คุณลักษณะของโปรตีเนสในปลากะตัก (*Stolephorus* spp.)

นางสาวพัชรินทร์ ศิริงาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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CHARACTERISTICS OF ENDOGENOUS PROTEINASES IN INDIAN ANCHOVY (*Stolephorus* spp.)

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พัชรินทร์ ศิริงาน : คุณลักษณะของโปรตีเนสในปลากะตัก (*Stolephorus* spp.) (CHRACTERISTICS OF ENDOGENOUS PROTEINASES IN INDIAN ANCHOVY (*Stolephorus* spp.)) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 99 หน้า. ISBN 974-533-429-4

วัตถุประสงค์ของการศึกษาเพื่อทำให้เอนไซม์บริสุทธิ์บางส่วน และศึกษาคุณลักษณะของเอนไซม์ในปลากะตัก (*Stolephorus* spp.)โดยศึกษาคุณลักษณะทางชีวเคมี คือ พีเอชและอุณหภูมิที่เหมาะสม ผลของเกลือ ความจำเพาะเจาะจงต่อสารตั้งต้น และสารยับยั้งกิจกรรมของโปรตีเนส พบว่า กิจกรรมย่อยสลายตัวเอง (autolytic activity) เกิดขึ้นสูงสุดที่ 60 องศาเซลเซียส อย่างไรก็ตามเมื่อเพิ่มความเข้มข้นของเกลือมีผลให้การย่อยสลายลดลง โปรตีเนสที่สกัดจากปลากะตักทั้งตัวสามารถย่อยสลายสารตั้งต้นสังเคราะห์สำหรับทริปซิน คือบิวทิลออกซิคาร์บอนิล-แอสพาร์ติก(เบนซิล)-โพรลีน-อาร์จีนีน-7-อะมิโด-4-เมทิลคลูมาริน (butyl-oxycarbonyl-Asp(oBzl)-Pro-Arg-7-amido-4-methylcoumarin) และถูกยับยั้งโดยสารยับยั้งทริปซินในถั่วเหลือง (soybean trypsin inhibitor) ลิวเพปติน (leupeptin) และ โทซิล-แอล-ไลซีน คลอโรเมทิล คีโตน (*N*-tosyl-L-lysine chloromethyl ketone) นอกจากนี้โปรตีเนสที่แสดงกิจกรรมสูงสุด คือ โปรตีเนสที่คล้ายทริปซิน (trypsin-like proteinase) และทริปซิน (trypsin) ที่สกัดจากเครื่องในของปลากะตัก ในขณะที่ในส่วนของกล้ามเนื้อปลา พบเฉพาะโปรตีเนสที่คล้ายทริปซิน โดยน้ำหนักโมเลกุลของโปรตีเนสในส่วนของเครื่องในของปลากะตัก คือ 31, 35, 44, 49 และ 57 กิโลดาลตัน ซึ่งทำการตรวจวัดกิจกรรมด้วยวิธีการย้อมสี ในสภาวะที่มีเกลือโซเดียมคลอไรด์ความเข้มข้น 4.0 โมลาร์ ในส่วนของกล้ามเนื้อ พบโปรตีเนสที่มีขนาด 56 กิโลดาลตัน เมื่อทำให้บริสุทธิ์บางส่วนด้วยวิธีการให้ความร้อน การตกตะกอนด้วยแอมโมเนียมซัลเฟตความเข้มข้นร้อยละ 30-60 การแยกด้วยเทคนิค การแลกเปลี่ยนประจุลบ (anion exchange) ความสามารถในการเกิดอันตรกิริยาไฮโดรฟอบิก (hydrophobic interaction) และแยกตามขนาดมวลโมเลกุล (gel filtration) สามารถแยกโปรตีเนสได้เป็น 4 ส่วน คือ P111 P21 P31 และ P4 โดยพบว่าทั้ง 4 ส่วนสามารถย่อยสลายสารตั้งต้นสังเคราะห์สำหรับทริปซิน มีอุณหภูมิและพีเอชที่เหมาะสมต่อกิจกรรมคือ 50-60 องศาเซลเซียส และ 8.5 ตามลำดับ นอกจากนี้ยังพบว่าสารยับยั้งทริปซินในถั่วเหลือง ลิวเพปติน และโทซิล-แอล-ไลซีน คลอโรเมทิล คีโตน สามารถยับยั้งกิจกรรมของโปรตีเนสที่ทำบริสุทธิ์บางส่วนได้ และยังพบว่าแคลเซียมไอออนไม่มีผลต่อกิจกรรมของเอนไซม์ที่แยกได้ยกเว้น P111 โดยโปรตีเนสที่ผ่านการทำให้บริสุทธิ์บางส่วนมีขนาด 27 ถึง 65 กิโลดาลตัน โปรตีเนสที่คล้ายทริปซินสามารถย่อยสลายเนื้อปลากะตักได้ที่อุณหภูมิ 35

ย่อยสลายเนื้อปลากะตักได้ที่อุณหภูมิ 35 องศาเซลเซียส และที่อุณหภูมิที่เหมาะสมของแต่ละส่วนในสภาวะที่มีเกลือโซเดียมคลอไรด์ความเข้มข้น 4.0 โมลาร์และพีเอช 8.5

พบโปรตีเนสที่คล้ายทริปซิน, ทริปซิน, ไคโมทริปซิน (chymotrypsin) และคาเทปซิน แอล (cathepsin L) ตลอดระยะเวลาการหมักน้ำปลา 12 เดือน ในขณะที่พบกิจกรรมของเอนไซม์ลิวซีน-อะมิโนเปปติเดส (leucine-aminopeptidase) ในช่วงเดือนแรกของการหมักเท่านั้น และโปรตีเนสที่พบในน้ำปลาช่วงระยะการหมักต่างๆ มีน้ำหนักโมเลกุลประมาณ 37, 47 และ 53 กิโลดาลตัน ในสภาวะที่มีเกลือโซเดียมคลอไรด์ความเข้มข้น 4.0 โมลาร์ซึ่งมีค่าใกล้เคียงกับเอนไซม์ในปลากะตัก

