union of Biochemistry defines one international unit of enzyme as the amount that catalyzes the formation of one micromole of product in one minute. Because enzymes are very sensitive to factors such as pH, temperature, and ionic strength, the conditions of assay must be specified. Another definition for units of enzyme activity is the katal. One katal is the amount of enzyme that converts of one mole of substrate to product in one second. Thus, one katal equals $6*10^7$ international units.

2.4.2 Effect of pH on enzymatic activity

Enzyme-substrate recognition and the catalytic reaction are greatly dependent on pH. An enzyme possesses an array of ionizable side chain and prosthetic groups that not only determine its secondary and tertiary structure but many also be intimately involved in its active site. Further, the substrate itself often has ionizing groups, and one or another of the ionic forms may preferentially interact with the enzyme. In general, enzymes are active only over a limited pH range and most have a particular pH at which their catalytic activity is optimal. Figure 2.4 illustrates the relative activity of four enzymes as a function of pH. Although the pH optimum of an enzyme often reflects the pH of its normal environment, the optimal may not be precisely the same. This difference suggests that the pH-activity response of an enzyme may be a factor in the intercellular of its activity.

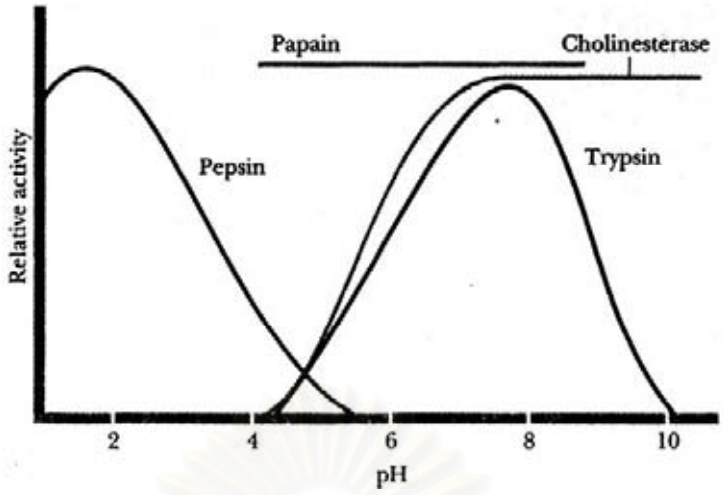
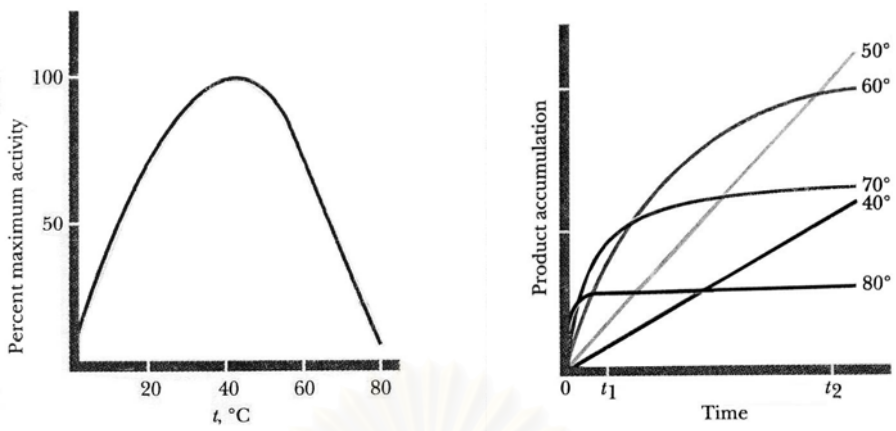


Figure 2.4 The effect of pH on enzyme activity. (Garrett, 1995)

2.4.3 Effect of temperature on enzymatic activity

Like most chemical reactions, the rates of enzyme-catalyzed reactions increase with increasing temperature. However, at temperatures above 50^{o}C to 60^{o}C, enzymes typically show decline in activity (Figure 2.5a). That due to two effects is occurred 1. the characteristic increase in reaction rate with temperature and 2. thermal denaturation of protein structure at higher temperatures. Most enzymatic reactions double in reaction rate for every 10^{o}C increase in temperature (that is, Q_{10}=2, where Q_{10} is defined as the ratio of activities at two temperatures 10^{o}C apart) as long as the enzyme is stable and fully active. Some enzymes that having very high activation energies, show proportionally greater Q_{10} values. The increasing rate with increasing temperature is causing the instability of higher orders of protein structure at elevated temperatures, so enzyme is inactivated. Figure 2.5b illustrates a typical progress curve for an enzymatic reaction at various temperatures. At lower temperatures, the rate is markedly enhanced as the temperature rises. However, at 50^{o}C, the enzyme is progressively inactivated over time. Figure 2.5a summarizes the overall situation described by the family of curve in Figure 2.5b. Not all enzymes are quite so thermally labile. For example, the enzymes of thermophilic bacteria (thermophilic meaning “heat-loving”) found in geothermal springs retain full activity at temperatures is excess of 85^{o}C.



a b

Figure 2.5 The effect of temperature on enzyme activity. (Garrett, 1995)

2.4.4 Enzyme inhibition

If the enzyme is inhibited by some compounds or the kinetics of the reaction has been interfered then the rate of an enzymatic reaction is decreased. The normal working of enzyme can be studied by systematic perturbations experimental. A systematic perturbation experimental proceeds by applied changes in the system and then observing the effects of the change. Accordingly, the study of enzyme inhibition has contributed a significantly information of enzymes.

Enzyme inhibitors are classified in several ways. The inhibitor may interact either reversibly or irreversibly with enzyme. Reversible inhibitors interact with the enzyme through noncovalent association/dissociation reactions. In contrast, the effects of irreversible inhibitors are usually manifested through stable, covalent alterations in the enzyme. The net effect of irreversible inhibitor is a decreasing of active enzyme.

2.5 Proteolytic enzymes (Walsh, 2002)

The proteolytic enzymes or proteases (protein-degrading) are the most important group of industrial enzymes currently in use. Their annual sales value is more than 50 percent of total sales revenue of all industrial enzymes. In the past, it was usually used in the food and detergent industries and more recently have been employed in leather processing and as the therapeutic agents.

Classification of proteases

Proteases can be classified in various criteria, but most often classified is based on two criterions: 1. the positioning of the peptide bond hydrolysed 2. the molecular mechanism (hydrolysis reaction). Based upon the relative position of the susceptible bond within the protein substrate, proteases may be described as exopeptidases or endopeptidases. Exopeptidases may be further classified into aminopeptidases, dipeptidyl peptidases, tripeptidyl peptidases, carboxypeptidases and peptidyl dipeptidases. Endopeptidases cleave peptide bonds which are found internally in the protein, usually some distance from the carboxyl or amino termini. Most such enzymes (both endo and exo) also display some level of selectivity towards the peptide bond they hydrolyse, for example chymotrypsin hydrolyses peptide bonds adjacent to aromatic amino acid residues.

On the basis of mechanism of action, proteolytic enzymes may be divided into four categories: serine proteases, aspartic proteases, cysteine proteases, and metalloproteases.

Serine proteases are characterized by the presence of a serine residue at the active site (Figure 2.6). They are the most common class of protease produced by archaea, bacteria, eukarya and viruses. Almost serine proteases have a molecular mass between 18 and 35 kDa and the maximum activity is occurred between pH 7 and 11. They may be divided into a number of subfamilies, based upon structural similarities. 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.

Aspartic proteases are a group of acidic proteases that contain an aspartic acid residue at the catalytic site (Figure 2.6). Most aspartic proteases show maximum activity at pH between 3 and 4, and have isoelectric points between 3 and 4.5. The generally molecular masses is around 30-45 kDa. Example of this group is pepsin and rennin (animal stomach-derived aspartic proteases). Microbial aspartic proteases are produced mainly by *Aspergillius*, *Penicillium*, *Rhizopus* and *Mucor* spp. and the best-known application of aspartic proteases is in cheese manufacture.

Cysteine proteases are commonly found in nature and characterized by the presence of cysteine and a histidine residue at the active site, which forms a catalytic dyad essential for biological activity. The best-known of this group is papain, generally active under reducing conditions and tend to show optimum activity at neutral pH values. In industrial application, they used mainly in the food industry.

Metalloproteases are a group of protease characterized by their requirement for (divalent) metal ions to sustain biological activity. Removal of these ions, by for example incubation of the enzyme with chelating agents, inhibit their catalytic activity. 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.

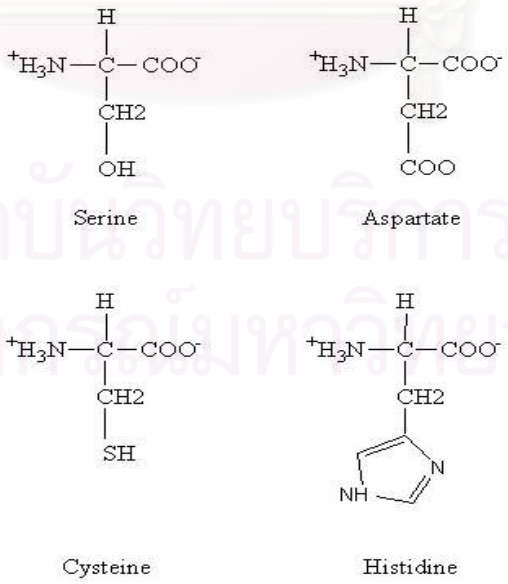


Figure 2.6 Structure of amino acids essential to the catalytic activity of proteases (Walsh, 2002).

2.5.1 Papain (Fox, 1991)

Papain is a cysteine proteases derived from papaya (see figure 2.7). The latex from papaya contains two proteolytic enzymes, papain and chymopapain. Papain is a single peptide chain made up from 212 of amino acid with three disulphide bonds and one cysteine side chain. The important catalytic side chain is Cys-25 (cysteine position 25) and His-159 (histidine position 159). The optimum working conditions are at 65-80^{o}C and pH 6-7. Papain is activated by cysteine sulfide and sulfite and inactivated by substances that react with sulfhydryl groups including heavy metals and H_2O_2.

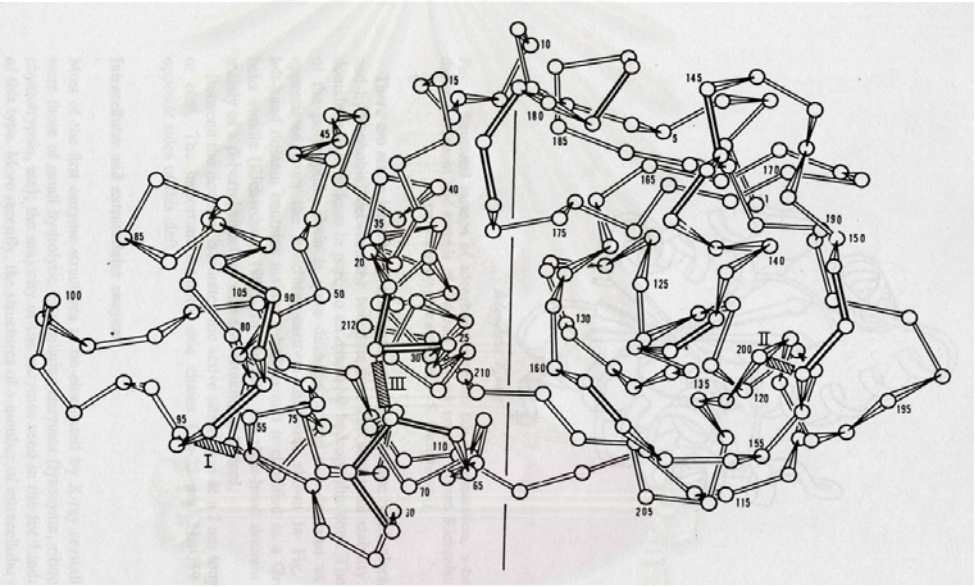


Figure 2.7 The folding of polypeptide chain in papain (Fox, 1991).

2.5.2 Neutrase (Product sheet, Novo Nordisk)

Netrase is produced by submerged fermentation of a selected strain of *Bacillus amyloliquefaciens* and can be used in protein cleaving to peptides. Neutrase is a metalloprotease (Zn), which is stabilized with Ca^{2+} and inhibited by EDTA. The optimum working conditions are at 45-55^{o}C and pH 5.5-7.5. Neutrase can be inactivated by heat treatment, e.g. 2 miuntes at 85^{o}C.

2.6 Protein determination (Sikorski, 2001)

There are many techniques of protein determination (gravimetric, refractometric, turbidimetric, spectrophotometric, colorimetric, etc.) 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, biuret and dye-binding.

2.6.1 Kjeldahl method

Kjeldahl method described in 1883 is frequently applied to determine the protein content, by using an appropriate conversion factor (Table 2.4) from the nitrogen content (as indirect method). The pure protein content is calculated by multiplied the nitrogen content with a conversion factor. In fact, it is not possible in analysis of food products, it is assumed that the product of the total amount of nitrogen in a sample (total-N) and conversion factor (e.g., 6.25) yields the amount of crude protein, the nitrogen determined in deproteinated extract of the sample being equivalent to the non-protein nitrogen (non-protein-N). Then (total-N) – (non-protein-N) = (protein-N) which, when multiplied by the same conversion factor, yields the amount of pure protein. The Kjeldahl method is the typical protein determination in foods and is also used as a reference for comparisons against all other methods. When properly conducted, the Kjeldahl assay ensures high precision and good reproducibility of results.

The major difference between the various modifications of Kjeldahl nitrogen assays is the catalyst used for mineralization and in the technique of ammonia distillation and titration. Regardless of those differences, the basic Kjeldahl procedure consists of five stages as follows: 1. preparation and weighing of sample, 2. addition of reagents, 3. mineralization, 4. steam distillation of ammonia and 5. titration.

Table 2.6 Nitrogen content and factors for calculation of crude protein.

Food	% N in Protein	Factor
Egg and egg products	14.97	6.68
Milk and daily products	15.66	6.38
Meat, fish and shellfish	16.00	6.25
Maize	16.00	6.25
Rice	16.80	5.95
Soya bean	17.51	5.71
Flour	17.54	5.7
Gelatin	18.00	5.55
Collagen	18.60	5.37

Weighing of sample

The sample should be as homogeneous as possible, weighed to 0.1 mg, and transferred to a glass tube without contact with its sides. The amount of sample is varied from 0.2 to 2.0g, depending on the protein content in product under assay. Example, at a protein content of 5-25%, an optimal sample weight is 0.5-1.0 g.

Addition of reagents

In Kjeldahl procedure, three types of reagents are added to the sample, each serving a certain function during mineralization.

1. Sulphuric acid causes oxidation of organic compounds to carbon dioxide and water and induces breakdown and transformation of nitrogen compounds to free ammonia, which is subsequently bound into ammonium sulphide.

2. Salts are necessary to increase the mineralization temperature (K_2SO_4, Na_2SO_4).

3. Catalysts accelerate oxidation. They include metal: Hg, Cu, Se (oxides): CuO, HgO, P_2O_5, TiO_2 (peroxides): H_2O_2: and salts: $CuSO_4$, $HgSO_4$.

Mineralization

Mineralization is used to transform all the nitrogen in the sample into ammonium ions. The duration of mineralization process is depends mainly on the sample type, acid volume used, catalysts applied and temperature. The presence of sulphuric acid only restricts mineralization temperature to that of sulphuric acid boiling point (338^{o}C). Addition of a salt increases the boiling point, decreases the decomposition time for organic compounds and facilitates decomposition of hard-to-mineralize substances. Because of that, they can be converted quantitatively into ammonia.

Alcacimetric titration

The distilled ammonia is titrated with hydrochloric acid solution if boric acid is used or with sodium hydroxide if hydrochloric acid is used. When titrating, it is convenient to use Tashiro indicator (0.2 g of methyl red and 0.19 g of methylene blue dissolved in 100 ml of ethanol), 0.1% bromocresol green-0.1% methyl red (10:2 v/v) in ethanol or a conductometer.

2.6.2 Lowry method

The method developed by Lowry is based on a color reaction produced by Folin-Ciocalteau reagent with peptide bonds of proteins and aromatic amino acids in appropriately alkaline medium. The reaction proceeds through two stages:

1. The biuret reaction, in which Cu^{2+} complexs with protein and peptides containing at least two peptide bonds.
2. Reduction of phosphomolybdic acid (the Folin agent) through the Cu^{2+}-protein complex to molybdenium blue.

The mechanism of Lowry reaction is described as follows. Firstly, in Folin reagent, the molybdate is reduced to molybdium blue by tyrosine and minor effect by cysteine, histidine and peptide bonds. The molybdium blue (which may include tungsten) has a blue color with maximum absorbance at 745-750 nm. Copper ions (Cu^{2+}) in alkaline solution accelerated the reduction process by forming a complex with the peptide bonds and reduced to Cu^{+}. Then Cu^{+} will react with Folin reagent, producing an unstable product which is slowly reduced to molybdate/tungsten blue.(Hall, 1996) The protein content can be calculated from the absorbance value by using Beer and Lambert's law (absorbance is linearly related to concentration, not obey at high concentration). The typical method used to evaluate the concentration of protein solution from Lowry method is calibration curve. The calibration curve is constructed by various known concentration of protein solution and showed the relationship of the protein concentration and absorbance.

2.6.3 Biuret method

The biuret reaction is based on the formation of a purple complex between copper ions and amide linkages or peptide bonds of protein in strongly alkaline solutions. The reaction affects all compounds which molecule contains two –CO-NH- groups interconnected. Tri- and oligopeptides and protein produce positive biuret reaction, but free amino acids and dipeptides can not detected by this method. Praline peptides do not form ultraviolet-absorbing complexes with copper. So this cause the less effective of reaction when used to determine gelatin (which has a high proportion of proline and hydroxyproline). The biuret method is a simple and rapid method but the disadvantage is its relatively low sensitivity and the reaction can be interfered when the sample contains various thiols, high concentrations of deoxyribonucleic acid (DNA), saccharides and lipids.

2.6.4 Dye-binding

The functional groups of proteins, particularly the basic groups in the side chains of arginine, lysine and histidine when reacting with dyes containing acid sulphonic groups ($-SO_3H$) under specific conditions, produce a color reduction in the dye solution (proportion to the protein content). The reaction is optimal in a strongly acidic medium. The concentration of protein in a sample can be determined by measuring absorbance of the dye solution before and after reaction or after separation of the insoluble dye-protein complex. Several dyes have been used as reagents to determine the protein content but the most popular in food analysis are Coomassie Brilliant Blue R250 and Orange G. Dye-binding method is very simple and rapid, convenient for routine use, highly sensitive and inexpensive. Color development occurs in less than 5 minutes and remains stable for at least 0.5-1 hour. An important advantage of dye-binding method is the lack of interference from many compounds that interfere with the Lowry method and elimination of the need for skillful manipulation and the use of corrosive reagent of the Kjeldahl method. The disadvantage of this method is according to a different affinity of dye for several pure proteins that react.

CHAPTER III

LITERATURE REVIEW

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

1. Enzymatic hydrolysis for protein hydrolysate production from various sources of animal protein.
2. Enzymatic hydrolysis for gelatin production from various sources of collagen.

1. Enzymatic hydrolysis for protein hydrolysate production from various sources of animal protein.

Taylor *et al*. (1989) studied the enzymatic treatment of offal from fleshing machines. The enzymatic treatments were performed by using Pancreatin, Enzeco, Alcalase and Neutrase. The digestion was shaken at 75 rpm for 3 hour at 40^{o}C and then for 0-18 hour at 60^{o}C at the appropriate amounts of water (50-100%). Enzyme concentration was varied in the range of 1-5% (w/w). In this study the experiments were carried out to determine the effect of time, raw material, enzyme and enzyme concentration on the protein and fat recovery. The fat from enzymatic treatments was characterized by the analyses saponification number, iodine number, peroxide value and the percentage of free fatty acids. The protein was determined by kjeldahl method. Treaments of pigskin and cattlehide fleshings with Pancreatin and Enzeco had the same result in a similar isolation of fat and hydrolyzed protein products. It was concluded that using of Pancreatin in 50% water at 40^{o}C for 3 hour and then 60^{o}C for 1 hour gave the highest percent recovery of fat and protein with the lowest amount of residue.