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2547

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
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PATCHARIN SIRINGAN : CHARACTERISTICS OF ENDOGENOUS PROTEINASES IN INDIAN ANCHOVY (*Stolephorus* spp.). THESIS ADVISOR : ASST. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D. 99 PP. ISBN 974-533-429-4

CHARACTERISTICS/ENDOGENOUS PROTEINASE/INDIAN ANCHOVY/ TRYPSIN-LIKE PROTEINASE

The objectives of this study were to characterize and partially purify endogenous proteinases from Indian anchovy. In addition, biochemical characteristics, including pH and temperature optimum, effect of NaCl concentration, substrate specificity, and inhibitor were elucidated. Autolytic activity was maximum at 60 ^{o}C and decreased with increasing NaCl concentration. Crude proteinases isolated from whole fish effectively hydrolyzed synthetic substrates of trypsin, especially butyloxycarbonyl-Asp(oBzl)-Pro-Arg-7-amido-4-methylcoumarin and was inhibited by soybean trypsin inhibitor (SBTI), leupeptin, and *N*-tosyl-L-lysine chloromethyl ketone (TLCK). Trypsin-like proteinase and trypsin from viscera exhibited the highest activity, while only activity of trypsin-like proteinases from muscle was predominant. Molecular weight (MW) of crude proteinases in viscera of Indian anchovy was estimated to be 31, 35, 44, 49, and 57 kDa by activity staining in the presence of 4.0 M NaCl. MW of crude proteinases extracted from muscle was 56 kDa. Trypsin-like proteinases were partially purified using heat treatment, 30-60% saturation ammonium sulfate fractionation, anion exchange, hydrophobic interaction, and gel filtration chromatography. Four fractions (P111, P21, P31, and P4) exhibiting

proteinase activity were obtained. All fractions preferably hydrolyzed synthetic substrates of trypsin. Optimal temperature and pH of all fractions were 50-60 °C and 8.5, respectively. SBTI, leupeptin, and TLCK also inhibited all partially purified proteinases activities. All purified proteinases were Ca^{2+} independent except for P111. Molecular weight of partially purified proteinases ranged from 27 to 65 kDa based on activity staining at 4.0 M NaCl, pH 8.5. Trypsin-like proteinases also hydrolyzed washed anchovy muscle mince in 4.0 M NaCl, pH 8.5, at either 35 °C or their optimal temperatures.

Trypsin-like, trypsin, chymotrypsin, and cathepsin L were found in commercial fish sauce samples throughout 12 months of fermentation. In contrast, leucine-aminopeptidase activity was detected only at the first month. MW of proteinases found in fish sauce at various fermentation periods was estimated to be 37, 47 and 53 kDa based on activity staining at 4.0 M NaCl, which was similar to that found in endogenous proteinases of fish.

School of Food Technology

Academic Year 2004

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I dedicate this work to my parents and my relatives, for their unconditional support and encouragement. They have provided me with every opportunity to succeed in life.

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Patcharin Siringan

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LIST OF ABBREVIATIONS

Ac	Actin
AMC	7-amido-4-methylcourmalin
BAPNA	*N*-benzoyl-L-arginine-p-nitroanilide
Boc	Butyloxylcarbonyl
BME	β-mercaptoethanol or 2-mercaptoethanol
BTEE	Benzyl-L-tyrosine-ethyl ester
BSA	Bovine serum albumin
Bzl	Benzyl
^{o}C	Degree Celsius
$CaCl_2$	Calcium chloride
cm	Centimeter
DEAE	Diethylaminoethyl
DFP	Diisopropylphosphofluoride
DMS	Dimethylsulfoxide
DTT	Dithiothreitol
E-64	*N*-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl-amido(4-guanidino)butane
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	Hour
HIC	Hydrophobic Interaction Chromatography

LIST OF ABREVIATIONS (Continued)

HPLC	High Performance Liquid Chromatography
kDa	Kilodalton
L	Liter
LAP	Leucine-aminopeptidase or Leu-aminopeptidase
M	Molar
MBSP	Myofibril-bound serine proteinase
MCP	Multicatalytic proteinase
MHC	Myosin Heavy Chain
min	Minute
mM	Milimolar
mL	Mililiter
mo	Month
MW	Molecular weight
NaCl	Sodium chloride
nm	Nanometer
nmole	Nanomole
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
pNA	*p*-Nitroaniline
SBTI	Soybean trypsin inhibitor
TAME	*N*-Tosyl-L-arginine-methyl ester
TCA	Trichloroacetic acid

LIST OF ABREVIATION (Continued)

TLCK	*N*-Tosyl-L-lysine chloromethyl ketone
TPCK	*N*-Tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris(hydroxyaminometane)
Tris-HCl	Tris-hydrochloride
SAPNA	*N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide
SDS	Sodiumdodecyl sulfate
SH	Sulhydryl
Suc	Succinyl
UV	Ultraviolet
× g	Gravitational acceleration
μg	Microgram
μL	Microliter
Z	Carbobenzoxy
ZPCK	Carbobenzoxy-L-Phe-chloromethyl ketone

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish is the most important source of protein, but it is susceptible to deterioration by indigenous and microbial enzymes after post-rigor (Saisithi, 1994). Biochemical and physico-chemical changes of fish take place during transportation, processing, and storage. Fish proteins are primarily degraded to oligopeptides and free amino acids by proteinases as fish undergo deterioration. However, proteolysis of fish protein is beneficial for some fisheries products, especially fermented and salted fish products. Fish sauce is a popular condiment among Asian countries. The product is prepared by mixing whole fish with 20-30% salts. The fish flesh is degraded during fermentation by the combined action of fish and microbial proteinases to form a clear liquid with high content of free amino acids and excellent flavor after 8-12 months (Saisithi, 1994).