Nakajima *et al*. (1992) studied the hydrolysis of fish proteins in an aqueous extract of Alaskan Pollack by enzyme Samoase using a free enzyme membrane reactor (FEMR). Batch experiments were carried out to determine the effects of stirring, temperature, and pH on the enzyme activity. Three stirring speeds were used in batch experiments 0 rpm, 100 rpm and 500 rpm. The enzyme activities at 10 min were measured at the temperature of 35 to 65^{o}C and pH 7.7. For determining the effect of pH, the enzyme activities were measured in the pH range of 6-9, by adding HCl or NaOH solutions. FEMR experiments were carried out in four different conditions. For batch reactor, the results showed that stirrer speed had no effect on the enzyme activity. Maximum enzyme activity was observed at 65^{o}C and at the pH range of 6.5-8.0. For FEMR, the results showed that, the productivity was not high due to a low permeate rate. From the analysis of both the models and the experiments, less than 1/10 of the enzyme was found to be effective during FEMR operations, which appears to be mainly due to reversible deactivation. In order to get an efficient FEMR, it is necessary to determine further the cause of the enzyme deactivation and to try other membrane modules.

Kida *et al*. (1995) studied the enzymatic hydrolysis of the horn and hoof of cow and buffalo. The mixture of horn and hoof was hydrolyzed by five kinds of proteinases (B.subtilis, Bacillus species, Bacillus licheniformis, Aspergillus melleus and Pancreas of pig). The experiments were carried out to determine the effects of pH, temperature, amount of enzyme, substrate concentration and conditions for pretreatment of horn and hoof. The digestion ratio was calculated from the residual. The results showed that alkaline proteinase from B.subtilis was superior to the other enzymes and the digestion ratio was 86.5%. For enzymatic hydrolysis, heat treatment was absolutely necessary prior to digestion. The optimum conditions for enzyme hydrolysis were determined to be as follows: reaction time 30-60 minute, pH 8.3, temperature 50^{o}C, weight ratio of substrate to enzyme 1/0.05 and concentration of substrate 62.5 g/l. The molecular weight of the obtained hydrolysate was around 1.7 kDa.

Raju *et al*. (1997) studied the enzymatic hydrolysis of tannery fleshing using chicken intestine protease. The experiments were carried out to determine the effects of acid, acid concentration and amount of enzyme. The protein content of hydrolysate was measured by Lowry's method. The rate of hydrolysis of tannery fleshing was measured by determining the amount of liberated tyrosine from the supernatant at different intervals (2, 4, 6, 8, 24, 48, and 72 hour). The results showed that a combination of 1 kg of tannery fleshing and 150 g of chicken intestines in the presence of 10 ml of formic acid and 5 ml of concentrated sulphuric acid when incubated at 37^{o}C for 72 hour was the optimum hydrolysis of tannery fleshing. The protein hydrolysate obtained could be incorporated in poultry of fish feed formulations.

Bajza *et al*. (2001) studied the thermal and enzymatic recovery of proteins from untanned leather waste. Thermal treatment was performed by indirect vapor warming within 2 hour. The enzymatic method was performed with alkaline protease (Protoderm 100T). Enzymatic treatments were carried out at pH values of 9 to 10 at 56^{o}C and duration period of 120 minutes. Concentrations of enzymes were prepared in the following order 500, 2500, 5000 and 15000 units per gram of leather waste. Total protein was determined via total nitrogen by a colorimetric method with the Braun and Luebbe's autolyser. The results showed that the increasing of enzyme concentration from 500 to 15000 unit/g was increased the solubility rate and the release of soluble proteins but the rate was higher at the beginning of the process and decreased during the process. The methods that were developed have shown that the enzyme concentration 500 unit per gram of leather waste was the most cost-effective. The result showed that the enzymatic treatment was a method of choice to recovering of proteins from untanned leather waste.

Guerard *et al*. (2001) studied the effects of Alcalase on the hydrolysis of proteins from tuna wastes and explored the possibility of obtaining hydrolysates of controlled molecular weight peptides that support the growth of microorganisms in culture. Hydrolysis experiments were carried out in a batch reactor at pH 8 and 50^{o}C using the pH-stat method in controlled hydrolysis conditions (pH, temperature, enzyme concentration and stirring speed). Enzyme concentration was varied in the range of 5.6, 14.4, 28.3, 45.3 and 85 Au/kg of wet tuna stomach. The effect of hydrolysis conditions was studied with regards to the extent of proteolytic degradation and the molecular weight distribution of peptides. The controlled hydrolysis of tuna stomach protein through the action of Alcalase provided a high proportion of peptides from 6.5 kDa to di-peptides and free amino acids. A linear correlation was found between the degree of hydrolysis (DH) and the enzyme concentration. After the addition of extra substrate during the hydrolysis reaction, the relative DH was proportional to the substrate added. It was concluded that the concentration of hydrolysable bonds was one of the main factors controlling the hydrolysis rate. Preliminary results showed that tuna hydrolysates performed effectively as nitrogenous source in microbial growth media.

Guerard *et al*. (2002) studied the ability of enzyme Umamizyme to solubilise the grounded tuna stomach. Enzymatic hydrolysis was investigated in a batch reactor at pH 7 and 45^{o}C. The influence of process variables (enzyme/protein substrate ratio range from 0.1-1.5% w/w) was studied with regards to the extent of the proteolytic degradation, the nitrogen released and the molecular weight distribution of the peptides. A degree of hydrolysis up to 22.5% was obtained with an enzyme/substrate ratio of 1.5% after 4 hour of hydrolysis. The controlled hydrolysis of tuna stomach protein through the action of Umamizyme provided a high proportion of small soluble peptides below 5 kDa. A linear correlation was found to exist between the degree of hydrolysis and the nitrogen was recovered. This study showed that the protease Umamizyme is suitable for use in the production of hydrolysate from tuna stomach proteins. Umamizyme performed as effectively as Alcalase for the tuna waste solubilisation. However, the Umamizyme stability is lower than Alcalase.

Morimura *et al*. (2002) studied the procedure for the extraction of protein and production of peptides by thermal and enzymatic hydrolysis from fish bone and pig skin wastes. Fat and inorganic components were first removed in pretreatment step. Pig skin from pretreatment step was used for the extraction of high molecular weight collagen protein under acidic conditions (pH 3) using a 1 hour reaction time at 60^{o}C. The results showed that molecular weight of obtained protein from thermal hydrolysis at pH 3 was around 100 kDa. The degradation efficiency of 16 commercially available enzymes was determined. The degradation efficiency was calculated as the ratio of degraded weight of substrate to initial weight. The results showed that *Bacillus sp*. had the highest degradation efficiency of 85.8%. The pretreated fish bone and de-fated pig skin were hydrolyzed with Bacillus sp. at 60^{o}C, pH 8 and 200 rpm for 1 hour. The enzyme hydrolysis was provided degradation efficiency at 75 to 80 % with pretreated fish bone and degradation efficiency at 90 to 95 % with de-fatted pig skin. The molecular weight of the extracted protein was around 350 kDa. The hydrolysate had suitable properties for use as a food additive and cosmetic material.

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

U.S. patent number 5,877,287 (1999) described a method for producing gelatin which can be used for different sources of collagen-containing raw material. The raw material was ground to a particle size not exceeding 1 mm and mixed with water to form slurry. The slurry was treated with an acid to adjust the pH to 2-5 and heated at 60^{o}C-130^{o}C for a sufficient time to expose the collagen in the raw material. The pH and the temperature of the slurry were adjusted once more. The slurry was separated into a liquid portion and a solid residue. The gelatin was recovered from the liquid portion. If an enzyme (Esperase, Novo) treatment involving pH and temperature is adjusted to a suitable working condition of enzyme instead of the acid process condition. This method provided cost-effective and environmentally-friendly production of high-quality gelatin (bloom > 250kDa), which would involve shorter residence times, less process water and chemicals and, hence, less waste.

U.S. patent number 6,100,381 (2000) described a process for the manufacture of gelatin which includes providing a collagen containing material and demineralizing the collagen containing material to produce ossein. It was preferred that the ossein was ground to a size of less than 0.25 inches. An enzyme solution containing protease at concentration of at least 10 ppb and most preferably at least 1 ppm was added to the ossein for a time sufficient to solubilize ossein to produce gelatin solution. A distribution of molecular weight of proteins was determined by aqueous size exclusion chromatography. The distribution of molecular weight was described as the following fractions: high molecular weight HMW (>250 kDa), Beta (250-150 kDa), Alpha (150-50 kDa), Subalpha (50-20 kDa), and low molecular weight or LMW (20-4 kDa). In general, high gel strength correlates with high gelatin alpha fraction content, and high viscosity correlates with high gelatin HMW faction content.

U.S. patent number 6,080,843 (2000) described a method for producing photographic gelatin by the enzymatic action of protease on residual ossein from alkaline process. The process was begun by liquefying (at 150^{o}F) or slurrying the residual ossein (particle size < 100 microns) to obtain gelatin slurry. Protease or a proteolytic enzyme was added to the gelatin slurry at a concentration of at least 10 ppb, preferably at least 40-100 ppm to form a mixture. The mixture was reacted for a time sufficient to achieve a viscosity of less than 9 cp, preferably between 2 and 9 cp. The protease was deactivated and the gelatin was clarified. The photographic gelatin from this method was composed of a high molecular weight fraction (>250 kDa) of from 0 to 25 weight percentage, a beta fraction (150kDa-250kDa) of from 0 to 20 weight percentage and an alpha fraction (50kDa-150kDa) of from 15 to 55 weight percentage. The gelatin had gel strength around 150 to 250g bloom and the absorbance of at least 0.069 at 420 nm, when measured at a gel concentration of 6.16%.

CHAPTER IV

EXPERIMENTAL WORK

The experimental work can be divided into three main parts: (1) Materials and reagents, (2) Equipments, (3) Experimental procedures. The experimental procedures are subdivided into eight parts: (i) Raw hide characterization, (ii) Buffer preparation, (iii) Activity assay, (iv) Hydrolysis reaction, (v) Gelatin purification, (vi) Protein determination, (vii) Gel strength determination (viii) Viscosity determination. All experimental procedures are summarized in Figure 4.1.

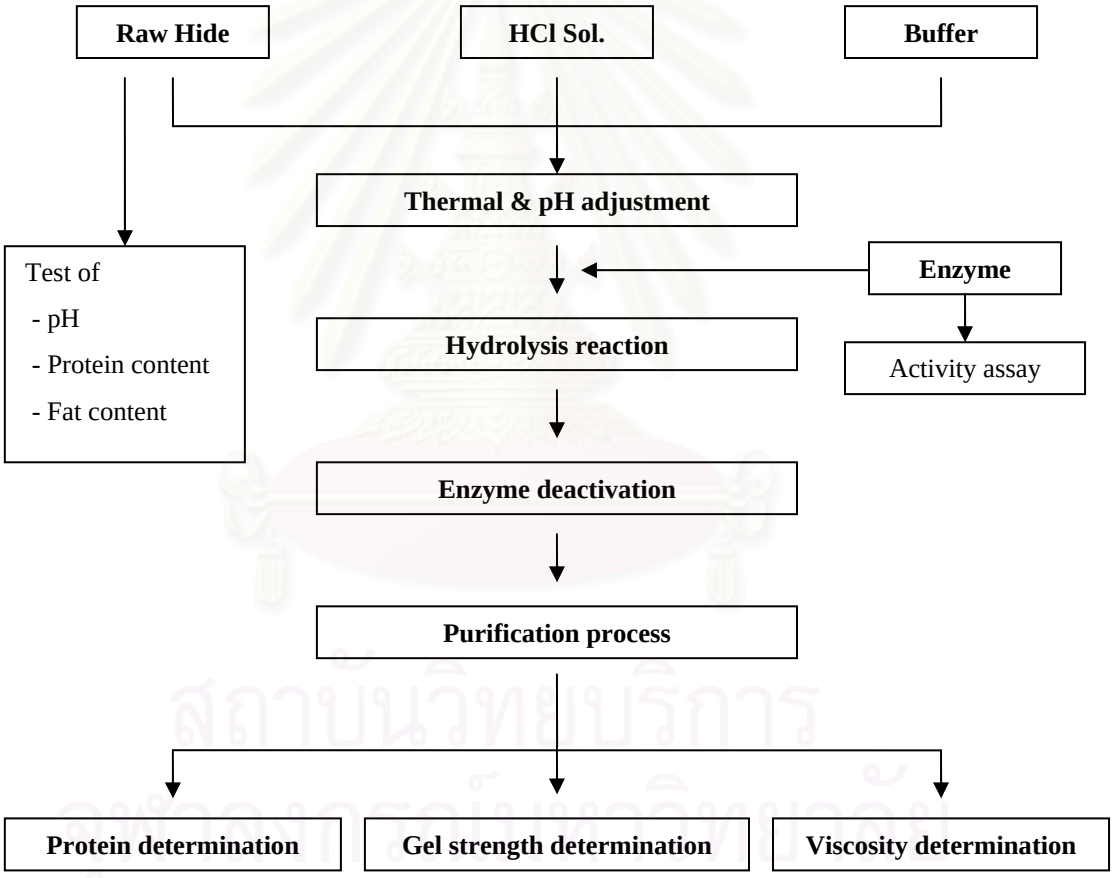


Figure 4.1 The schematic diagram of experimental procedures

4.1 Materials and Reagents

1. Raw hide

Raw hide material used in this work was limed split (grade C), kindly provided by World Pet International Co., Ltd. The dried split was obtained as two-separated lots, noted as C and C1. Each lot of dried split was ground into an average size of not exceed 1.18 mm (mesh number 16). Since there is a variation in raw hide split due to differences in original hide source and the amount of chemicals used in liming process, each lot of dried split was sampled to check pH, protein and fat contents. The pH of raw hide was used in the adjustment of pH of hide slurry by buffer and HCl solution. The results of pH of raw hide were reported in Table 4.2 and the results of protein and fat content were reported in section 5.1.2.

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 pH is at 6-7. However, before uses, the activity of papain as the function of pH and temperature was determined according to the method, which modified from assay of endo-protease using azo-casein (Megazyme), detailed in section 4.3.3. The results of the papain activity were reported in section 5.1.1.1.

3. Neutrase

Neutrase was supplied by Novo Nordisk (East Asiatic Co., Ltd) as brown solution. Neutrase is a bacterial protease produced by submerged fermentation of a selected strain of *Bacillus amyloliquefaciens*. The activity of neutrase is reported at 0.8 Anson Units/gram (Au/g) and the optimal working conditions are 45-55^{o}C and pH 5.5-7.5. The method to determine the activity of neutrase was similar to papain. The results of the neutrase activity were reported in section 5.2.1.1.

4. Copper II sulphate (anhydrous) was supplied by Unilab.
5. Sodium carbonate (anhydrous) and Potassium sodium tartrate were supplied by Univar.
6. NaOH, Dihydrogen phosphate monohydrate and Trichloroacetic acid (TCA) were supplied by Merck.

7. Di-sodium hydrogen phosphate heptahydrate and Azo-casein were purchased from Fluka.
8. HCl conc. 37 %, Chloroform and Methanol were purchased from Lab-Scan.
9. Folin-ciocalteu's reagent Casein was supplied by Carlo Erba.
10. Gelatin (Laboratory grade) was purchased from Khurusapha.
11. Gelatin (Laboratory grade, gel strength was reported) was purchased from Labchem.
12. Gelatin (Food grade) was purchased from Siam square.

4.2 Equipments

1. pH meter (PP-50) from Sartorius
2. Centrifuge (Kubota 7640) from Kubota Corporation
3. Centrifuge (Kubota 5100) from Kubota Corporation
4. UV-Spectrophotometer (6405 UV/VIS) from Jenway
5. Rheometer (Rheolab MC1) from Parr Physica
6. Texture analyzer (TA-XT2i) from Stable micro systems
7. Reactor set (1 L reactor with semi-circle stirrer and heater jacket)
8. Nitrogen analyzer set from Buchi

4.3 Experimental procedures

4.3.1 Raw hide characterization

4.3.1.1 Protein determination

In this work the protein content of raw hide was determined by the Nitrogen analyzer set (kjeldahl method). Raw hide (0.1 g) was digested with 10 ml of concentrated H_2SO_4 solution and then 0.5 g of selenium oxide was added as a catalyst (to increase the rate of organic break-down during the acid digestion and reduce the reaction time to 90 minute). The acid digested mixture was left to cool down at room temperature then back titrated with NaOH (50% w/w), boric acid (2% w/w) and concentrated H_2SO_4. The percentage of nitrogen content that calculated from back titration was multiplied by a specific protein factor (6.25) to convert to the percentage of protein content in raw hide.

4.3.1.2 Fat determination (http://www.cyberlipid.org)

The most popular fat extraction procedure is the method of Folch. The fat content of raw hide was determined by mixing raw hide with chloroform/methanol solvent (2/1 v/v). The ratio of chloroform/methanol volume and the weight of raw hide sample was 20:1. 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 (0.2 times volumes of chloroform/methanol solvent) was added to the solution to separate methanol from chloroform. After vortexing for a few seconds, the solution was centrifuged at low speed (2000 rpm) for 15 minute to separate the two liquid phases. The upper phase (water/methanol) was removed by a dropper. The lower phase (chloroform/fat) was filled into a foil dish and then left in an oven for 4 hour to evaporate chloroform. The dried fat was cooled in a desiccator at room temperature. The weight of dried fat was measured using a 4-digit balance.

4.3.2 Buffer preparation

Buffer solution used in this work was phosphate-based buffer. The 0.2 M buffer solution was prepared from sodium phosphate monobasic and dibasic solutions. Sodium phosphate monobasic solution was prepared by mixing 13.9 g of sodium phosphate monobasic with distillated water and the final volume was adjusted to 500 ml. Sodium phosphate dibasic solution was prepared by mixing 26.8 g of sodium phosphate dibasic with distillated water and the final volume was adjusted to 500 ml. The pH of the buffer solution depended on the mixing volume of sodium phosphate monobasic and dibasic solutions as shown in Table 4.1. The relationship between the volume of sodium phosphate monobasic and the obtained pH can be plotted as shown in Figure 4.2. After mixing the desired amount of sodium phosphate monobasic and dibasic solutions, the volume of the buffer solution was then adjusted to be 200 ml with deionized water.

In this work, HCl solution was used to neutralize the pH of hide slurry. The HCl solution was prepared by diluting HCl conc. 37% with deionized water to obtain the volume of 200 ml. The volume of HCl conc. 37% depended on the operating pH and the lots of raw hide as shown in Table 4.2.