Limitation of fish sauce production is a long fermentation time and large production area required. The quality of fish sauce is difficult to control due to the natural fermentation process. Traditional fish sauce fermentation is actually a combination of salting and protein hydrolysis. The salting process is used for preventing fish spoilage. However, high salt content inhibits proteolytic activities of endogenous enzymes (Noda, Vo Van, Kusukabe, and Murakami, 1982). Due to a natural fermentation process, pH and temperature are not controlled during fish sauce fermentation. After fresh fish is mixed with salts, pH decreases from the initial value

of 5.9 to 5.3 during fermentation (Vo Van, Kusakabe, and Murakami, 1984). Under such condition, the activity of the digestive proteinase is minimal because the optimal pH of these enzymes is about 7-8 (Gildberg, Hermes, and Orejana, 1984). Furthermore, fermentation of fish sauce takes place at ambient temperature, which varies between 30 and 44 ^{o}C. Therefore, pH and temperature during natural fermentation are well below the optimum condition for endogenous proteinases. Protein hydrolysis induced by endogenous proteinases could be increased if the optimum conditions (pH, temperature, and salt concentration) of these proteinases are determined. This would in turn accelerate fermentation process without an additional cost.

Endogenous proteinases found in fish include digestive proteinases in stomach and intestine, such as pepsin, trypsin, chymotrypsin, carboxypeptidase, aminopeptidase, etc. In addition, proteinases found in muscle tissue include cathepsins, calpain, trypsin-like and alkaline proteinases, etc.(Haard,1994). Classifications of proteinase are based on an amino acid at the active site, which are serine, aspartic, cysteine, and metallo proteinase (Haard and Simpson, 1994). Each catalyzes protein hydrolysis at different pH and temperatures. It was reported that cathepsin B, L and D were endogenous proteinases that degraded fish muscle, resulting in fish muscle tenderization in many species, such as arrowtooth flouder (Visessanguan, Menino, Kim, and An, 2001; Visessanguan, Benjakul, and An, 2003), herring (Nielsen and Nielsen, 2001), chum salmon (Yamashita and Konagaya, 1990), and mackerel (Jiang, Lee, and Chen, 1994). Other possible enzymes are alkaline proteinase in menhaden (Choi, Lanier, Lee, and Cho, 1999), trypsin-like enzyme in hake (Martone, Busconi, Folco, and Sanchez, 1991), trypsin in crayfish (Kim,

Meyers, and Godber, 1996), trypsin in menhaden (Pavlisko, Rial, and Coppes, 1999), and serine trypsin-like in threadfin bream (Kinoshita, Toyohara, and Shimizu, 1990). It is observed that proteinases responsible for muscle protein degradation vary with species.

Protein hydrolysis during fish sauce fermentation has been studied. Gildberg and Shi (1994) recovered tryptic enzymes during fermentation of fish sauce and applied it to herring maturation. Orejana and Liston (1982) showed that proteolysis of fish sauce (patis) was induced by trypsin-like enzyme in fish. Vo Van et al. (1984) showed that activity of the aminopeptidase was detected during the first two months of fermentation. Aminopeptidase extracted from sardine fish sauce showed similar biochemical characteristics to aminopeptidase (I) extracted from internal organs of sardine. Therefore, endogenous proteinases appeared to play an important role in protein hydrolysis during initial fermentation period.

Raw material used for fish sauce production is a small fish, such as anchovy, round scad, sardine, etc. Anchovy (*Engraulis* spp.) is a raw material for fish sauce production in Korea. According to Heu, Pyeun, Kim, and Godber, (1991), proteolytic activity in intestine of anchovy (*E. japonica*) was resulted from alkaline proteinases. The enzymes were later purified and identified as trypsin and chymotrypsin, exhibiting an optimal pH between 8-9 (Heu, Kim, and Pyeun, 1995). However, actual pH of fish sauce fermentation is about 5.5-5.8. Therefore, it is unlikely that these enzymes were responsible for proteolysis during fermentation. Heu, Kim, Cho, Godber, and Pyeun (1997) purified cathepsin L-like from the same anchovy. Optimal pH of the enzyme was later determined at pH 6, which was closed to pH of fish sauce. However, the effect of salt concentration on activities of proteinase was not

investigated. Furthermore, Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguoz-Jerez, and Mora-Ventura (1999) showed that alkaline proteinase was dominant in fish muscle and brine during the ripening of salted anchovy (*E. encrasicholus*). However, the sort of endogenous proteinases in Indian anchovy (*Stolephorus* spp.) involved in proteolysis during fish sauce production remain unclear. Many different enzymes might contribute to hydrolysis of anchovy during fermentation. When biochemical characteristics of predominant endogenous proteinases are identified, such knowledge can be applied to accelerate protein hydrolysis during fish sauce fermentation.

This research was aimed at investigating autolysis of anchovy (*Stolephorus* spp.), which is a major raw material for fish sauce production in Thailand. Additionally, proteinases responsible for autolysis will be partially purified and characterized. The effect of salt concentration, pH, and temperature on proteolysis will also be investigated. The outcome of this research will be critical for further understanding of biochemical characteristics of major proteinases responsible for autolysis of anchovy. In addition, the knowledge of this study may be applied to fish sauce for reduction of fermentation time.

1.2 Research objectives

The overall objective of this research was to study protein hydrolysis of Indian anchovy (*Stelophorus* spp.) induced by endogenous proteinases. Additionally, to characterize both crude and partially purified endogenous proteinases and to investigate the effect of NaCl concentration, temperature, and pH on activity of these proteinases.

Specific objectives of this research are:

(1) To investigate the effect of temperature, and salt on autolysis of whole Indian anchovy mince,

(2) To characterize endogenous proteinases in crude extract and to investigate the effect of salt, pH, and temperature on activity of enzymes,

(3) To investigate predominant proteinases in fish sauce at various fermentation periods,

(4) To partially purify major proteinases responsible for proteolysis and to investigate the effect of salt, pH and temperature on activity of partially purified endogenous proteinases in Indian anchovy.