Table 4.1 The ratio of sodium phosphate monobasic and sodium phosphate dibasic in 0.2 M phosphate buffer

ml of sodium phosphate monobasic	92.0	81.5	73.5	62.5	51.0	39.0	28.0	19.0	13.0	8.5	5.3
ml of sodium phosphate dibasic	8.0	18.5	26.5	37.5	49.0	61.0	72.0	81.0	87.0	91.5	94.7
pH	5.8	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0

Table 4.2 The relationship of HCl solution volume and pH of raw hide slurry

Lot of hide	C	C	C1	C1	C1	C1	C1
Volume of HCl 37% (ml)	0	20	0	20	6	4	2
Obtained pH (± 0.5)	8.65	6.0	8.95	5.0	6.0	7.0	8.0

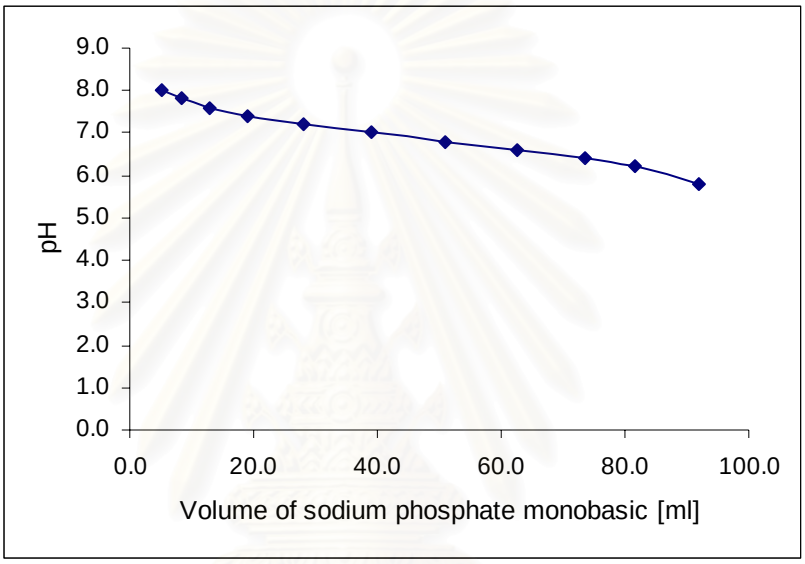


Figure 4.2 The relationship of volume of sodium phosphate monobasic and pH of buffer solution

4.3.3 Papain and neutrase activity assay (http://secure.megazyme.com/downloads/en/data/S-AZCAS.pdf**)**

Two types of proteolytic enzyme, papain and neutrase, were used for the investigation of enzymatic reaction. Table 4.3 shows the activity assay conditions that used in this work and the test method was modified from assay of endo-protease using azo-casein (Megazyme). Firstly, 0.1 ml of neutrase or 1% w/v of papain was added to the mixture solution of 1 ml of substrate solution (0.2% w/v) and 0.9 ml of 0.2 M phosphate buffer. Secondly, the solution was stirred on a vortex mixer and incubated at the operating temperature for 20 minutes. After that, the solution was put in a water bath at a controlled temperature of 4^{o}C to terminate the reaction and non-hydrolyzed substrate was precipitated by the addition of 2 ml of TCA (10% w/v). Finally, the

solution was centrifuged at 3000 rpm for 20 minutes to separate the solid and liquid phases. The liquid phase was then taken to determine the absorbance of the UV spectrum at the wavelength of 440 nm using a UV spectrometer. Blank tests were prepared by adding the TCA to the substrate solution immediately before enzyme was added.

Table 4.3 The conditions of papain and neutrase activity assay

Enzyme	Operating Temp. (^{o}C)	Operating pH	Substrate
Papain	50-70	pH 6-8	Azo-Casein
Neutrase	30-60	pH 6-8	Azo-Casein

4.3.4 Hydrolysis reaction

The hydrolysis reaction was carried out in a 1 L reactor. Since, pH of the hide slurry changed during the hydrolysis process, phosphate buffer was used to maintain the desired pH. The ratio of raw hide to buffer solution was 1:4 (200g:800g). The amount of papain and neutrase in hydrolysis reaction was 0.3 g and 0.117 ml per 200 g of raw hide, respectively. For papain, the ratio of enzyme to raw hide substrate was selected from the results reported in section 5.1.2. The hydrolysis reaction was performed by first mixing 200 g of raw hide with 200 ml of HCl solution. The mixture was stirred until well mixing was obtained, then 400 ml of phosphate buffer was added. The speed of over-head stirrer was adjusted to 75 rpm and the slurry was heated to a desired temperature. The enzyme solution was prepared by mixing 200 ml of phosphate buffer with enzyme. After the temperature of hide slurry reached the hydrolysis temperature, the enzyme solution was added. Samples were collected at 6, 10, 20, 40, 60 and 90 minute periodically. The collected samples were kept in a freezer (-10^{o}C) to inhibit the hydrolysis reaction. Finally the collected samples were heated to 90^{o}C for 15 minutes to deactivate the activity of enzyme.

4.3.5 Gelatin purification

In purification process, gelatin solution was separated from hide residue by centrifugation and filtration techniques. In centrifugation step, the treated slurry was filled into centrifuge bottles. Centrifuge machine that was used in this study was Kubota 7640 and the operating conditions were set at 3000 rpm for 15 minutes at the room temperature. After centrifugation, the supernatant was separated from residue by a vacuum filtration kit using Whatman filtration paper grade 4 (20-25μm). The 0.25 ml of filtered gelatin solution was brought to determine the protein content by Lowry method. The remaining solution was preheated in a microwave oven at 100 W for 15 minutes, then dried in a conventional oven at 60^{o}C for 10 hours and then kept in a desiccator. The obtained gelatin was taken to determine the gel strength and viscosity afterwards.

4.3.6 Protein determination

In this study Lowry method was used to determine the percentage of protein content in gelatin solution.

4.3.6.1 Reagents

Reagents used in Lowry test are composed of

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

4.3.6.2 Test procedures

Solution E was prepared by mixing 49 ml of solution C, 49 ml of solution D, 1 ml of solution A and 1 ml of solution B together. Solution E must be freshly prepared for each test because of the precipitation of copper. The 0.25 ml of gelatin solution was adjusted to 100 ml by distillated water. The 0.5 ml of diluted gelatin solution was mixed with 2.5 ml of solution E and shaked in a vortex mixer. After 10 minute, 0.25 ml of solution F was added and the solution was further shaked. Reaction was allowed to occur at room temperature for 30 minute. The absorbance of the sample was measured at the wavelength of 750 nm by a UV Spectrometer. Diluted gelatin solution was replaced by distillated water and used as a blank. The standard curve was prepared by using commercial gelatin solution (laboratory grade from khurusapha) at various concentrations in the range of 0.2-1 mg/ml (see Appendices). In this work the reported protein content in gelatin solution was determined from the average of 3-repeated test value and the error was around ±1-2%.

4.3.7 Gel strength Determination

4.3.7.1 Gel preparation

The method of gel strength determination in this work was modified from the British Standard (BS EN ISO 9665:200). The concentration of gelatin solution used to form gel was 12.5% (w/w). The 5 g ± 0.01 g of gelatin was dissolved in 35 ml of water in a test bottle. The bottle was swirled vigorously to completely wet the sample then placed in an oven at 50^{o}C for 15 minute. The solution was manually stirred and put in the oven again for 15 minute. 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. If the gelation occurred, the gel strength of the sample was measured.

4.3.7.2 Gel strength determination

In this study the texture analyzer was used to determine the gel strength of the gelatin. The gel strength of the sample was determined by measuring the force required to depress the gel sample vertically to a depth of 4 mm ± 0.01 mm, either at a constant rate of loading not exceeding 40 g/s or at a constant rate of penetration not exceeding 0.8 mm/s. Test bottle was placed on the platform of the texture analyzer, then the center of gel surface was adjusted to be underneath the plunger. The measurement was performed as suggested in the instrument manual. The type of the plunger that used for measuring the force in this study is hemisphere (P/0.5 HS). The measured gel strength was double bloom and the conditions of the texture analyzer were shown in Figure 4.3. In this work the gel strength was reported as the averaged value of the two measurements.

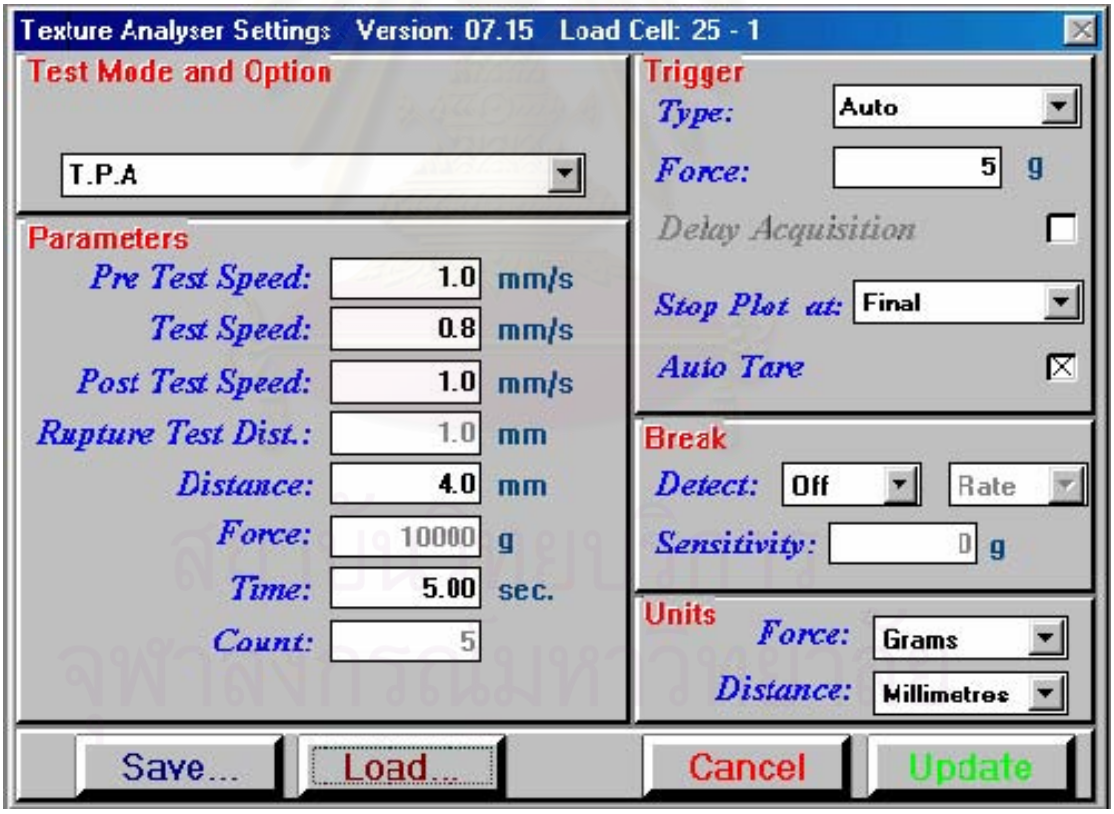


Figure 4.3 The conditions of texture analyzer for gel strength determination

4.3.8 Viscosity determination

The method of viscosity determination in this work was modified from The British Standard (BS EN ISO 9665:200). The concentration of the gelatin solution used to measure viscosity was 12.5% (w/w). The gelatin solution was prepared as described in section 4.3.7.1. The gelatin solution was heated at 60^{o}C for 30 minute before the viscosity test. The gelatin solution (30 ml) was filled in a rheometer and the test was proceeded with the sequence of the operations required. The shear rate was adjusted to 0.1-500 s^{-1} and the viscosity was recorded every 10 second (30 points). The type of rheometer head was Z1 DIN (double gap). In this work the viscosity was reported as the averaged value of the two measurements.

CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Papain

5.1.1 Test of Papain

5.1.1.1 Activity

The activity of papain at various temperatures and pH was presented in Figure 5.1.

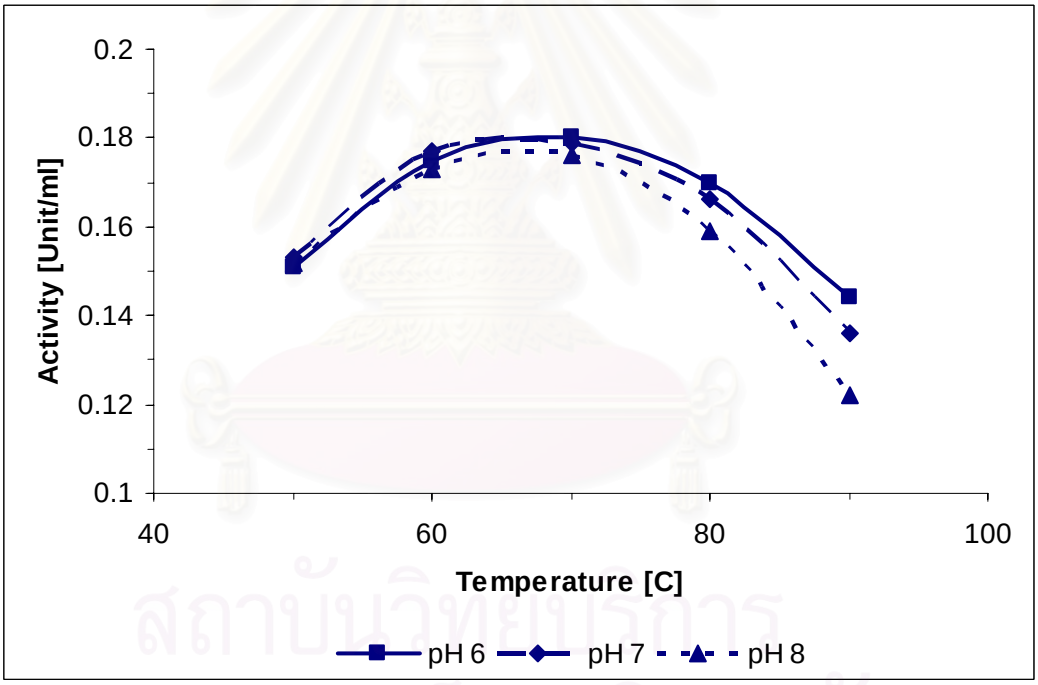


Figure 5.1 Activity of papain at various pH and temperatures (substrate: Azo-casein)

At pH 6, the activity of papain increased as the temperature increased from 50°C to 70°C. On the contrary, that of papain started to decrease as the temperature was higher than 70°C. This could be due to the reason that enzyme was denatured at high temperature. When the pH was increased to 7 and 8, papain showed the same characteristic of activity as pH 6 but at pH 8 the activity decrease significantly. The

result shows that the optimum pH and temperature of enzyme papain are at pH 6-7 and 60-70^{o}C, respectively.

5.1.1.2 Optimum ratio of enzyme to raw hide

The objective of this section was to find the proper amount of papain to hydrolyze 200 g of raw hide. The amount of papain was increased until the recovery of gelatin was not increased.

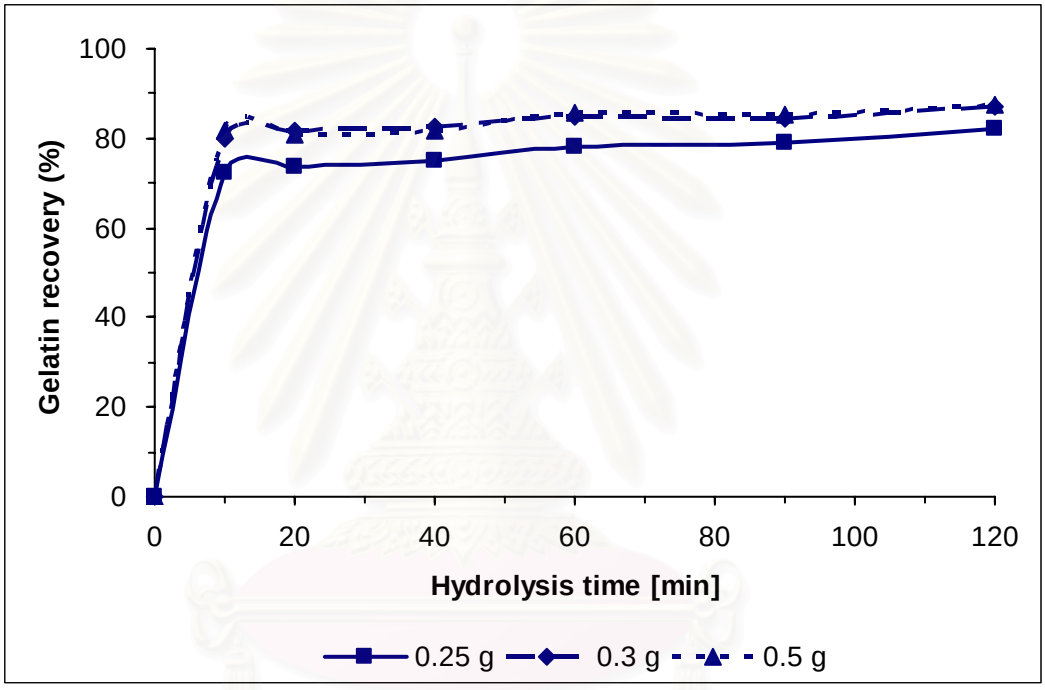


Figure 5.2 Gelatin recovery from raw hide (C) at various amounts of papain

The hydrolysis of hide C was carried out at 70^{o}C, 75 rpm and pH 6. The gelatin recovery at various amounts of enzyme from 0.25 g to 0.5 g per 200 g of raw hide was presented in Figure 5.2. The average of gelatin recovery increased 8.8 % when the amount of enzyme was increased from 0.25 g to 0.3 g (20 % increase). The result showed that the average of the gelatin recovery did not significantly change when the amount of enzyme was increased from 0.3 g to 0.5 g (66.67 % increase). It can be noticed that as the amount of papain was higher than 0.3 g, the percentage of gelatin recovery did not significantly increase. This could be concluded that the optimum amount of papain used to hydrolyze a 200 g of raw hide was 0.3 g.

Therefore the ratio of enzyme papain to raw hide used in further investigation in this work was fixed at 0.3 g to 200 g, respectively.

5.1.1.3 Stability of papain activity

The stability of papain activity during hydrolysis reaction was investigated using a blank hydrolysis reaction batch (without raw hide) at 70^{o}C, 75 rpm and pH 6. The enzyme solution in the reactor was taken to test its activity at different hydrolysis times of 1, 10, 20, 30, 40 and 50 minute. The activity of enzyme papain was presented in Figure 5.3.

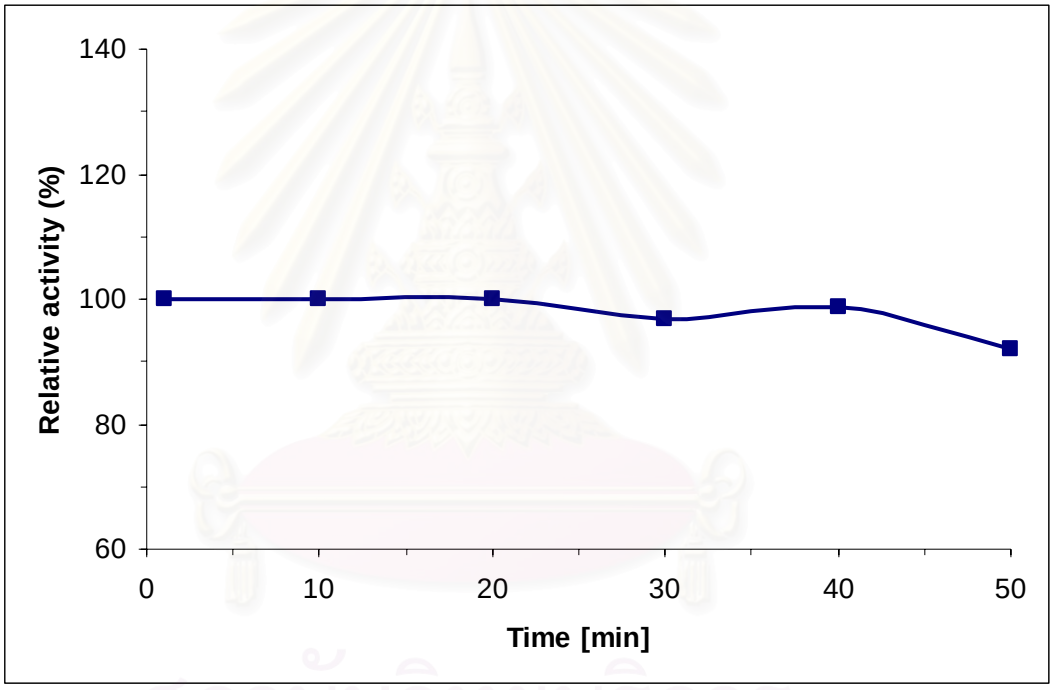


Figure 5.3 Activity curve of papain at 70^{o}C and pH 6 (substrate: Azo-casein)

The result showed that papain activity was stable during the first 20 minute. However, the activity of the enzyme decreased by 1.25% (20-40 minute) and by 6.88% (40-50 minute). This indicated that papain can be used in hydrolysis for approximately 40 minute under the tested condition (70^{o}C, pH 6). (At longer period of time, the enzyme activity reduced. As a result, the hydrolysis decreased.)

5.1.2 Protein and fat contents in raw hide

Two lots of raw hide (C and C1) were used in this work. It was possible that these two lots were from different sources and tanning treatment. Therefore protein and fat contents of the sample must be determined and were used to characterize the raw hide. The amount of lime and liming time in the tannery treatment were the main factors that could cause the difference in protein and fat contents in raw hide. The protein and fat contents from two lots of raw hide were presented in Figure 5.4.