1.3 Research hypothesis

Autolysis of anchovy is induced by endogenous proteinases in either viscera or muscle or both sources. Some proteinases could catalyze protein degradation at high salt content, which would in turn play an important role in fish sauce fermentation.

1.4 Scope of the study

Endogenous proteinases in Indian anchovy was extracted and partially purified. Biochemical characteristics, such as optimal pH and temperature, substrate specificity, and proteinase inhibition were studied. Changes of proteolytic activity during fish sauce fermentation were investigated.

1.5 Expected results

1. To understand autolytic activity of Indian anchovy and changes of proteolytic activity during fish sauce fermentation.
2. To gain the knowledge of biochemical characteristics of endogenous proteinases in Indian anchovy.

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CHAPTER II

LITERATURE REVIEWS

2.1 Classification of proteinase

Proteinase or peptidase is a group of enzyme that catalyzes hydrolysis of protein to oligopeptides and free amino acids. Proteinase can be divided into two groups based on the action on polypeptide chain. Cleavage on internal bond of polypeptide chain and only near the ends of polypeptide is classified as endopeptidase and exopeptidase, respectively. Furthermore, proteinase can be divided into serine, aspartic, cysteine, and metallo proteinase according to an amino acid at the active site (Barrett, 2001). Typically, cysteine and metello proteinases are active at neutral pH, while serine and aspartic acid proteinases are active at alkaline and acidic pH, respectively (Barrett, 2001). Moreover, proteinase can be classified based on its optimal pH, as alkaline proteinase, acidic proteinase and neutral proteinase. Subsite specificity of proteinase is considered from the amino acid residues at the active site of enzyme that binds to the polypeptide substrate. These subsites on proteinase are called S (for subsites) and the substrate amino acid residues are called P (for peptide) (Schechter and Berger, 1967). The amino acid residues of the substrate at both sites of the cleaved amide bond are denoted P_1 and P_1' for the amino acid residue in the N-terminal and in the C-terminal, respectively. The subsites on the proteinase are denoted S_1 and S_1' for the N-terminal and C-terminal, respectively (Figure 2.1).

The substrate specificity is defined as the ability of each proteinase to bind at specific amino acid residues on substrate. For example trypsin, which is serine

endopeptidase, preferably cleaves substrate containing Arg and Lys at P_1 position. This property can be used to classify characteristics of proteinase.

Substrate: NH_3^+--P_3-P_2-P_1+ P_1'-P_2'-P_3'--COO^-

Enzyme: NH_3^+--S_3-S_2-S_1* S_1'-S_2'-S_3'--COO^-

Figure 2.1-Schematic representation of Schechter and Berger nomenclature, *is catalytic site of enzyme and + is the site of peptide bond cleavage
Ref. (Barrett, 2001)

2.2 Endogenous proteinases in fish

Many endogenous proteinases were found in both viscera and muscle of various fish species (Table 2.1). Fish viscera including digestive tract and hepatopancrease was a significant source of endo- and exopeptidase, such as pepsin, trypsin, chymotrypsin, aminopeptidases, and carboxypeptidases (Haard, 1994). Proteinases are also found in sarcoplasmic fluid and myofibrillar protein of fish muscle. These included trypsin-like, chymotrypsin-like, cathepsin B and L, calpain, alkaline proteinase, aminopeptidase, carboxypeptidase-like and myofibril-bound serine proteinase (MBSP) etc. (Shahidi and Kamil 2001). In addition, muscle soluble serine proteinase was a novel serine proteinase that was purified from soluble fraction of lizardfish (Ohkubo, Miyagawa, Osatomi, Hara, Nozaki, and Ishihara, 2004b).

Table 2.1-Endogenous proteinases found in different species

Category group	Proteinases	Fish	Sources	References
Cysteine	Cathepsin L	Chum salmon	Muscle	Yamashita and Konagaya (1990)
Proteinase		Pacific whiting	Muscle	Seymour et al. (1994)
		Mackerel	Muscle	Jiang et al. (1996)
		Anchovy	Muscle	Heu et al. (1997)
		Carp	Hepatopancrease	Aranishi et al. (1997)
		Arrowtooth	Muscle	Visessanguan et al. (2003)
	Cathepsin B	Mackerel	Muscle	Jiang et al. (1994)
Serine	Trypsin and	Milkfish	Muscle	Jiang et al. (1990)
proteinase	trypsin-like	Hake	Muscle	Martone et al. (1991)
		Anchovy	Viscera	Heu et al. (1995)
		Hybrid tilapia	Intestine	El-shemy and Levin (1997)
		Tilapia	Plasma	Inaba et al. (1997)
		Menhaden	Pyloric caeca	Pavlisko et al. (1999)
	Alkaline proteinase	Anchovy	Viscera	Heu et al. (1991)
		Atlantic salmon	Muscle	Stoknes and Rustad (1995)
		Atlantic menhaden	Muscle	Choi et al. (1999)
	Chymotrypsin and	Anchovy	Viscera	Heu et al. (1995)
	chymotrypsin - like	Sardine	Muscle	Lugo-Sanchez et al. (1997)
	MBSP*	Carp	Muscle	Osatomi et al. (1997); Cao et al. (1999a, 1999b)
		Lizardfish	Muscle	Cao et al. (2000); Ohkubo et al. (2004a)
Aspartic	Cathepsin D	Tilapia	Muscle	Jiang et al. (1991)
proteinase		Carp	Muscle	Goldman-Levkovitz et al. (1995)
		Herring	Muscle	Neilsen and Neilsen (2001)
	Pepsin	Sardine	Viscera	Castillo-Yanez et al. (2004)
Metallo	Calpain	Salmon	Muscle	Geesink et al. (2000)
proteinase	Carboxy-peptidase A, B	Sardine	Muscle	Lugo-Sanchez et al. (1997)
	Cathepsin A	Milkfish	Muscle	Jiang et al. (1990)
	Leu-aminopeptidase	Sardine	Muscle	Lugo-Sanchez et al. (1997)