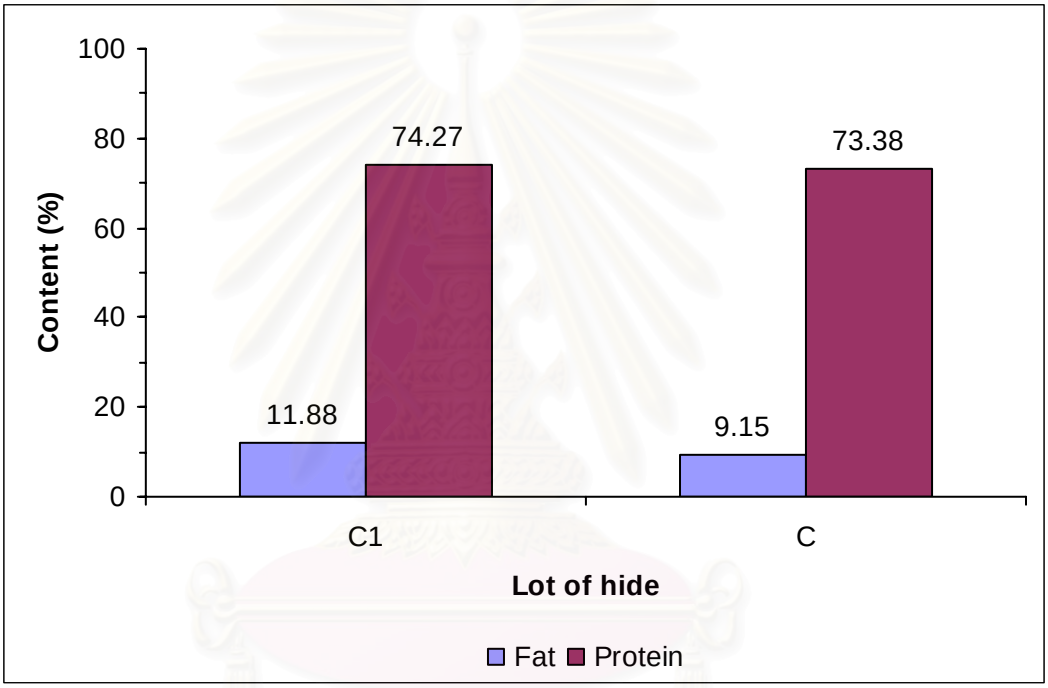


Figure 5.4 Protein and fat contents in raw hide from lot C1 and C

From Figure 5.4, it can be seen that the protein contents in hide C and C1 were slightly different and the fat content in hide C1 was higher than that in hide C by 2.7 %. The result implied that the amount of lime used in the tannery treatment of hide C was more than that of hide C1 or the liming time in the tannery treatment of hide C was longer than that of hide C1. Normally, liming chemical was used to preserve raw hide. However, at the same time, the liming hydrolysis of raw hide occurred so the exposure of hide in concentrated lime or long time treatment could cause the loss of protein and fat in raw hide.

In order to compare the effects of protein and fat contents on the enzymatic hydrolysis of raw hide, two different lots of raw hide materials (C and C1) were tested under the same hydrolysis conditions.

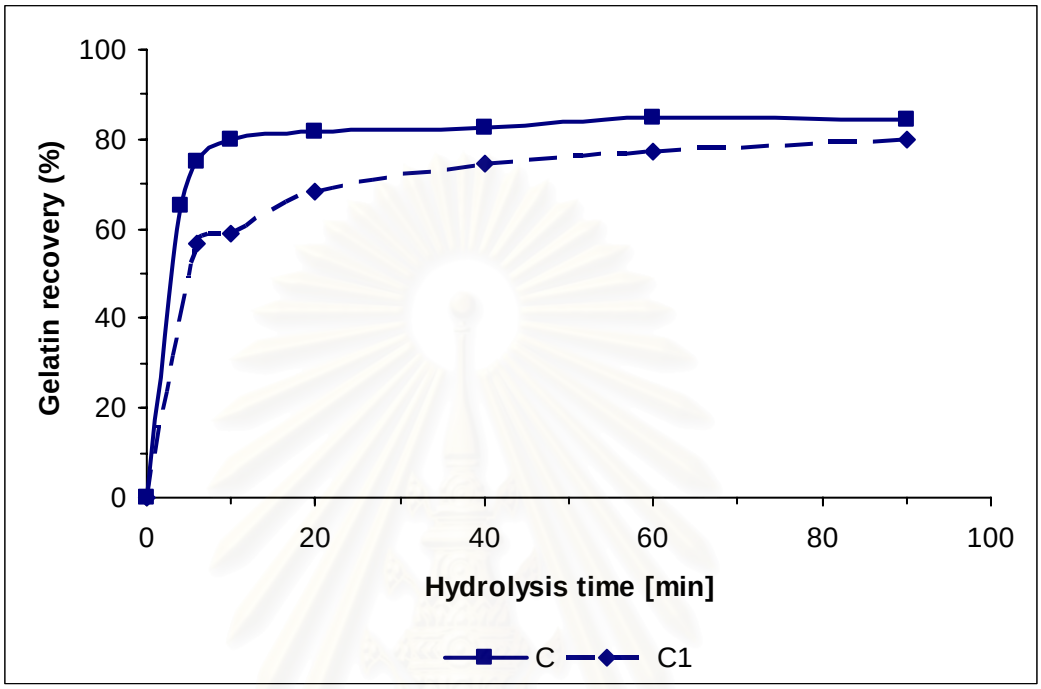


Figure 5.5 Gelatin recovery of two different lots of raw hide (C and C1) at 70°C and pH 6

The hydrolysis condition of this experiment was carried out at 70°C, 75 rpm, and pH 6. The gelatin recovery of two samples of hide (C and C1) was presented in Figure 5.5. The gelatin recovery from hide C was always higher than that from hide C1, especially from the beginning of the reaction up to 10 minute. This could be explained by the reason that lipid molecules in the structure of raw hide interfere the action of enzyme. For example, lipid molecule might obstruct or obscure the hydrolysis site. Therefore, the gelatin recovery from hide C1 which has higher fat content was lower than that from hide C. The results therefore implied that fat content in raw hide was one of the main factors controlling the hydrolysis rate of raw hide, especially at the beginning of the hydrolysis reaction.

5.1.3 Effects of hydrolysis conditions on gelatin recovery

5.1.3.1 Effects of temperature on gelatin recovery

The effects of hydrolysis temperature at each fixed pH on gelatin recovery are presented in this section. Figure 5.6 shows the gelatin recovery from papain hydrolysis reaction. The hydrolysis condition of hide C in this experiment was carried out at pH 6 and 75 rpm.

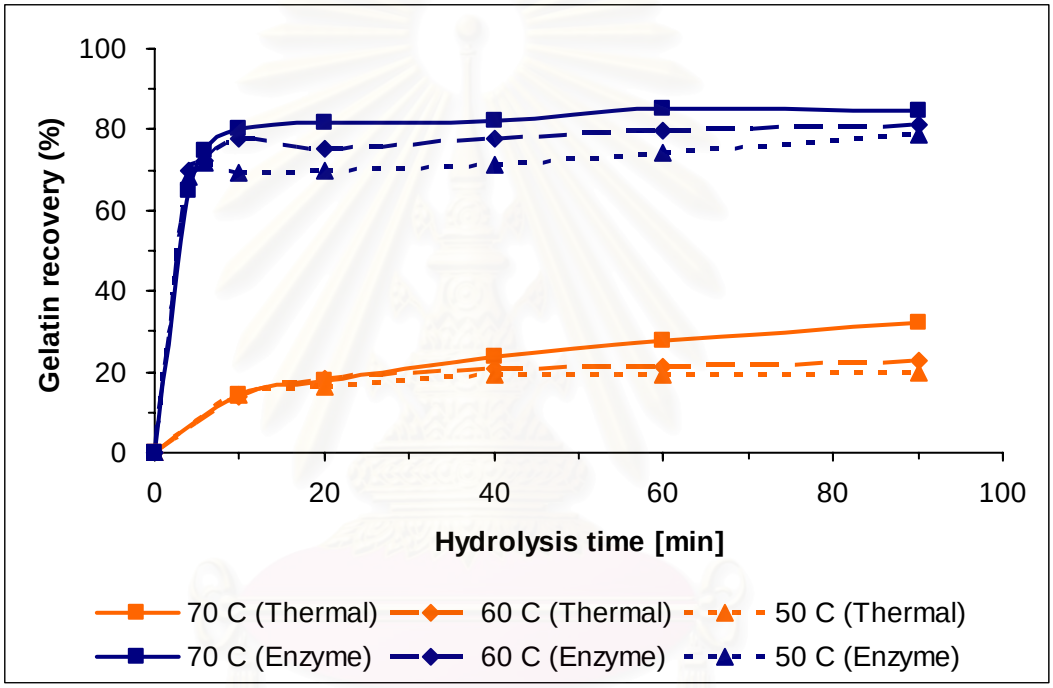


Figure 5.6 Gelatin recovery from enzymatic hydrolysis and thermal hydrolysis at pH 6 and various temperatures (raw hide: C)

Considering each gelatin recovery curve at constant temperature, one can notice that the initial rate of gelatin recovery from enzyme hydrolysis was very high (within 4-6 minute). After that the recovery rate was gradually lower as the hydrolysis reaction progressed. Therefore at the beginning of the hydrolysis reaction, the percentage of gelatin recovery was increased sharply up to about 6 minute, then started to slightly increase. This might be caused by the depletion of protein source, the enzyme deactivation and the product inhibition. From section 5.1.1.3, it was showed that the activity of enzyme did not decrease until 40 minute of reaction so the

possible reason for decreasing of recovery rate might be due to the depletion of protein or the low concentration of protein substrate.

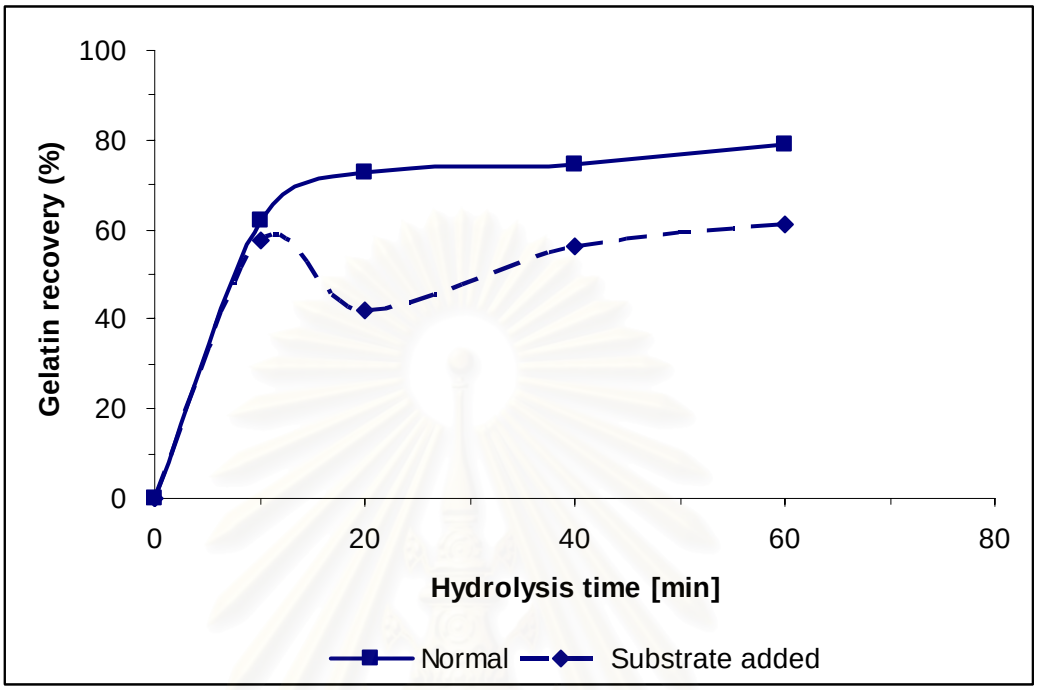


Figure 5.7 Gelatin recovery from enzymatic hydrolysis at pH 7 and constant temperature 70^{o}C (raw hide: C1)

Additional experiment was performed to verify this point by adding raw hide substrate in the reactor after 10 minute of hydrolysis, so the substrate concentration was about the same as at the starting of hydrolysis reaction. The gelatin recovery was compared with the normal hydrolysis (no substrate added) in Figure 5.7. After the substrate was added in the reaction, it can be seen that the percentage of gelatin recovery was decreased. This was because the higher concentration of substrate. If the concentration of substrate was the factor controlling the hydrolysis rate, the percentage of gelatin recovery should be increased when the reaction proceeded. From the results, it was found that in the case of adding substrate the gelatin recovery was increased comparing to the case of no substrate adding. So the depletion of protein or the low concentration of protein substrate should be the main factor controlling the hydrolysis rate. The effect of product inhibition might be a minor effect and can be negligible as reported by the work of Guerard *et al* (2001). Guerard *et al*. (2001) have studied the enzymatic hydrolysis of proteins from yellowfin tuna wastes using Alcalase, have suggested that the hydrolysis curve downward tendency

could be attributed to one of the following phenomena: a decrease in concentration of peptide bonds available for hydrolysis, an enzyme activity decrease and a product inhibition. They have proved that the main reason was the decrease in the concentration of peptide bonds, corresponding to our results.

When varying the temperature (50^oC, 60^oC and 70^oC) of the enzyme hydrolysis reaction at pH 6, it was found that at the beginning of reaction (within 6 minute) the percentages of gelatin recovery at 50^oC, 60^oC and 70^oC were similar. As the hydrolysis time was longer than 6 minutes, the percentages of gelatin recovery at 70^oC were highest and the percentages of gelatin recovery at 50^oC were lowest. When the hydrolysis time proceeded longer than 60 minute, the gelatin recovery at 70^oC was rather consistent at around 85%. While the gelatin recovery at 60^oC and 50^oC was still slowly increased, moving closed to the result at 70^oC. In other words, the higher enzyme hydrolysis temperature was, the higher gelatin recovery was observed. This was the result from the activity of papain at pH 6 showing the optimum hydrolysis temperature of enzyme papain was at 70^oC (see Section 5.1.1.1). However, the effects of enzyme hydrolysis temperature mentioned earlier could not be noticed during the first 6 minute of the reaction, possibly due to the fat content in raw hide structure that inhibited the binding step of active site of enzyme with peptide bond.

In this work the enzyme hydrolysis reaction was carried out at high temperature of which collagen can be thermally hydrolyzed. So the gelatin recovery in Figure 5.6 might be the result of both enzyme and thermal hydrolysis reaction. Therefore to clarify the influence of thermal hydrolysis reaction, the thermal hydrolysis of raw hide at each constant temperature and pH 6 was performed. From Figure 5.6, at the beginning of reaction (within 10 minute), the percentages of gelatin recovery from thermal hydrolysis at pH 6 (50^oC, 60^oC and 70^oC) were similar at around 14%. This implied that during the first period of thermal hydrolysis the temperature had no effects on the gelatin recovery. As the time was longer than 10 minute, the percentages of gelatin recovery at 50^oC and 60^oC were rather consistent, but the percentages of gelatin recovery at 70^oC were gradually increased. When the thermal hydrolysis reaction proceeded longer than 60 minute, it can be obviously noticed that the gelatin recovery at 70^oC was much higher than those at 60^oC and 50^oC while the gelatin recovery at 60^oC was slightly higher than that at 50^oC. This

was because the structure of collagen molecules in muscle remains relatively stable due to the crystallization energy of the triple helix until the temperature reaches 64°C at which the triple helix begins to break down (Powell, 1999). The result on thermal hydrolysis corresponds with the work of Linus *et al*. (1996). The percentages of gelatinous protein extracts recovered from the mechanically deboned turkey residue (MDTR) by thermal hydrolysis were increased when the hydrolysis temperature was increased (55-85°C).

The actual gelatin recovery from only the enzyme hydrolysis was calculated by subtracting the gelatin recovery from enzyme hydrolysis with that from thermal hydrolysis, and presented in Figure 5.8.

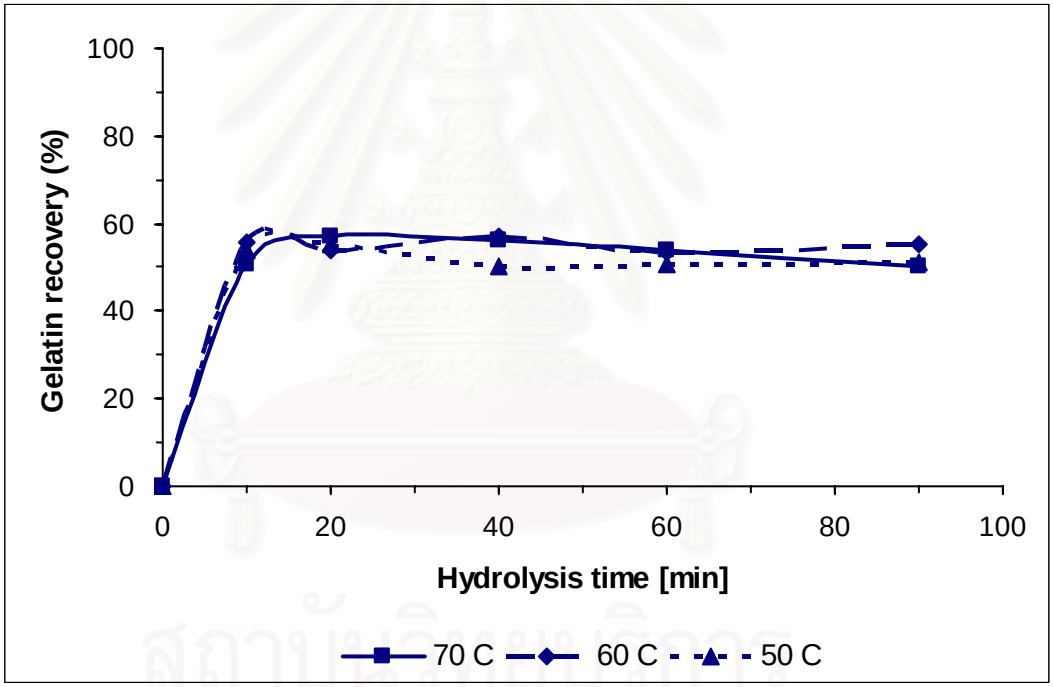


Figure 5.8 Gelatin recovery resulted from only the enzyme hydrolysis at various temperatures and pH 6

From Figure 5.8, it can be seen that the gelatin recovery from only the enzyme hydrolysis at each constant temperature was increased sharply from the beginning up to 10 minute. As the hydrolysis time was longer than 10 minute, the gelatin recovery (from only the enzyme hydrolysis) at 60°C and 70°C was rather consistent because of the depletion of protein in enzyme reaction and the accumulation of gelatin recovery from thermal hydrolysis. But at 70°C, it can be noticed that at the longer hydrolysis

time the gelatin recovery from only the enzyme hydrolysis was slightly decreased. This could be due to the effect of thermal on the gelatin recovery at high temperature (70°C) which was stronger than that at lower temperatures (60°C and 50°C) and the decreasing of activity of papain (see Figure 5.3).

The gelatin recovery from papain hydrolysis reaction at various temperatures and constant pH 7 was presented in Figure 5.9.

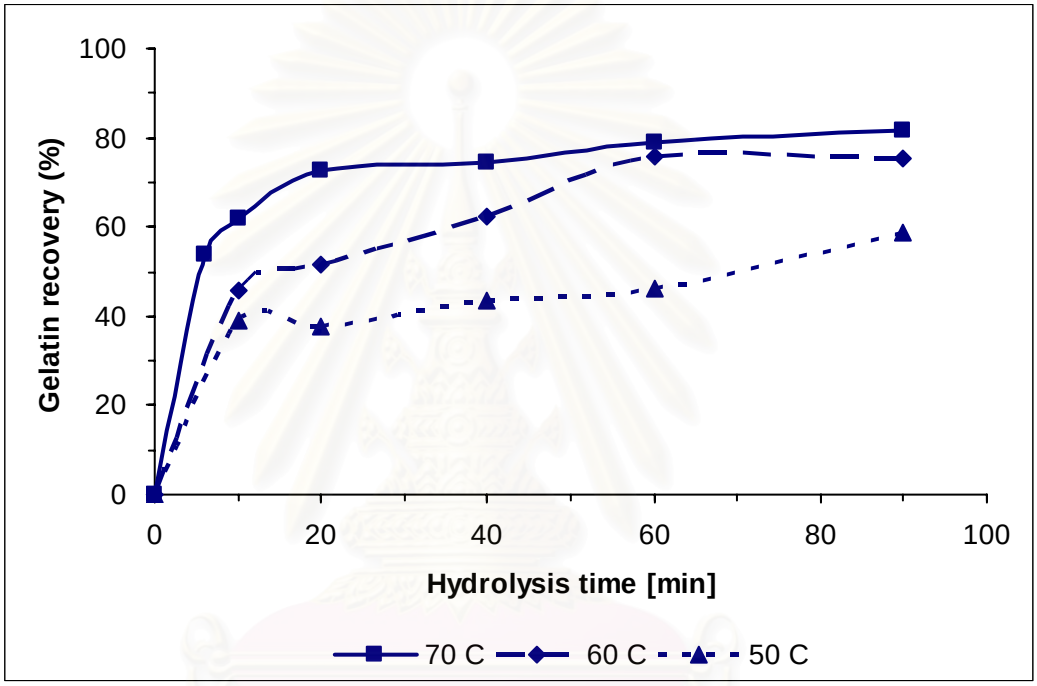


Figure 5.9 Gelatin recovery from enzymatic hydrolysis at pH 7 and various temperatures (raw hide: C1)

The raw hide used in this condition was C1 instead of C that used in pH 6 because of the shortage of hide supply. From Figure 5.9, similarly to the gelatin recovery at pH 6, the percentages of gelatin recovery at 70°C were higher than the one at 60°C and 50°C. This was because of the activity of papain at pH 7 was increased when increasing the temperature and the optimum hydrolysis temperature of enzyme papain was at 70°C (see section 5.1.1.1). However, in this case, the initial gelatin recovery rates at three different temperatures were different. The initial recovery rates were in the order of 70°C>60°C>50°C. As hydrolysis time was longer than 10 minutes, at 60°C, the gelatin recovery was gradually increased until 60 minutes. After that the gelatin recovery was rather consistent because the effect of fat in raw hide

structure was decreased by heat and agitation that discussed in section 5.2. But at 50°C, the gelatin recovery started to slowly increase after 20 minutes of enzyme hydrolysis reaction, up to 90 minutes it was still much lower than the ones at 60°C and 70°C. It might be due to the hydrolysis condition that was not suitable for the enzyme hydrolysis reaction.

The gelatin recovery from papain hydrolysis reaction at various temperatures and constant pH 8 was presented in Figure 5.10.

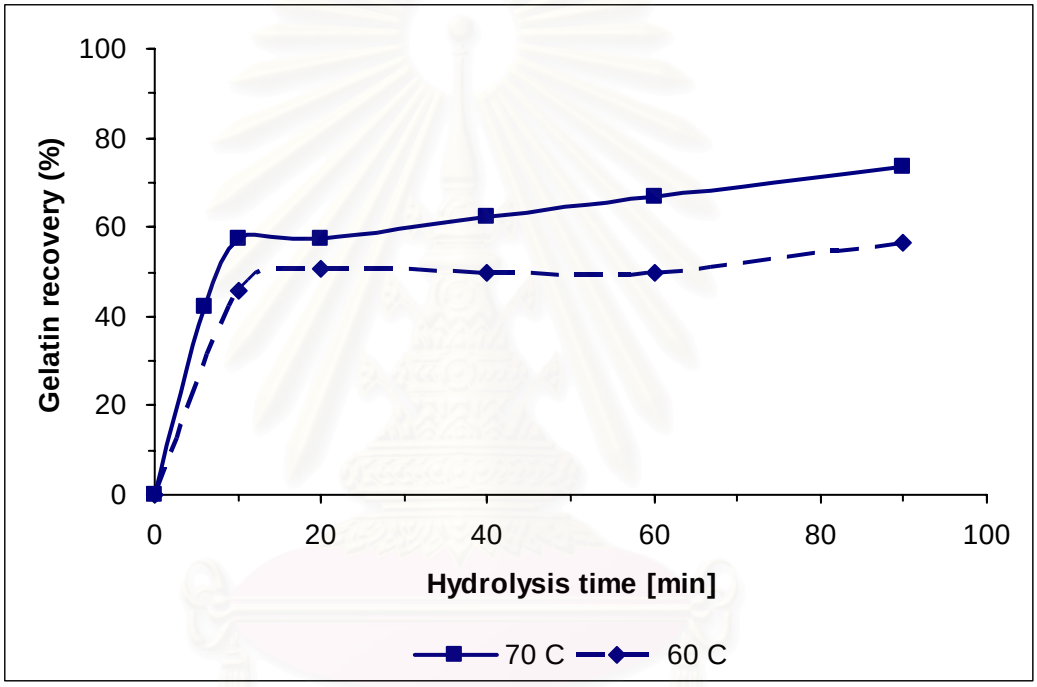


Figure 5.10 Gelatin recovery curve at various temperatures constant pH 8 (raw hide: C1)

The pattern of gelatin recovery at pH 8 was similar to the one at pH 7. From Figure 5.10, the results showed that the gelatin recovery at 70°C was higher than the one at 60°C. This was due to the reason of the activity of papain at pH 8 (see section 5.1.1.1). In this case of study, the gelatin recovery at 50°C was not measured since the papain hydrolysis reaction at this condition was extremely slow, i.e. after 90 minutes of reaction the raw hide was still in the solid form. This might be due to the activity of papain was very low at this condition.

From the results of the effects of enzyme hydrolysis temperature on the gelatin recovery at constant pH 6, pH 7 and pH 8, it can be concluded that the maximum gelatin recovery at each constant pH was reached when the temperature of the reaction was at 70^{o}C, the optimum working temperature of papain. The percentages of gelatin recovery decreased when temperature was decreased from 70^{o}C to 60^{o}C and 50^{o}C, respectively. This was mainly resulted from the effect of thermal hydrolysis that was stronger at higher temperature (see Figure 5.6). The other reason was the decreasing of papain activity at lower temperature.

5.1.3.2 Effects of pH on gelatin recovery

From section 5.1.3.1, the results showed that the optimum temperature for highest gelatin recovery at various pH was 70°C. Therefore, in this section, the effects of pH (5 to 8) on the gelatin recovery from hide C1 at constant hydrolysis temperature of 70°C were compared. The gelatin recovery from papain hydrolysis reaction at various pH from 5 to 8 and constant hydrolysis temperature of 70°C, was presented in Figure 5.11.

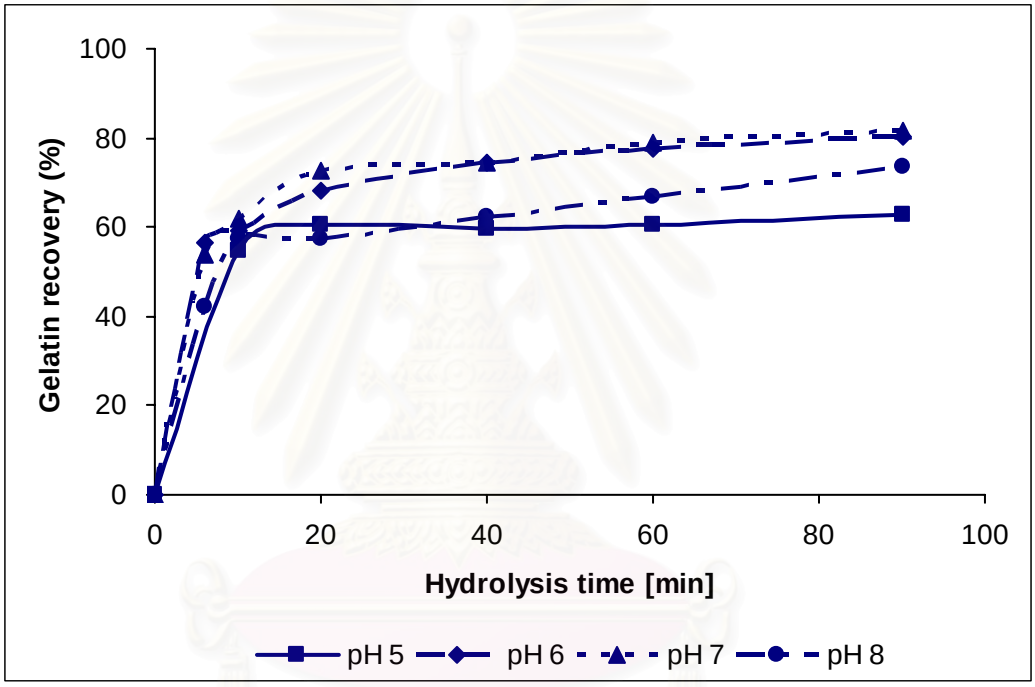


Figure 5.11 Gelatin recovery curve at various pH, constant temperature of 70°C (raw hide: C1)

In this case the effect of fat in raw hide was neglected because hide C1 was all used. From Figure 5.11, it can be noticed that the initial rate of gelatin recovery (within 10 minute) at pH 6 and pH 7 was very similar and higher than those at pH 5 and pH 8. This was because the activity of papain at pH 6 was very similar to that of pH 7 and it was at the highest value comparing to those at pH 8 and pH 5. As the hydrolysis time was longer than 10 minute, the percentage of gelatin recovery at pH 6 and pH 7 was gradually increased with the same trend until the maximum of gelatin recovery at the hydrolysis time of 90 minute (≈80 %) was reached. After 90 minute of reaction, the percentage of gelatin recovery was slightly increased (≈1-2% per

hour) so the reaction was stopped at 90 minute. At pH 5, the percentages of gelatin seem to be consistent as the hydrolysis time progressed. This might be due to the activity of papain was very low at pH 5. At pH 8, the percentages of gelatin recovery was slowly increased but less than at pH 6 and pH 7.

It can be seen that the optimum pH of enzyme hydrolysis reaction at 70^{o}C was at pH 6-7 which is the optimum working pH of papain. The activity of papain was decreased at pH 8 and pH 5. At pH 5, it seems that the rate of gelatin recovery was very low indicating that this condition was not suitable to recover gelatin from raw hide by enzyme papain.

5.1.4 Effects of hydrolysis conditions on gelatin properties

5.1.4.1 Effects of temperature on gelatin properties

The effects of enzyme hydrolysis at each condition on the gelatin properties are presented in this section. The gelatin properties studied in this work were the viscosity and gel strength. The methods of preparation and determination were described in section 4.3.7 and 4.3.8. The viscosity and gel strength of gelatin solutions from papain hydrolysis reaction at pH 6 of hide C were showed in Figure 5.12 and 5.13, respectively.

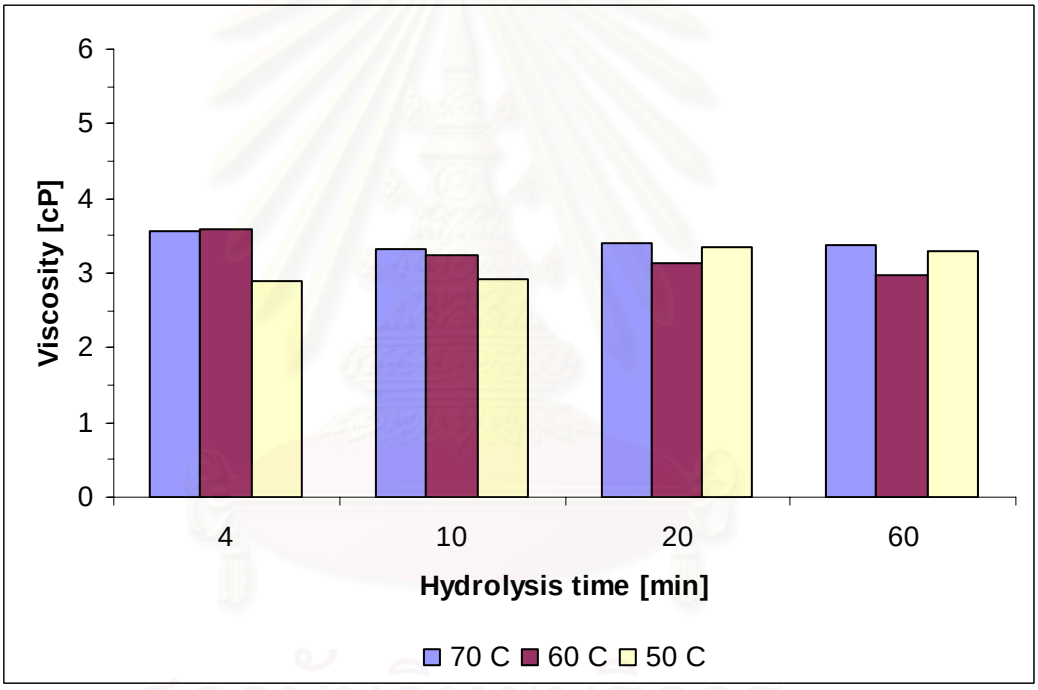


Figure 5.12 Viscosity of gelatin solution obtained from hydrolysis reaction at various temperatures, pH 6 (raw hide: C)

From Figure 5.12, it can be seen that the viscosity of gelatin solution at any temperature was not significantly different from each other. The viscosity was in the range of 2.9 cP to 3.6 cP. The effects of enzyme hydrolysis temperature at pH 6 on the viscosity of gelatin could not be obviously noticed for all hydrolysis time.

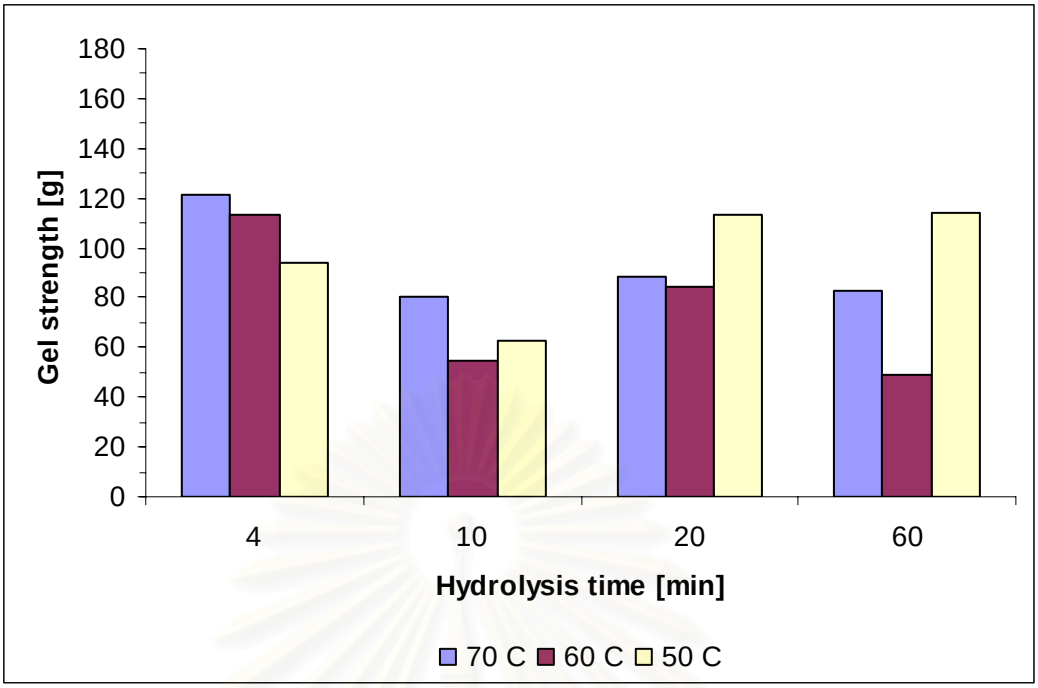


Figure 5.13 Gel strength of gelatin obtained from hydrolysis reaction at various temperatures, pH 6 (raw hide: C)

From Figure 5.13, it can be seen that, the effects of enzyme hydrolysis temperature on the gel strength of gelatin could not be clearly determined and the variation of the gel strength of gelatin solution was much higher than that of the viscosity. The gel strength was varied in the range of 48 g to 121 g.

The viscosity and gel strength of gelatin from papain hydrolysis reaction at various temperatures, constant pH 7 were presented in Figure 5.14 and 5.15, respectively. From Figure 5.14, when considering the viscosity of gelatin solution at the same hydrolysis time for each temperature, it can be seen that the values of viscosity were not significantly different. But the average viscosity of gelatin solution obtained at 90 minute of reaction was higher than the one obtained at 10 minute of reaction. The average viscosity of gelatin obtained at 90 minute and 10 minute of hydrolysis time were 4.6 cP and 3.3 cP, respectively.

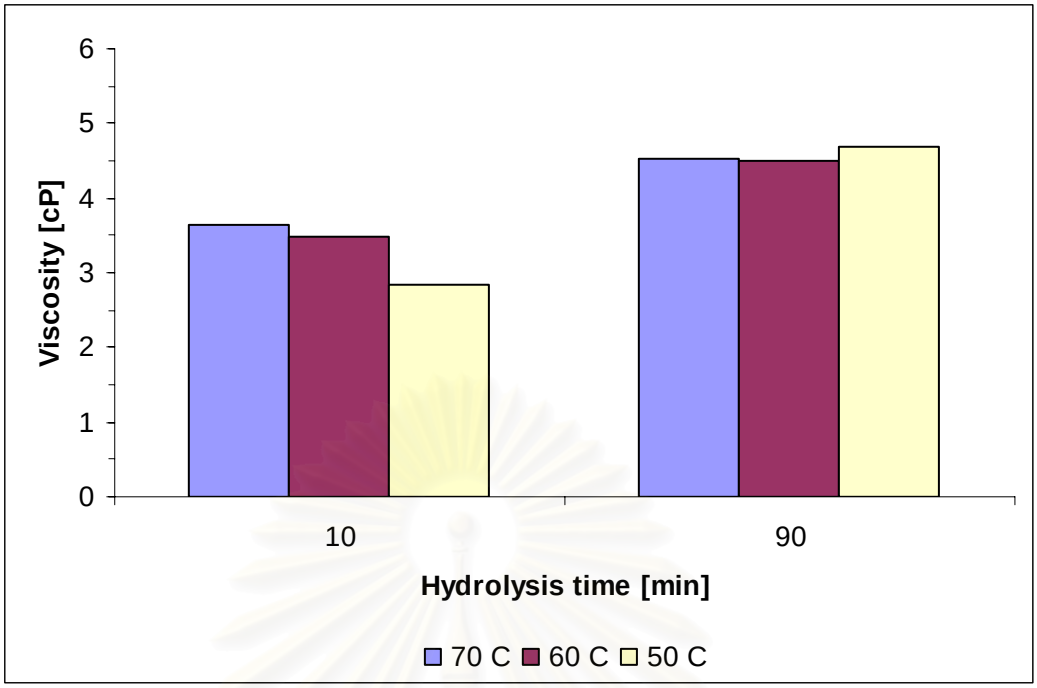


Figure 5.14 Viscosity of gelatin solution obtained from hydrolysis reaction at various temperatures, pH 7 (raw hide: C1)

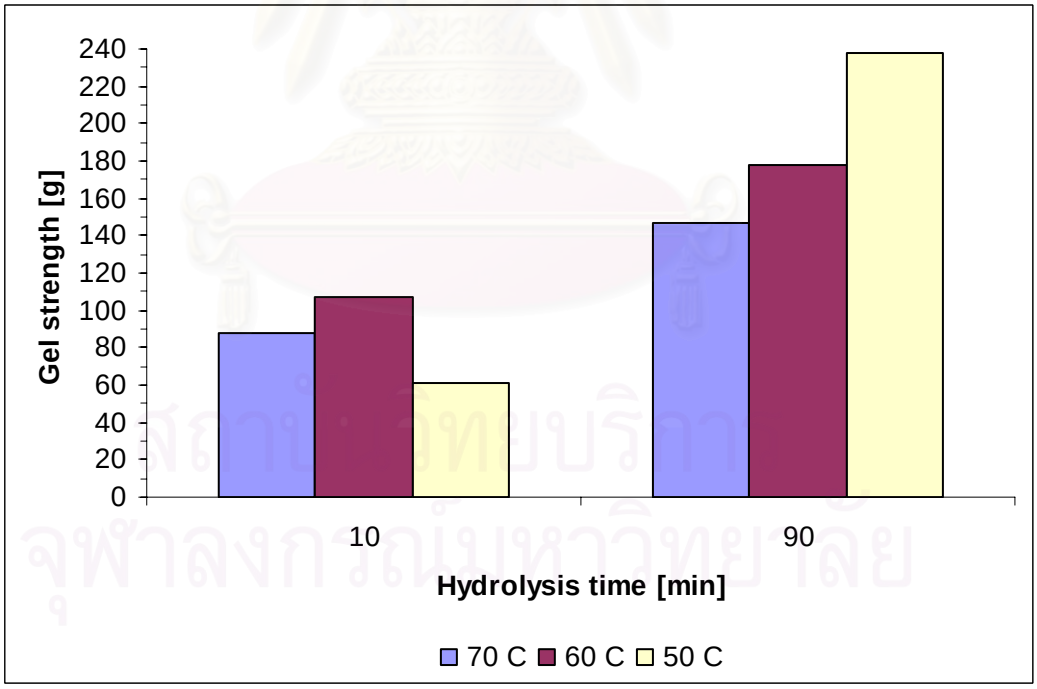


Figure 5.15 Gel strength of gelatin obtained from hydrolysis reaction at various temperatures, pH 7 (raw hide: C1)

From Figure 5.15, for each fixed temperature, it can be clearly observed that the gel strength of gelatin solution obtained at 90 minute of reaction was higher than the one obtained at 10 minute of reaction. The average gel strength of gelatin obtained at 90 minute and 10 minute of hydrolysis time were 187 g and 85 g, respectively.