* MBSP = Myofibril-bound serine proteinase

2.2.1 Trypsin and trypsin-like proteinase

Trypsin is a serine proteinase, which is found in digestive tract. Trypsin-like proteinases are present in fish muscle. Trypsin-like proteinases were purified from sarcoplasmic fraction in skeletal muscle of hake (Martone, Busconi, Folco, and Sanchez, 1991) and white croaker (Cao, Hara, Weng, Zhang, and Su, 2005). Moreover, trypsin-like proteinase was also isolated from plasma of tilapia (Inaba, Buerano, Natividad, and Morisawa, 1997). Biochemical characteristics of trypsin and trypsin-like proteinases have been reported. Optimal pH of trypsin is typically found at alkaline range.Trypsin purified from digestive tract of anchovy (*Engraulis encrasicholus*) had pH optimum of 8-9 for *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) (Martinez, Olsen, and Serra, 1988). However, optimal pH of this enzyme was 9.5 with using casein and myofibrillar protein as substrates. Trypsin isolated from Japanase anchovy (*E. japonica*) also exhibited maximum activity at pH 9.0 for casein and at pH 8.0 for BAPNA (Heu, Kim, and Pyeun, 1995). Trypsin purified from pyloric caeca of rainbow trout showed a maximum activity towards BAPNA at pH 9.0-10.0 (Kristjansson, 1991). Variation of optimal pH depended on type of a substrate used. Two trypsins, A and B purified from heptopancreas of carp, had optimal temperature at 40 and 45 ^{o}C, respectively. The optimal temperature of trypsin-like proteinase obtained from milkfish muscle was 60 ^{o}C (Jiang, Tsao, Wang, and Chen, 1990), which was similar to trypsin-like proteinases isolated from threadfin bream muscle (Kinoshita, Toyohara, and Shimizu, 1990). Trypsin and trypsin-like proteinase prefer to cleave a substrate contained Arg or Lys at P_1 positions (Barrett, Rawlings, and Woessner, 1998). Synthetic substrates used for characterization of these proteinases included chromogenic and fluorogenic peptide substrates, such as

BAPNA, *N*-tosyl-L-arginine-methyl ester (TAME), butyloxycarbonyl (Boc)-Gln-Ala-Arg-7-amido-4-methylcoumarin (AMC), Boc-Val-Leu-Lys-AMC etc. In addition, peptides used for determining substrate specificity were oxidized insulin B chain, neurotensin, methionyl-lysyl-bradykinin, etc. Degradation of peptides products was separated by high performance liquid chromatography (HPLC) and subsequently analyzed by an amino acid analyzer. Amino acids at the cleaved peptide bond were identified. For example, trypsin-like proteinase purified from skeletal muscle of white croaker preferably cleaved the peptide bond where carboxyl side was either Arg or Lys.

Soybean trypsin inhibitor and *p*-tosyl-L-lysyl-chloromethylketone (TLCK) inhibited trypsin and trypsin-like proteinase. In additon, these enzymes are also inhibited by general serine proteinase inhibitors, such as diisopropyl fluorophosphate (DFP), aprotinin, leupeptin, and phenylmethylsulfonyl flouride (PMSF). Molecular weight (MW) of these proteinases in fish was estimated to be 24-30 kDa (Heu et al., 1995; Pavlisko, Rial, and Coppes, 1999; Martinez et al. 1988). Trypsin-like proteinase in plasma of tilapia, which involved in degradation of vitellogenins showed MW of 170 kDa based on SDS-PAGE (Inaba et al., 1997). Trypsin showed both cationic and anionic forms in many species due to distinctive amino acid sequence. Four different trypsin isoforms were isolated from Atlantic salmon. These included three anionic isoforms with pI of 4.70, 4.60, and 4.55, and one cationic isoform with pI of 9.3. (Outzen, Berglund, Smalas, and Willassen, 1996). Anchovy trypsin also exhibited two anionic isofoms with pI of 4.9 and 4.6 (Martinez et al., 1988).

2.2.2 Chymotrypsin and chymotrypsin-like proteinase

Chymotrypsin is a digestive serine proteinase. It effectively hydrolyzes peptide bonds with the carboxyl side being hydrophobic amino acids, such as Try, Trp, Phe, or Leu. Chymotrypsin purified from anchovy (*E. japonica*) showed an optimal pH and temperature of 8.0 and 45 ^{o}C, respectively (Heu et al., 1995). Several substrates can be used for monitoring its activity including chromogenic and fluorogenic peptide substrates, such as *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPNA), benzoyl-L-tyrosine ethyl ester (BTEE), Boc-Ala-Ala-Pro-Phe-AMC, and Boc-Leu-Leu-Val-Tyr-AMC. Specific inhibitors of chymotrypsin are *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK) and carbobenzoxy-L-Phe- chloromethyl ketone (ZPCK). Moreover, activity of chymotrypsin-like proteinase extracted from Monterey sardine was reduced by EDTA, $CuSO_4$, iodoacetamide and cysteine (Lugo-Sanchez, Pacheco-Aguilar, Yepiz-Plascencia, 1997). Chymotrypsin required Ca^{2+} for activation. MW of chymotrypsin purified from anchovy was estimated to be 26 kDa (Heu et al., 1995).

2.2.3 Cathepsin L and B

Cathepsin L and B are cysteine proteinases and found in lysosome. These proteinases involve muscle degradation of various fish species. Cathepsin L and B were predominant proteinases in Pacific whiting muscle and involved in myosin degradation during conventional heating of fillets and surimi (An, Weerasinghe, Seymour, and Morrissey, 1994). Aoki and Ueno (1997) reported that mackerel cathepsin L hydrolyzed myosin, troponin T, troponin I, and tropomyosin, while mackerel cathepsin B did not hydrolyze myofibrillar protein from mackerel.