The viscosity and gel strength of gelatin solution from papain hydrolysis reaction at 70^{o}C and 60^{o}C, constant pH 8 were presented in Figure 5.16 and 5.17, respectively. At pH 8, the viscosity and gel strength of gelatin solution at 50^{o}C was not measured as described in section 5.1.3.1.

From Figure 5.16 and 5.17, it can be seen that the pattern of viscosity and gel strength of gelatin solution was similar to the ones at pH 7. The viscosity and gel strength at 90 minute of reaction time was slightly higher than those ones at 10 minute of reaction time. The viscosity was varied in the range of 3.9 cP to 5.6 cP and the gel strength was varied in the range of 116 g to 200 g.

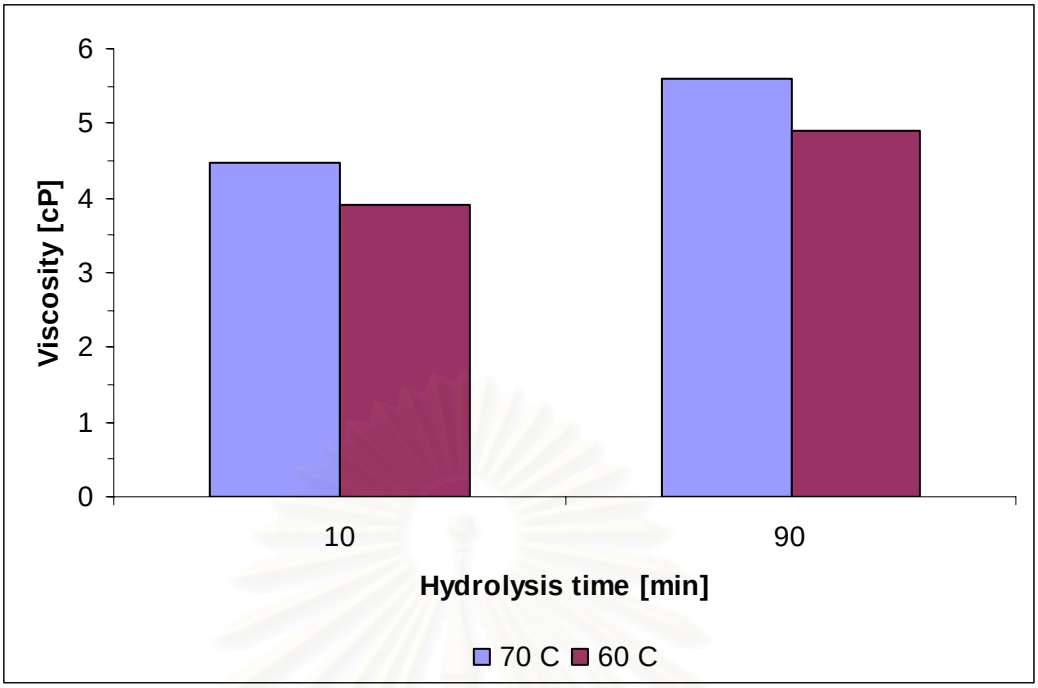


Figure 5.16 Viscosity of gelatin solution obtained from hydrolysis reaction at various temperatures, pH 8 (raw hide: C1)

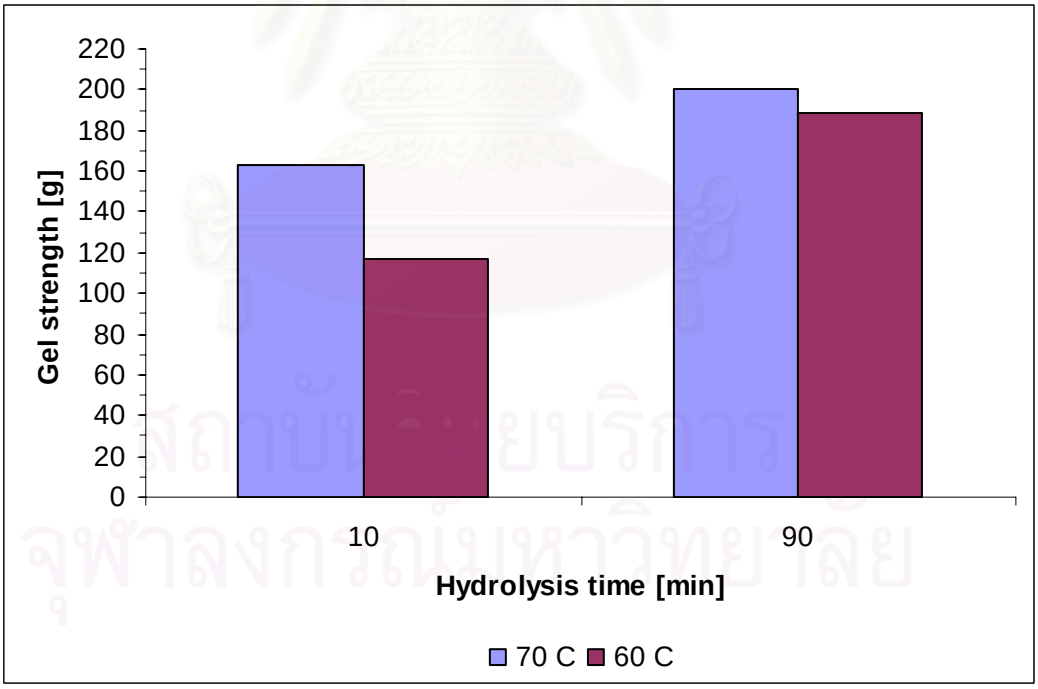


Figure 5.17 Gel strength of gelatin obtained from hydrolysis reaction at various temperatures, pH 8 (raw hide: C1)

5.1.4.2 Effects of pH on gelatin properties

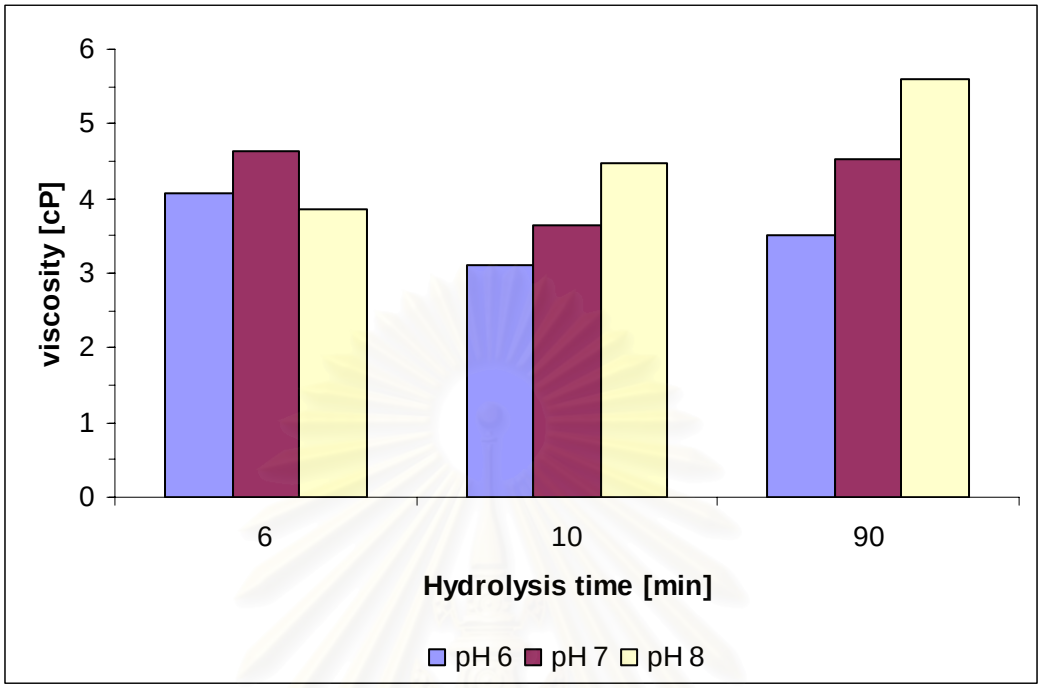


Figure 5.18 Viscosity of gelatin solution obtained from hydrolysis reaction at various pH, 70^{o}C (raw hide: C1)

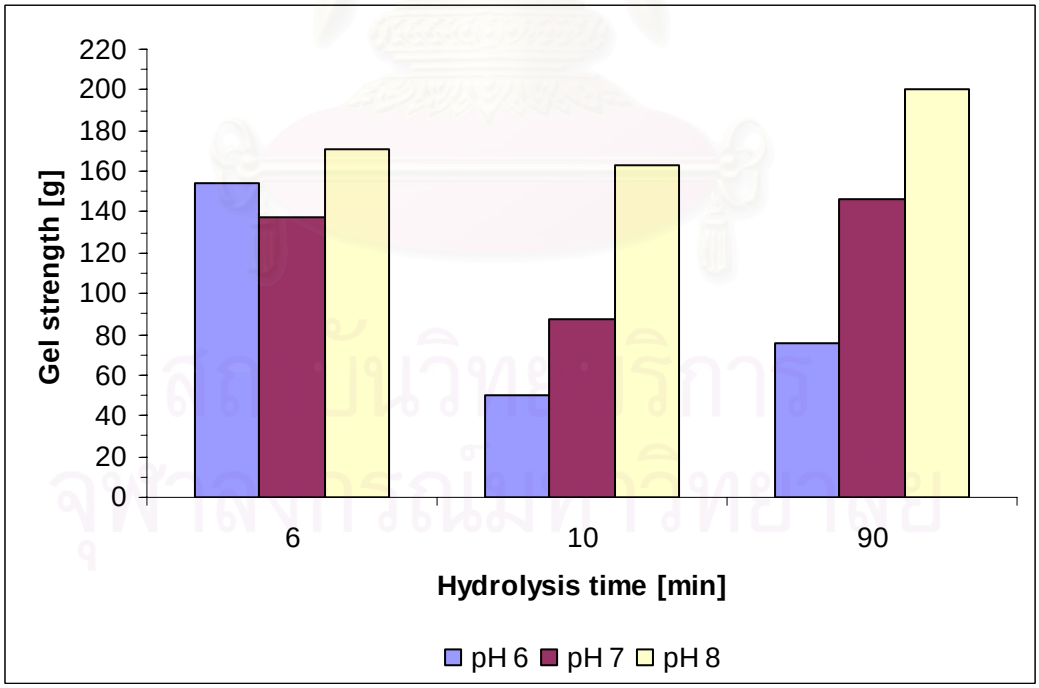


Figure 5.19 Gel strength of gelatin obtained from hydrolysis reaction at various pH, 70^{o}C (raw hide: C1)

The viscosity and gel strength of gelatin solution obtained from hide C1 at various pH constant temperature 70^{o}C were presented in Figure 5.18 and 5.19. When considering the gelatin property at optimum temperature of hydrolysis reaction (70^{o}C) and various pH, it can be found that at the beginning of reaction (within 6 minute), the gelatin property was not significantly different. But as the hydrolysis time was longer than 6 minute, the difference of gelatin property can be clearly observed. The viscosity and gel strength of gelatin were increased when the hydrolysis pH was increased.

Considering the properties of gelatin obtained at 10 minute and 90 minute of hydrolysis time, one can see that the viscosity and gel strength of gelatin obtained at 90 minute of hydrolysis time were higher than those at 10 minute of hydrolysis time. Low viscosity and gel strength of gelatin obtained at 10 minute of hydrolysis indicating short peptide chain of gelatin which was the result of high activity of papain. At longer hydrolysis time, the activity of papain decreased as discussed earlier in section 5.1.1.1. So the chain length of gelatin recovered at longer time should be longer than the one recovered at the beginning of the hydrolysis. The fraction of longer peptide chain could result in higher viscosity and gel strength of gelatin.

From U.S patent 6,100,381 (2000), the relationship of gelatin properties (gel strength and viscosity) and molecular weight distribution was shown. Due to variable bond breakage in gelatin processing, gelatin is composed of a distribution of protein of varying lengths. The distribution is described as containing the following fraction: high molecular weight HMW (>250 Kdaltons); beta (250-150 Kdaltons); alpha (150-50 Kdaltons); subalpha (50-20 Kdaltons) and low molecular weight or LMW (20-4 Kdaltons). In general, high gel strength correlates with high gelatin alpha fraction content and high viscosity correlates with high HMW fraction content.

From the results on the gelatin recovery and gelatin property, the percentages of gelatin recovery and the gelatin property were compared in Table 5.1. It can be observed that the property of gelatin from the hydrolysis batch with lower protein recovery was better than the one from the batch with higher protein recovery. At optimum hydrolysis temperature (70^{o}C), the property of gelatin obtained from pH 8 (lower activity of papain) was better than the one obtained from pH 6 and pH 7

(higher activity of papain). The gelatin received from 90 minute of hydrolysis reaction at pH 8 showed the highest viscosity and gel strength.

Table 5.1 The relationship of percentages of protein recovery and the gelatin property at the hydrolysis temperature of 70^{o}C (raw hide: C1)

pH	Protein recovery (%)		Viscosity (cP)		Gel strength (g)	
	10 min	90 min	10 min	90 min	10 min	90 min
6	59.0	80.1	3.1	3.5	49.6	75.4
7	62.1	81.6	3.6	4.5	87.4	146.5
8	57.4	73.6	4.5	5.6	163.0	200.6

Table 5.2 Comparison of the viscosity and gel strength of gelatin obtained in this work and commercial gelatin

Gelatin grade	Viscosity (cP)	Gel strength (g)
Food grade (Siam square)	17.2	627.5
Laboratory grade (Labchem)	13.2	558.2
Our work (Ave. range)	2.9-5.6	48.8-238.2

From Table 5.2, the averaged viscosity and gel strength of gelatin obtained in this work were compared with those of the commercial grades. It can be seen that the viscosity and gel strength of gelatin solution from enzyme hydrolysis reaction in this work was greatly lower than those from the commercial gelatin produced from acid process (Type A gelatin). This might be mainly resulted from the effect of enzyme that reducing the length of peptide chain. Other two possibilities are that gelatin extracted in this work was not purified and there might be some additives in commercial gelatin. The property of gelatin in this work should be improved if it was purified or if any proper additives were used (U.S patent 5,877,287, 1999).

5.2 Neutrase

5.2.1 Test of neutrase

5.2.1.1 Activity

From product sheet (Novo Nordisk), the activity of neutrase was determined by the Anson method (AF 4), which is based on denatured hemoglobin. It was suggested that the optimal working conditions for neutrase are at 45°C -55°C and pH 5.5 - 7.5. In this study the activity of neutrase was assayed by the method, which modified from assay of endo-protease using azo-casein (Megazyme) as described in section 4.3.3, using azo-casein as the substrate. The activity of enzyme neutrase at various temperatures and pH was presented in Figure 5.20.

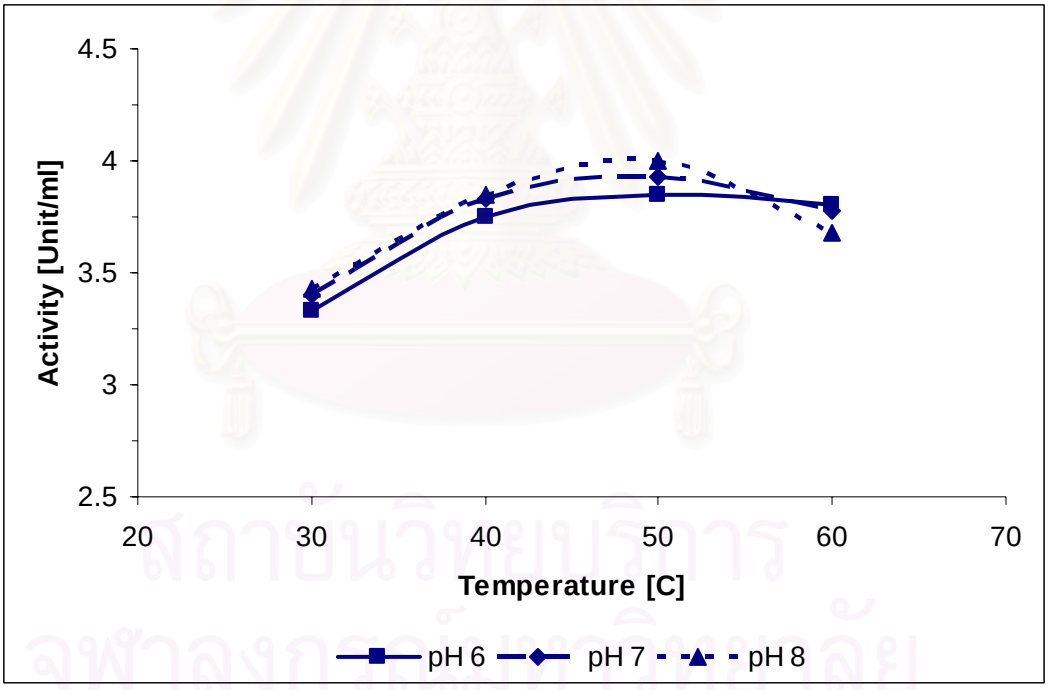


Figure 5.20 Activity of neutrase at various pH and temperatures (substrate: Azo-casein)

As pH 6, the activity of neutrase increased as the temperature increased from 30°C to 50°C. After that, the activity of neutrase was consistent when the temperature was higher than 50°C. At pH 7 and 8, the activity of neutrase increased as the temperature increased from 30°C to 50°C. But when the temperature was higher than

50°C, the activity of neutrase started to decrease. Considering the activity at various pH, it can be seen that the activity of neutrase at pH 6, pH 7 and pH 8 are similar. The result showed that the optimum pH and temperature of enzyme neutrase are at pH 6-8 and 40-50°C, respectively.

5.2.1.2 Optimum ratio of enzyme to raw hide

The optimum amount of neutrase used to hydrolyze 200 g of raw hide was determined, same as in the case of papain described in section 5.1.1.2. The protein recovery at various amounts of enzyme from 0.05-0.15 g per 200 g of raw hide was presented in Figure 5.21.