Two cathepsin L isoforms were purified from Pacific whiting muscle, and showed pH optimum at 5.5 and 6.0 and temperature optimum at 55 and 60 ^{o}C, respectively (Seymour, Morrissey, Peters, and An, 1994). Purified cathepsin L from arrowtooth flouder muscle also showed maximum activity at pH 5.5 and 60 ^{o}C (Visessanguan, Benjakul, and An, 2003). In addition, purified catehpsin L from hepatopancreas of carp exhibited a maximum activity at pH 5.5-6.0 and 50 ^{o}C (Aranishi, Ogata, Hara, Osatomi, and Ishihara, 1997). MW of cathepsin L was estimated to be 25-27 kDa. Z-Phe-Phe-CHN_2 was a specific inhibitor for cathepsin L. General cysteine proteinase inhibitors, such as leupeptin, E-64, chymostatin, also inhibited cathepsin activities. These enzymes were activated by reducing reagents, such as dithiothreitol, cysteine, glutathione, 2-mercaptoenthanol since these compounds prevented disulfide bond formation of SH group at active site. Cathepsin-L like proteinase purified from muscle of anchovy (*E. japonica*) was able to hydrolyze oxidized insulin B chain at Phe_1, Asn_3, Val_{13}, Glu_{14}, Val_{19} and Gly_{24} (Heu, Kim, Cho, Godber and Pyeun, 1997).

Cathepsin B was purified from white muscle of chum salmon and ordiary mackerel muscle (Yamashita and Konagaya 1990; Jiang, Lee, and Chen, 1994). MW of this enzyme was estimated to be 28 kDa. Isoelectric point (pI) of cathepsin B was estimated to be 4.9. In addition, Cu^{2+} and Hg^{2+} greatly inhibited cathepsin B activity because these ions exhibited high affinity towards the SH groups at the active site. Optimal pH and temperature of cathepsin B isolated from mackerel were 6.5 and 55 ^{o}C, respectively (Jiang et al., 1994). Cathepsin B isolated from Pacific whiting showed maximum activity between 20-37 ^{o}C (An et al., 1994).

2.2.4 Acid proteinases

Pepsin is an aspartic proteinase and found in fish stomach. Its optimal pH was about 2.0-4.0. General inhibitors of acid proteinases are pepstatin, diazoketones, and phenylacryl bromides (Beynon and Bond, 2001). Pepsin purified from Monterey sardine displayed the optimal pH and temperature at 2.5 and 45 ^{o}C, respectively. MW of pepsin was estimated to be 33 kDa (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, and Navarrete-Del Toro, 2004). Pepsin purified from viscera of bolti fish (*Tilapia nilotica*) was stable at pH 3-6 and unstable at neutral and alkaline pH (El-Beltagy, El-Adway, Rahma, and El-Bedawey, 2004).

Cathepsin D is a lysosomal proteinase, which is also classified as aspartic proteinase. It is typically found in fish muscle. MW of cathepsin D purified from herring and carp muscle was estimated to be 36-39 kDa (Nielsen and Nielsen, 2001; Goldman-Levkontz, Rimon, and Rimon, 1995). However, cathepsin D purified from tilapia mucle was approximated to be 55 kDa (Jiang, Wang, and Chen, 1991). Optimal pH and temperature of this enzyme were 2.5-3.7 and 37 ^{o}C, respective, using hemoglobin as a substrate (Nielsen and Neilsen, 2001; Goldman-Levkontz et al., 1995; Jiang et al., 1991). Barrett and Kioschke (1981) described that cathepsin D seemed to have limited activity on protein, whereas cysteine proteinases showed broad activity. The major cleavage of oxidized insulin B by carp cathepsin D were 56% at Tyr_{16}-Leu_{17} and 40% at Phe_{25}-Tyr_{26} (Goldman-Levkontz et al., 1995). Cathepsin D isolated from herring muscle preferentially cleaved at Leu_{15}-Tyr_{16} (47%), Tyr_{16}-Leu_{17} (34%) and Ala_{14}-Leu_{15} (18%) of oxidized insulin B chain (Neilsen and Neilsen, 2001). Cathepsin D was completely inhibited by pepstatin, and partially

inhibited by p-chloromercuri-benzoate (PCMB) and EDTA. Acid proeinase was also inhibited by Hg^{2+} and Fe^{3+} and activated by Ca^{2+}, Ni^{2+}, and Mg^{2+} (Jiang et al., 1991).

2.2.5 Myofibril-bound serine proteinase (MBSP)

MBSP is a serine proteinase found in myofribrillar fraction and was solubilized from myofibril fraction by acid and salt solution. MBSP was purified from ordinary muscle of carp using 0.6 M KCl, pH 4.0 (Osatomi, Sasai, Cao, Hara, and Ishihara, 1997). MW of purified MBSP from carp muscle was estimated to be 30 kDa by SDS-PAGE and gel filtration, while that purified from skeletal muscle of lizardfish was estimated to be 29-30 kDa under reducing condition, and 50-60 kDa under non-reducing condition (Cao, Osatomi, Hara, and Ishihara, 2000; Ohkubo, Miyagawa, Osatomi, Hara, Nozaki, Ishihara, 2004a). These results suggested that MBSP in lizardfish was a dimer composed of two homologous subunits linked by disulfide bonds. These enzymes showed maximum activity at pH 8.0 and 55-60 ^{o}C. MBSP hydrolyzed Boc-Phe-Ser-Arg-AMC and Boc-Val-Pro-Arg-AMC that are synthetic substrates for trypsin. The enzyme was inhibited by DFP, SBTI, and TLCK (Osatomi et al., 1997; Cao et al., 2000). Therefore, the enzyme was considered to be trypsin-like proteinase associated with myofibril. MHC was hydrolyzed by MBSP at 0.5 M NaCl, 55-60 ^{o}C. These results suggested that activity of MBSP was involved in muscle degradation and responsible for gel-weakening phenomenon.

2.2.6 Alkaline proteinases or multicatalytic proteinase

Alkaline proteinases play an important role in muscle degradation of Atlantic menhaden and Atlantic salmon (Boye and Lanier, 1988; Choi, Lanier, Lee, and Cho,

1999). Alkaline proteinases are located in the muscle sarcoplasmic, microsomal, and also bound to myofibrils (Shahidi and Kamil, 2001). Alkaline proteinases were composed of multiple subunits ranging from 20-35 kDa (Haard, 1994). MW of two alkaline proteinases purified from Atlantic menhaden muscle was estimate to be 707 and 450 kDa (Choi et al., 1999). pH and temperature optimum towards casein and actomyosin were 7.5-8.0 and 55-60 ^{o}C, respectively (Boye and Lanier, 1988; Choi et al., 1999). A distinct feature of alkaline proteinase is that activation the enzyme is achieved by heat and protein denaturants, such as urea, fatty acid, and detergents (Shahidi and Kamil, 2001). Alkaline proteinases usually possess cysteine at the active site (Haard, 1994). However, two alkaline proteinases purified from Atlantic menhaden were inhibited by DFP, TPCK and TLCK and strongly hydrolyzed BTEE and TAME, which were characteristics of serine proteinases (Choi et al., 1999).

Multicatalytic proteinase (MCP) is a high molecular weight non-lysosomal proteinase with a complex multisubunit structure containing several distinct catalytic sites. The MCP cleaved bonds on the carboxyl side of basic, hydrophobic, and acidic amino acid residues. Optimal pH of MCP was 7.5-9.5 depending on the substrate (Rivett, 1989). MCP was found in fish muscle and showed high MW with 13-15 subunits. MW of MCP purified from Atlantic salmon under non-denaturing conditions was estimated to be 600 kDa, while MW under denaturing conditions was 25-30 kDa (Stoknes and Rustad, 1995). pI of the enzyme was about 7.3. The MCP hydrolyzed substrates for trypsin and chymotrypsin-like proteinase. The enzyme was also inhibited by several serine proteinase inhibitors. The enzyme had at least two different active sites (Stoknes and Rustad, 1995). MCP in rat's liver had at least three catalytic components termed chymotrypsin-like, trypsin-like, and peptidylglutamyl

peptide-hydrolyzing, and were associated with the proteinase according to Pereira, Yu, and Wilk (1994). In addition, MCP was inhibited by urea and acid treatment due to dissociation of multisubunits (Rivett, 1989). MCP was responsible for muscle degradation at high temperature (60 ^{o}C).

2.2.7 Other proteinases

Aminopeptidase and carboxypeptidase are metallo exoproteinases that contain metal ion at active sites. Lugo-Sanchez et al. (1997) reported that carboxypeptidase A and B in sarcoplasmic fluid of Monterey sardine was activated by Cu^{2+} and inhibited by EDTA. Carboxypeptidase B purified from starfish had an optimal pH and temperature at 7.5 and 55 ^{o}C, respectively and MW of 34 kDa. It was activated by Co^{2+} and inhibited by EDTA (Kishimura, Hayashi, and Ando, 2005). However, leucine-aminopeptidase (LAP) found in Monterey sardine and anchovy was not inhibited by EDTA (Martinez and Serra, 1989). Cathepsin A-like (called carboxypeptidase A) purified from milkfish muscle was able to hydrolyze Z-Gly-Phe at pH 7.0 and 40-50 ^{o}C. The enzyme was unstable at high temperature and inhibited by pepstatin A and Hg^{2+} (Jiang et al., 1990). Caboxylpeptidase A and B preferably hydrolyzed Phe/Try and Arg/Lys from carboxyl terminus of proteins, respectively (Haard, 1994; Kishimura et al., 2005). Moreover, Neutral proteinases were found in fish muscle and exhibited optimal activity at neutral pH. Calpains or Ca^{2+}-activated proteinases were neutral proteinases found in skeletal muscle. μ-Calpain and *m*-calpain were activated in vitro by micromolar and millimolar Ca^{2+} concentration, respectively (Barrett et al., 1998). Calpain was found in sarcoplsmic fraction that was released from myofilament by degradation of Z-disk. Ladrat, Chaplet, Verrez-Bagnis,

Noel, and Fleurence (2000) reported that calpains accelerated post-mortem degradation of muscle protein. Two neutral serine proteinases purified from muscle of salted fish also involved in muscle degradation during ripening (Ishida, Niizeki, and Nagayama, 1994; Ishida, Sugiyama, Sato, and Nagayama, 1995). The first enzyme showed MW of 25 kDa and exhibited maximum activity at 45 ^{o}C and pH 6.8, while the other showed MW of 37 kDa with maximum activity at 50 ^{o}C and pH 7.0-7.5.

2.3 Role of endogenous proteinases in fish sauce fermentation

Effects of endogenous proteinase on biochemical and physiochemical changes of fish take place during transportation, preparation, processing, and storage. Fish proteins are degraded to oligopeptides and amino acids by endogenous proteinases, resulting in tissue softening, and loss of functional properties of muscle protein. However, proteolysis is a desirable reaction for fermented and salted fish products. Amino acids and small peptide produced from protein degradation are important precursors for flavor and aroma development.