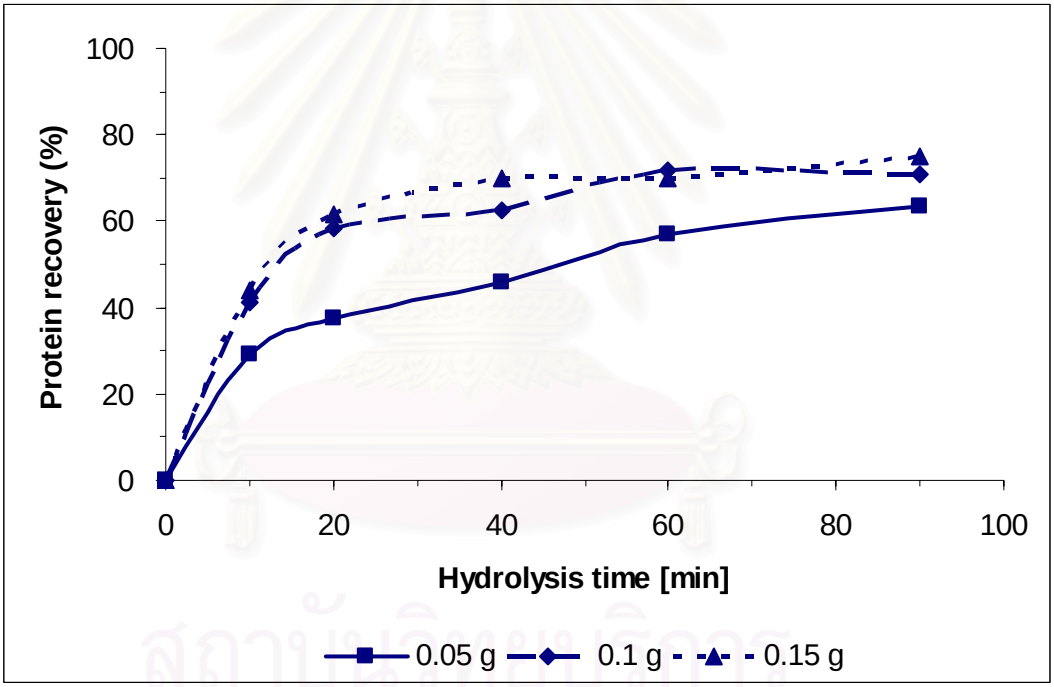


Figure 5.21 Protein recovery at various amounts of enzyme per 200 g of raw hide (raw hide: C1)

The hydrolysis condition of this experiment was carried out at 50°C, 75 rpm, pH 6 and hide C1 was used. The average of protein recovery increased 14.2 % when the amount of enzyme was increased from 0. 05 g to 0.1 g (increased 100 %) and the average of protein recovery increased 3.3 % when the amount of enzyme was increased from 0.1 g to 0.15 g (increased 100 %). It can be noticed that when the amount of neutrase was increased from 0.1 g to 0.15 g, the average percentage of

protein recovery slightly increased when compared to those one from 0.05 g to 0.1 g so the optimum amount of papain used to hydrolyze 200 g of raw hide should be 0.1 g. But in this study the amount of neutrease used in hydrolysis reaction was 0.15 g and the effect of excessive enzyme will be discussed in the last section of this chapter.

5.2.1.3 Stability of neutrase activity

The stability of neutrase activity in hydrolysis reaction was investigated using a blank hydrolysis reaction batch (without raw hide) at pH 6, 75 rpm and neutrase 2.5 g was used. Two different hydrolysis temperature were compared, i.e. 40°C and 50°C. The enzyme solution in the reactor was taken to test the activity at the hydrolysis time of 0, 10, 20, 30, 40, 50 and 60 minute. The activity of enzyme neutrase was illustrated in Figure 5.22.

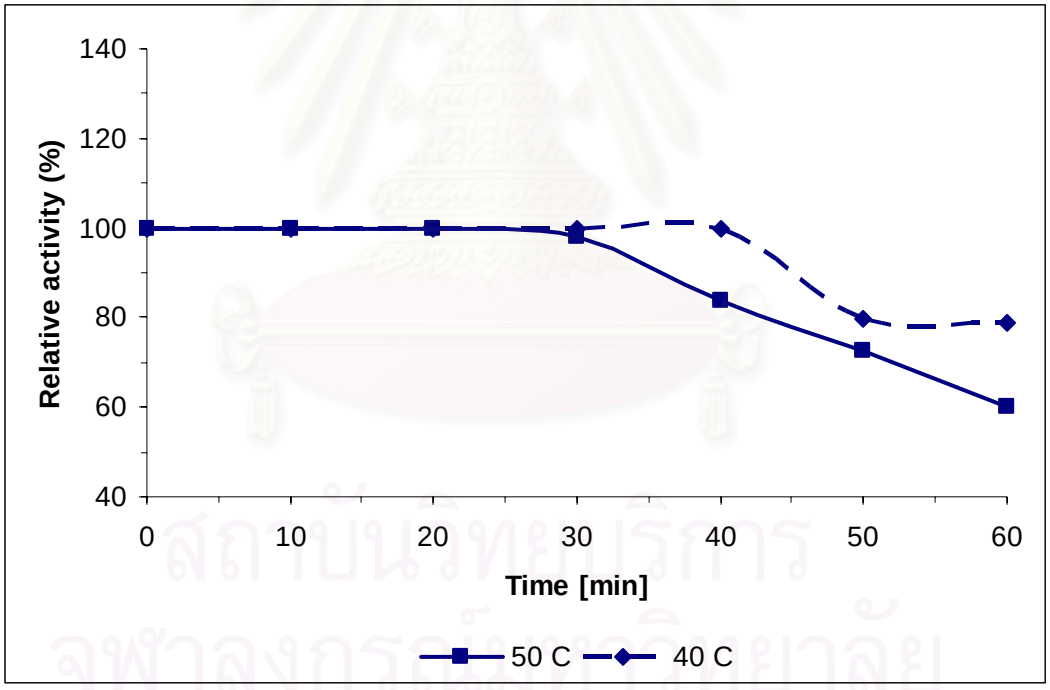


Figure 5.22 Activity curve of neutrase at 50°C, 40°C and pH 6 (substrate: Azo-casein)

At 50°C, the neutrase activity was stable during the first 20 minute. The neutrase activity decreased by 1.8 % (20-30 minute) and by 38.2 % (30-60 minute). The result showed that the neutrase can be used in hydrolysis for approximately 30 minute under tested condition at 50°C, pH 6. When the hydrolysis time was longer

than 30 minute, the neutrase activity decreased sharply. As a result, the hydrolysis rate was decreased. At 40°C, the activity of neutrase was stable during the first 40 minute. The neutrase activity decreased by 20.3 % (40-50 minute) and by 0.7 % (50-60 minute). The result showed that the neutrase can be used in hydrolysis for approximately 40 minute under tested condition at 40°C, pH 6. This indicated that the activity of neutrase at 40°C was more stable than at 50°C.

5.2.2 Effect of raw hide on neutrase hydrolysis reaction

As described in section 5.1.2, the effects of fat and protein contents on enzymatic hydrolysis were determined by hydrolyzing two different lots of raw hide materials (C and C1) at the same conditions. The protein recovery from two lots of hide was presented in Figure 5.23.

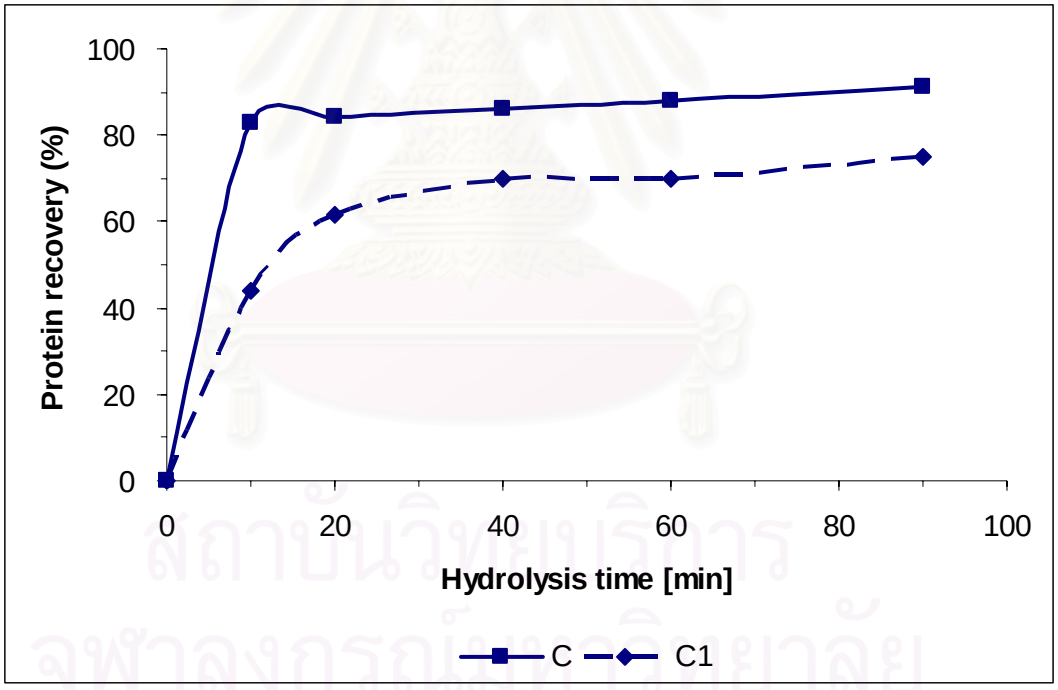


Figure 5.23 Protein recovery of two different lots of raw hide (C and C1) at 50°C, pH 6

The hydrolysis condition of this experiment was carried out at 50°C, 75 rpm, pH 6. It can be seen that the protein recovery from hide C was always higher than the protein recovery from hide C1. This was similar to the case of papain hydrolysis presented in section 5.1.2. From the beginning of the reaction up to 20 minute, the

protein recovery from hide C was greatly higher than that from hide C1. It might be due to the effect of fat contents in hide C1 structure as described in section 5.1.2.

5.2.3 Effects of hydrolysis conditions on protein recovery

5.2.3.1 Effects of temperature on protein recovery

The effects of hydrolysis temperature at constant pH on protein recovery are presented in this section. The protein recovery from neutrase hydrolysis reaction is presented in Figure 5.24. The hydrolysis condition of this experiment was carried out at pH 6, 75 rpm, and two lots of raw hide (C and C1) were used.

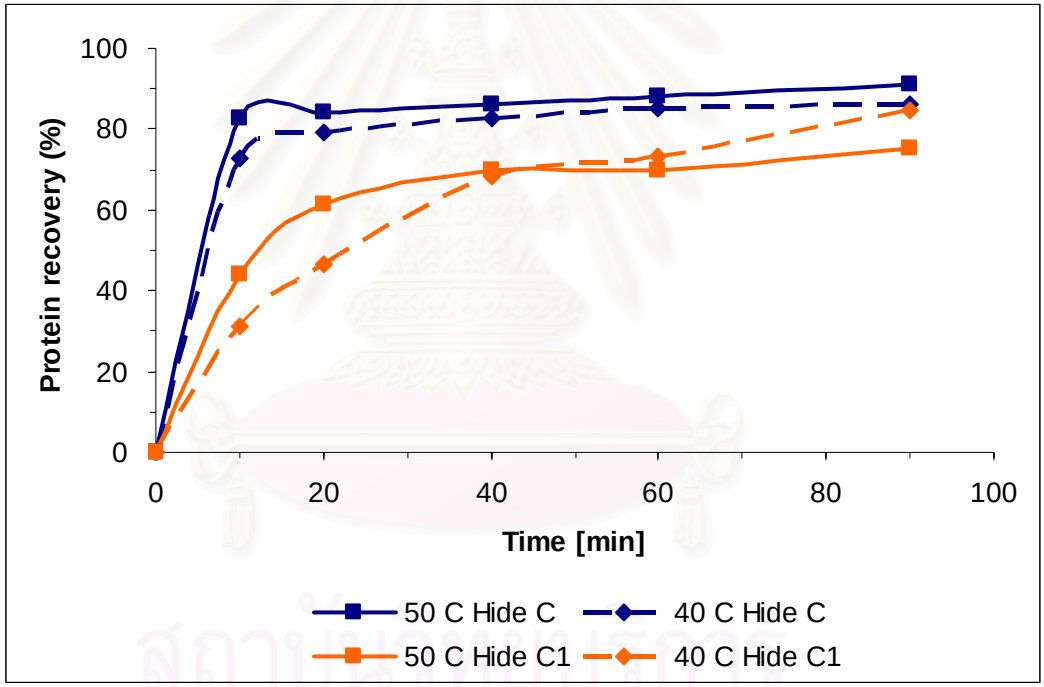


Figure 5.24 Protein recovery from enzymatic hydrolysis at pH 6 and various temperatures (raw hide: C and C1)

When considering the protein recovery from hide C at each constant temperature, it can be found that the initial rate of protein recovery from enzyme hydrolysis was very high (within 10 minute). After that the recovery rate was gradually lower as the hydrolysis reaction progressed. The percentage of protein recovery was sharply increased at the beginning of reaction (within 10 minute) then started to slightly increase. This might be caused by the depletion of protein source or

the enzyme deactivation. From section 5.2.1.3, it was showed that the activity of enzyme did not decrease until 30 minute of reaction so, in this study, the decreasing of recovery rate should be mainly resulted from the depletion of protein or the low concentration of protein substrate. The results showed that the percentage of protein recovery at 40^{o}C was similar to the one at 50^{o}C. It should be because the activity of neutrase at 40^{o}C and 50^{o}C was similar as showed in section 5.2.1.1.

For the protein recovery from hide C1, it can be seen that the initial rate of protein recovery at 50^{o}C was higher than the one at 40^{o}C. At 50^{o}C, the percentage of protein recovery was sharply increased at the beginning of reaction (within 20 minute) then started to slightly increase. But at 40^{o}C, the percentage of protein recovery was continuously increased until 40 minute of hydrolysis time then started to gradually increase. At the beginning of reaction, the percentage of protein recovery at 50^{o}C was higher than the one at 40^{o}C but after 40 minute of hydrolysis time the percentage of protein recovery at 50^{o}C was lower than the one at 40^{o}C. It might be due to the reducing of activity of neutrase as showed in section 5.2.1.3.

It can be noticed that the percentage of protein recovery from hide C was higher than the one from hide C1. When hydrolysis time was increased the differences of protein recovery from hide C and C1 was not as much as that in the first period of hydrolysis. This might be caused by the effect of higher fat contents in hide C1 structure as described in section 5.1.2. In this case of study, the protein recovery at 30^{o}C and 60^{o}C was not performed since the neutrase hydrolysis reaction at these conditions was extremely slow, i.e. after 90 minute of reaction the raw hide was still in the solid form. This might be due to the low activity of papain.

The protein recovery from neutrase hydrolysis reaction at various temperatures and constant pH 7 is presented in Figure 5.25. The hydrolysis condition of this experiment was carried out at pH 7, 75 rpm and hide C was used.

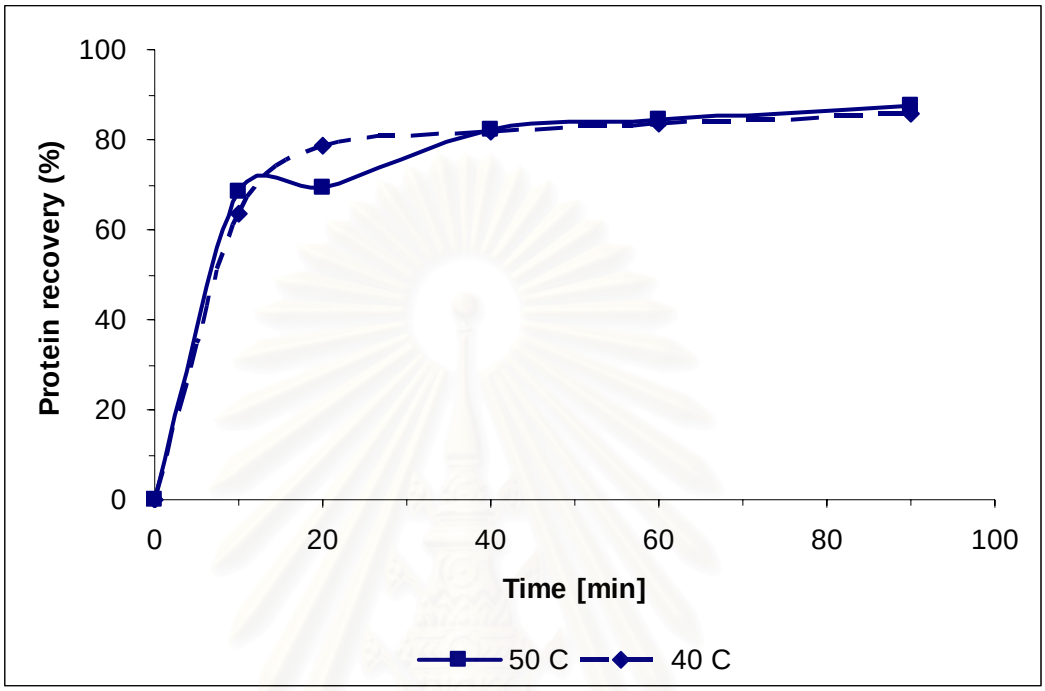


Figure 5.25 Protein recovery at pH 7 and various temperatures (raw hide: C)

From Figure 5.25, similarly to the protein recovery from hide C at pH 6, the percentages of gelatin recovery at 50^{o}C was similar to the one at 40^{o}C. This was because the effect of thermal hydrolysis was low at low temperature (40-50^{o}C) and the activity of neutrase at 50^{o}C was not significant different to the one at 40^{o}C. The percentage of protein recovery was sharply increased at the beginning of reaction (within 10 minute). As the hydrolysis time was longer than 10 minute, the percentage of protein was started to slightly increased. This might be caused by the depletion of protein or the low concentration of protein substrate.

The protein recovery from neutrase hydrolysis reaction at various temperatures and constant pH 8 is presented in Figure 5.26. The hydrolysis condition of this experiment was carried out at pH 8, 75 rpm and hide C was used.

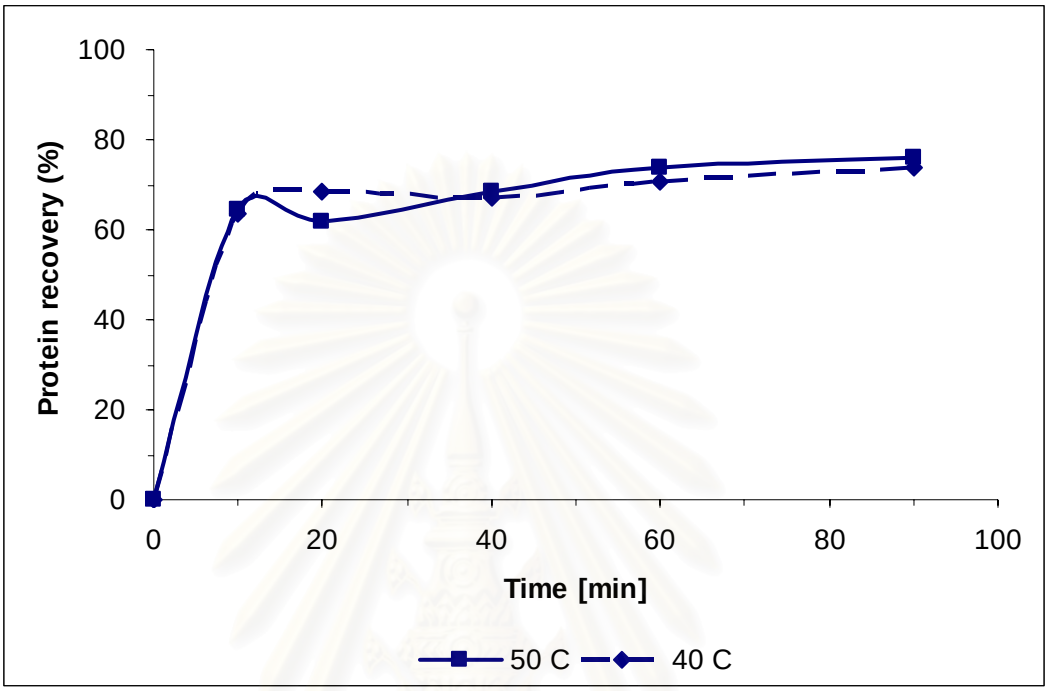


Figure 5.26 Protein recovery at pH 8 and various temperatures (raw hide: C)

It can be found that the pattern of protein recovery at pH 8 was similar to the one at pH 6 and pH 7. The percentage of protein recovery was sharply increased at the beginning of reaction (within 10 minute) then slightly increased as the hydrolysis time was longer than 10 minute. It can be noticed that the protein recovery at 50^{o}C was similar to the one at 40^{o}C.

From the results on the effects of enzyme hydrolysis temperature on the protein recovery from hide C at constant pH 6, pH 7 and pH 8, it can be concluded that the percentage of protein recovery at 50^{o}C was similar to the one at 40^{o}C for all pH of hydrolysis reaction. This was because the effect of thermal hydrolysis was low at low temperature (40-50^{o}C) and the activity of neutrase at 40-50^{o}C and pH 6-8 was not significant different. But for the protein recovery from hide C1 at constant pH 6, the percentage of protein recovery at 40^{o}C was higher than the one at 50^{o}C as the hydrolysis time was longer than 40 minute. Because the deactivation of neutrase at 50^{o}C (within 30 minute).

5.2.3.2 Effects of pH on protein recovery

From section 5.2.3.1, the results showed that the optimum temperature for highest protein recovery at various pH was 40-50^{o}C. In this section, the effects of pH (6 to 8) on the protein recovery from hide C and C1 at constant hydrolysis temperature (50^{o}C and 40^{o}C) were compared. The protein recovery from neutrase hydrolysis reaction at various pH from 6 to 8, constant hydrolysis temperature (50^{o}C and 40^{o}C) are presented in Figure 5.27 and 5.28, respectively.

The results showed that the percentage of protein recovery was sharply increased at the beginning of reaction (within 10 minute) then slightly increased as the hydrolysis time was longer than 10 minute. It can be seen that the percentage of protein recovery at pH 6 and pH 7 was very similar and higher than those at pH 8.

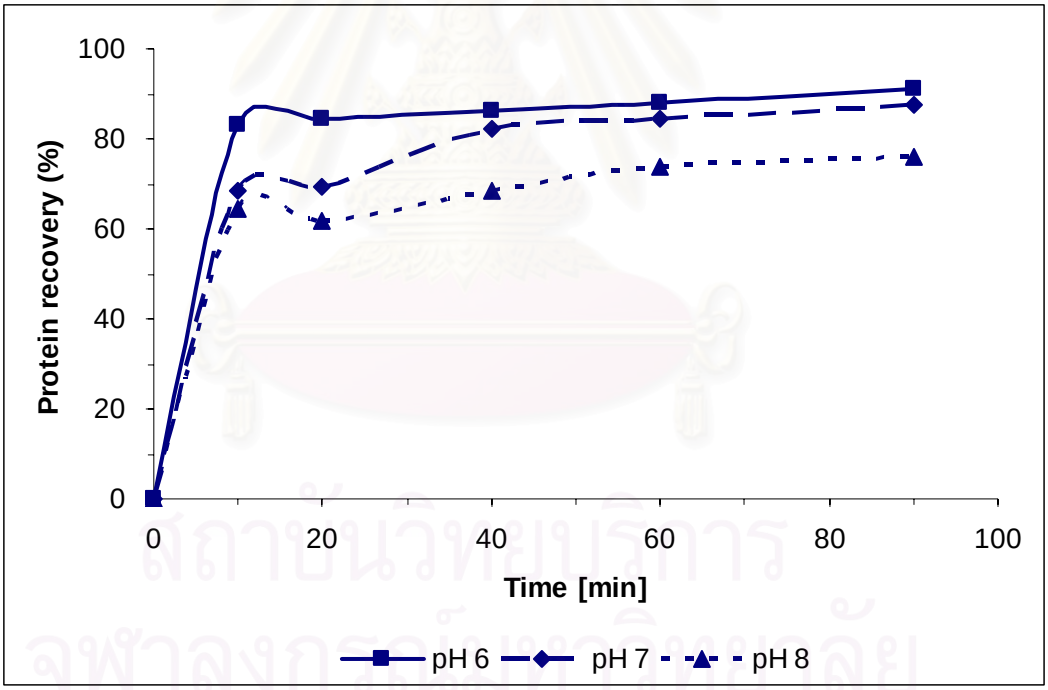


Figure 5.27 Protein recovery curve at various pH, constant temperature of 50^{o}C (raw hide: C)

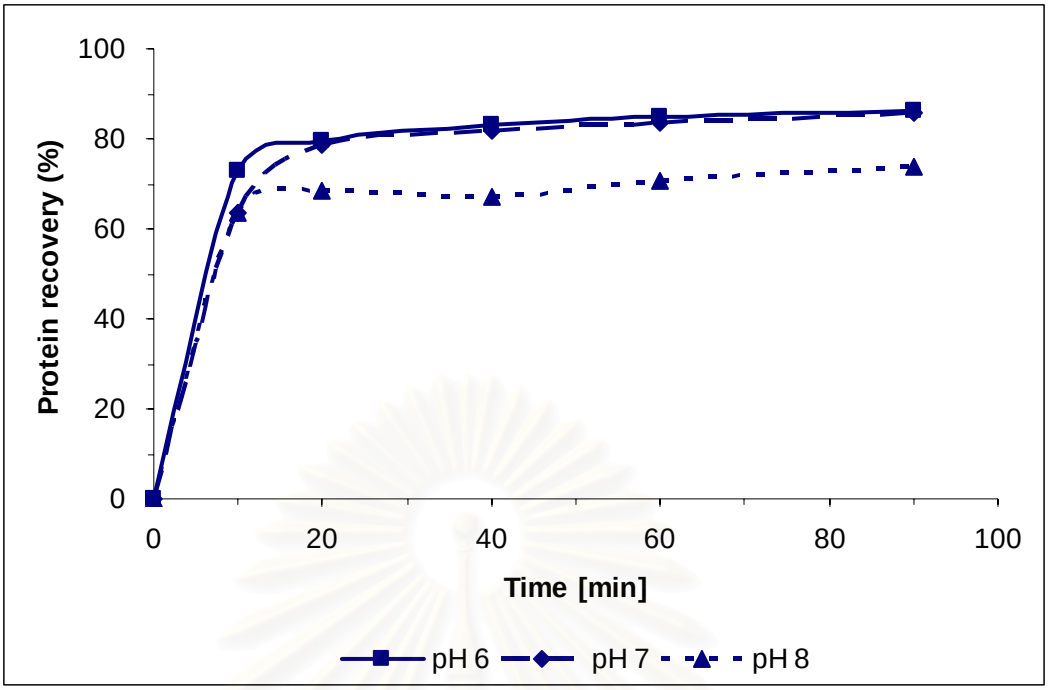


Figure 5.28 Protein recovery curve at various pH, constant temperature of 40°C (raw hide: C)

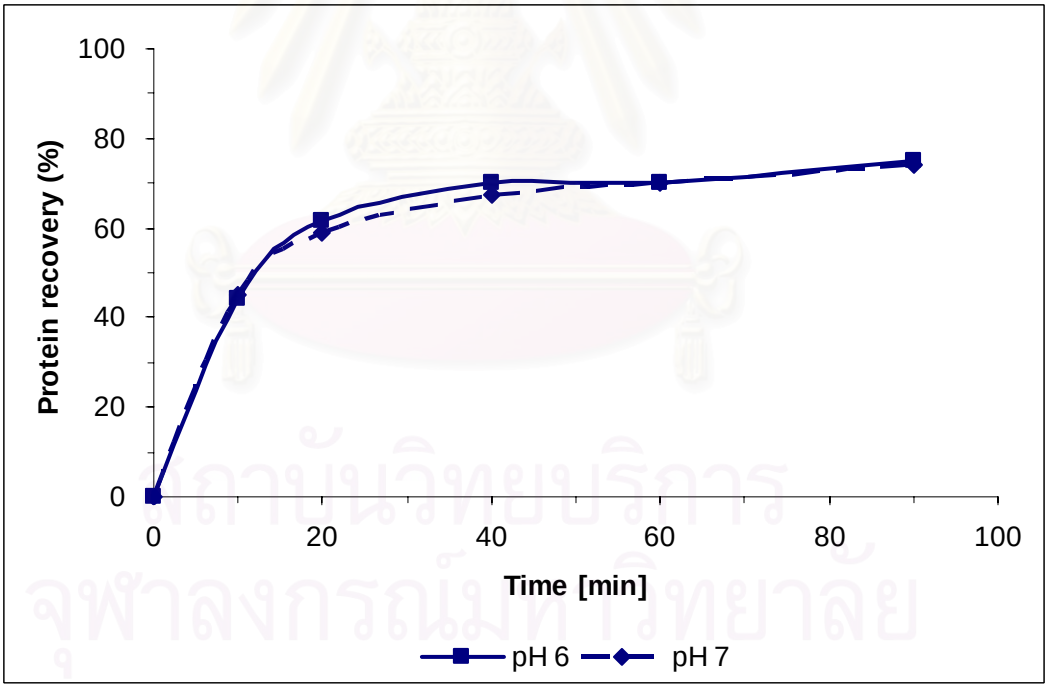


Figure 5.29 Protein recovery curve at two pH, constant temperature of 50°C (raw hide: C1)

The effect of pH on protein recovery from raw hide C1 was performed. The protein recovery from hide C1, two pH (6 to 7) and constant hydrolysis temperature 50^{o}C was presented in Figure 5.29. The results showed that the effect of pH on protein recovery from raw hide C1 was similar to the one from raw hide C. The percentage of protein recovery was sharply increased at the beginning of reaction (within 10 minute) then slightly increased and the percentage of protein recovery at pH 6 and pH 7 was very similar.

From the results of effects of pH on protein recovery, it can be seen that the optimum pH of neutrase hydrolysis reaction at 50^{o}C and 40^{o}C was at pH 6-7 due to the maximum of neutrase activity. As a result, the highest protein recovery was reached.

5.2.3.3 Effects of amount of neutrase on the properties of recovered hydrolysate

In this work, it was found that the protein recovered from neutrase hydrolysis reaction of raw hide at the ratio of neutrase to raw hide (0.15:200) can not form gel. This might be due to the excessive amount of neutrase used in the hydrolysis reaction. Therefore, additional experiments were performed using less amount of neutrase (0.05-0.1 g) to hydrolyse 200 g of raw hide. The gelatin property of recovered protein was investigated. After keeping 12.5% protein solution for 17 hours at 4^{o}C, it was found that the solution could not form gel even at 0.05 g of neutrase (within 10 minute of hydrolysis time, $\approx$30 % of gelatin recovery). This implied that the recovered protein from neutrase hydrolysis reaction from raw hide was in hydrolysate form (can not form gel) for all hydrolysis conditions and all ratios of enzyme to raw hide (0.05-0.15 g to 200 g). This might due to the specific attack of neutrase on collagen molecules. The degradation of collagen by metalloprotease such as neutrase was started from the exterior. Enzyme binds tightly to triple helix at the surface, whereas molecules in the interior become accessible to enzymes in the course of the progressive degradation from the outside. After the triple helix is cracked, the primary fragments were cleaved into small peptides and amino acids. (Friess, 1998) On the other words, neutrase specifically destroyed the intramolecular crosslink of collagen. So the chain length of protein recovered from neutrase hydrolysis reaction was very short, i.e. it was in the hydrolysate form and gelation could not occur. The viscosity of

the protein solution was varied in the range of 1.5 cP to 3 cP. Neutrase was suggested to be suitably used for protein hydrolysate or collagen hydrolysate from raw hide. This corresponds to the work of Taylor *et al*. (1989). They have used neutrase in the enzymatic treatment of offal from fleshing machines to produce protein hydrolysate.

5.3 Comparing of papain and neutrase hydrolysis

When comparing the activity of enzymes, papain and neutrase, used in hydrolysis reaction of raw hide in Table 5.3, at maximum enzyme activity, it can be seen that the activity of papain used in hydrolysis reaction was much higher than the one from neutrase. After the properties of recovered gelatin from papain and neutrase were determined, it was found that the recovered gelatin from papain hydrolysis can form gel but the one from neutrase can not form gel. This might suggest different mechanism of papain and neutrase hydrolysis. Papain might be able to attack intermolecular crosslink of collagen so the peptide chain was not too short and might still be in the helix form. Sol-gel transition of protein solution could occur. On the other hand, neutrase might attack intramolecular crosslink of collagen. The short peptide chain of collagen hydrolysate was then achieved and gelation could not occur.

Table 5.3 Comparison of the activity of papain and neutrase used in hydrolysis reaction at pH 6

Enzyme	specific activity (Unit/g)	amount (g)	total activity (Unit)
papain	177.5	0.3	53.25
neutrase	3.1	0.15	0.465

So from the results of papain and neutrase hydrolysis of raw hide, it can be suggested that papain could be used to produce gelatin from raw hide with low gel strength and viscosity. But neutrase could be used to produce protein hydrolysate.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This work aims to studying the effects of enzymatic conditions (temperature, pH and time) for gelatin production on the yield of gelatin and gelatin properties such as gel strength and viscosity. The two types of commercial enzymes (papain and neutrase) were used to hydrolyzed raw hide.

Papain

From the results of gelatin recovery, it was showed that the percentage of gelatin recovery at the beginning of reaction was increased sharply up to about 10 minute, then started to slightly increase. The optimum working conditions of papain for highest gelatin recovery are at 70^{o}C, pH 6-7 and 90 minute of hydrolysis time. The optimal ratio of papain per raw hide is 0.3:200. The main factor that controlled the reaction is the concentration of raw hide substrate. The recovered gelatin from papain hydrolysis reaction can form gel after chilled at 4^{o}C for 17 hours. This might due to the attack of papain on the intermolecular bonding of collagen molecules. The gel strength and viscosity of gelatin from papain hydrolysis is varied in the ranged 48.8 - 238.8 g and 2.9-5.6 cP, respectively. The properties of recovered gelatin were relatively low when compared with food grade and laboratory grade Type A gelatin.

Neutrase

From the result of protein recovery, it was showed that the optimum working conditions of neutrase for highest gelatin recovery are at 40-50^{o}C, pH 6-7 for 40 minute of hydrolysis time. From the results of protein properties, it was showed that the recovered protein can not form gel after chilled at 4^{o}C for 17 hours and the viscosity of protein solution is very low. This might due to the breakdown of intramolecular of collagen caused by neutrase. The product from neutrase hydrolysis reaction is classified as protein hydrolysate.

6.2 Recommendations

Although several points concerning about studying the effect of enzymatic conditions for gelatin production from raw hide have been dealt with in this work, there still be some interesting points which can be further investigated. These are some recommendation.

1. The effect of protein and fat contents in raw hide on gelatin recovery and gelatin properties have to be further investigated.

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

3. The effect of drying process on gelatin properties should be further investigated.

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

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APPENDICES

A1. Activity calculation

From method described in section 4.3.3, the activity of enzyme is calculated from the change of absorbance at the wavelength of 440 nm (the absorbance change 0.1 is referred to 1 Unit of activity). For example, 0.1 ml of enzyme sample and controlled sample (no enzyme) have an absorbance 0.4 and 0.1, respectively. The activity of 0.1 ml of enzyme is 3 Unit. So if 1 ml of enzyme sample is used, the activity will equal to 30 Unit. When comparing with the incubation time (20 minute), the activity per time is 1.5 Unit per minute. The calculation is showed as follows:

Activity of 0.1 ml of enzyme = (0.4-0.1)*10 = 3 Unit

Activity of 1 ml of enzyme = $\frac{1*3}{0.1} = 30$ Unit/ml

when comparing with incubation time (20 minute), the activity per time (1 minute) is

$\frac{30}{20} = 1.5$ Unit/ml

A2. 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 (Figure A2). The calibration curve is constructed using a standard gelatin solution (lab grade, Khurusapha).

The concentration of recovered gelatin is calculated from this formulation.

$$\text{Gelatin concentration, } C \text{ (g/cc)} = \frac{A * B}{D}$$

where A is the absorbance value (at 750 nm).

B is the volume of water used to dilute 1 g of protein sample (0.4 l).

D is the conversion factor (received from calibration curve, 1.2193).

The percentage of gelatin recovery is calculated from this formulation.

$$\text{Percentage of gelatin recovery (\%)} = \frac{C * 100}{E}$$

where C is gelatin concentration (g/cc).

E is total protein in raw hide per solution volume at the beginning of reaction (0.1835 g/cc for hide C and 0.1841 for hide C1)

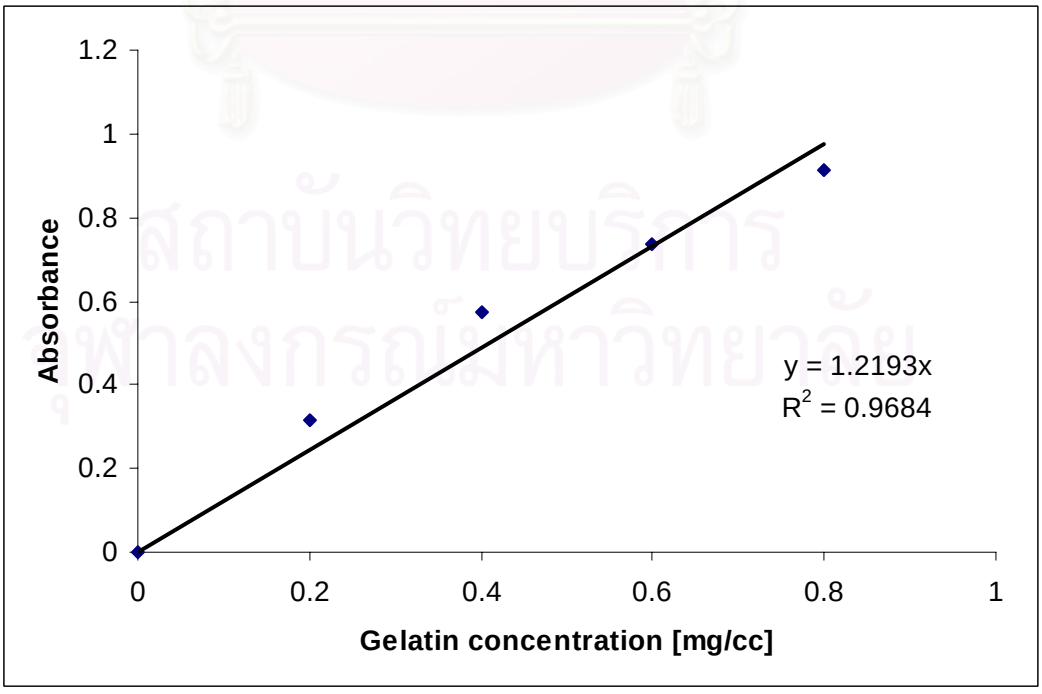

Figure A2 The calibration curve of standard gelatin solution.

A3. The comparison of protein determination (Kjeldahl and Lowry)

In this study the protein content in raw hide was determined by Kjeldahl method. But in gelatin solution, the protein content was determined by Lowry method. So the difference of the two methods was evaluated by using the same commercial gelatin solution. The results were showed in Table A3.

Table A3 The difference of measured protein from Kjeldahl and Lowry.

Sample (mg/cc)	Kjeldahl, K (mg/cc)	Lowry, L (mg/cc)	Difference (L/K)
0.20	0.24	0.23	0.95
0.40	0.43	0.40	0.93
0.60	0.69	0.65	0.94

The result showed that the amount of protein from Kjeldahl method is more than the one from Lowry method around 6%. This is because Kjeldahl method was calculated from the total amount of nitrogen in sample but Lowry method was calculated from the amount of peptide bonds and aromatic amino acids in sample. So if we multiple the concentration of raw hide protein obtained from Kjeldahl method by 0.94, the value of gelatin recovery reported in this work will be increased 6.37%.

CURRICULUM VITAE

Mr. Kongpob Ratanathammapan was born in Bangkok, Thailand on September 15, 1980. He graduated the secondary school from Chulalongkorn Demonstration School in Bangkok and received the Bachelor degree of Engineering (Chemical Engineering), Mahidol University, Thailand in 2000. He entered the Master of Engineering in Chemical Engineering Program at Chulalongkorn University in 2001.