Fish sauce is a liquefied fermented product produced by the hydrolysis of fish protein in the presence of high salt concentration (Chaveesuk, Smith, and Simpson, 1993). It was prepared by mixing small fish, such as anchovies, sardines, and round scad with 25-30% salt and fermented for 8-12 months. Proteolysis of fish protein is a primary process during fish sauce fermentation. The role of various endogenous proteolytic enzymes in fish sauce has been investigated. Orejana and Liston (1982) reported that the main proteinase involved in protein degradation appeared to be trypsin-like enzymes. Trypsin-like proteinase which was separated from laboratory fish sauce and commercial fish sauce, was inhibited by soybean trypsin inhibitor and

exhibited maximum activity at pH 8.2 and 60-65 ^{o}C. The enzyme was also inhibited by end products, such as amino acids and small peptides. However, activity of trypsin-like proteinase was only maximal during the first month of fermentation and gradually decreased afterward. Noda, Vo Van, Kusakabe and Murakami (1982) elucidated protein hydrolysis of sardine fish sauce by endogenous proteinases and found that alkaline and acid proteinases were stable at 25% NaCl for three months of fish sauce fermentation. Gildberg and Shi (1994) reported that trypsin, chymotrypsin, and elastase were recovered from fish sauce made from cod viscera. Chymotrypsin was the most important enzyme during fermentation as its activity was higher than that of trypsin and elastase. Trypsin activity was only observed at the initial stage of fermentation. However, proteinase activity was not detected after 25 days of fermentation. Cathepsin L was a predominant proteinase in Pacific whiting and involved its muscle degradation (An et al., 1994). Cathepsin L and B were found in the first month of fish sauce prepared from whole Pacific whiting and a mixture of fish and surimi byproducts, and their activities gradually decreased afterward (Tungkawatchara, Park, and Choi, 2003). Chymotrypsin, trypsin, and cathepsin L-like were purified from anchovy (*E. japonica*), which is a major raw material used for fish sauce fermentation in Korea (Heu et al., 1995; Heu et al., 1997). These enzymes were suspected to play an important role in protein hydrolysis during fish sauce fermentation. Choi, Heu, Kim, and Pyeun (2004) reported that chymotrypsin and cathepsin L-like activities contributed to degradation of myofibrillar protein to a grater extent than trypsin. These results indicated that chymotrypsin and cathepsin L-like were likely to be responsible for autolysis of muscle protein. However, these proteolytic activities decreased with increasing NaCl concentration. Aminopeptidase

is also another important enzyme in fish sauce fermentation because it contributes to an increase of free amino acids. Aminopeptidase I isolated from sardine fish sauce was stable at 15% NaCl with residual activity of 70% and showed similar biochemical characteristics to aminopeptidase I purified from sardine. However, activities of these aminopeptidases were only found in the first two months of fish sauce fermentation (Vo Van, Kusakabe, and Murakumi, 1984). These research works suggested that many types of endogenous proteinases are involved in protein hydrolysis only at the initial stage of fish sauce fermentation. This explains why fish sauce fermentation takes about 1-1.5 years. Thus, a proper measure to activate endogenous proteinases activity for a longer period would lead to extensive protein hydrolysis which could in turn accelerate fish sauce fermentation.

2.4 References

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CHAPTER III

AUTOLYTIC ACTIVITY AND BIOCHEMICAL CHARACTERISTICS OF ENDOGENOUS PROTEINASES IN INDIAN ANCHOVY (*Stolephorus* spp.)

Abstract

Maximum autolytic activity of Indian anchovy (*Stolephorus* spp.) was found at 60 ^{o}C. Autolytic activity decreased with increased NaCl concentration. Remaining autolytic activity at 25% NaCl (w/w) was 52%. Crude proteinase extracts exhibited the highest activity at 60 ^{o}C using either casein or acid denatured hemoglobin (dHb) as a substrate. Optimal pH of crude extracts was found at 8.5 for casein and 9.5 for dHb. Activity of crude proteinases decreased > 50% when NaCl concentration was greater than 0.1 M. Crude extract was stable up to 8 h at 4, 30, and 60 ^{o}C. Crude proteinase hydrolyzed several synthetic substrates of trypsin, including Boc-Asp(oBzl)-Pro-Arg-AMC, Boc-Val-Leu-Lys-AMC, and Boc-Gln-Ala-Arg-AMC. Soybean trypsin inhibitor (SBTI), leupeptin, phenylmethylsulphonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone (TLCK) inhibited activities of proteinase, while 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), ethylenediaminetetraacetic acid (EDTA), bestatin, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) slightly inhibited the activity. Pepstatin A did not inhibit proteinase activity. Molecular weight of proteinases exhibiting casinolytic activity at

4.0 M NaCl was estimated to be 63, 53, 46, 40, 35, and 31 kDa using electrophoresis activity staining.

Keywords: Indian anchovy (*Stolephorus* spp.), autolytic activity, endogenous proteinase, trypsin-like proteinase

3.1 Introduction

Fish sauce is a clear amber liquid containing free amino acids, oligopeptides with distinct aroma and flavor. Indian anchovy (*Stolephorus* spp.) is widely used as a raw material for fish sauce manufacturing in Southeast Asia. Fish is typically mixed with 20-30% solar salt and left in a concrete tank at ambient temperature for 8-12 months (Saisithi 1994). Major limitation of fish sauce industry is a long fermentation time. Fish endogenous proteinases and microbial enzymes play an important role in protein hydrolysis during fish sauce fermentation. Understanding biochemical characteristic of endogenous proteinases would lead to a means for fish sauce acceleration.

Anchovy (*Engraulis encrasicholus*), a raw material used for salted fish in Europe, was susceptible to proteolytic degradation by digestive enzymes from pyloric caeca and intestine (Martinez and Gilberg 1988). Two trypsin-like enzymes purified from pyloric caeca and intestine of anchovy had pH optimum at 8-9.5 (Martinez, Olsen, and Serra, 1988). Major proteinases in pyloric caeca and intestine were trypsin, chymotrypsin, elastase, and aminopeptidase (Martinez and Serra 1989). Furthermore, Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguoz-Jerezz, and Mora-Ventura (1999) showed that alkaline proteinase was predominant in fish muscle and brine during the ripening of salted anchovy (*E. encrasicholus*). According

to Heu, Pyeun, Kim, and Godber (1991), proteolytic activity in intestine of anchovy (*Engraulis japonica*) was resulted from alkaline proteinases. These enzymes were later purified and identified as trypsin and chymotrypsin, exhibiting an optimal pH between 8-9 (Heu, Kim, and Pyeun, 1995). Ishida, Sugiyama, Sato, and Nagayama (1995) reported that neutral serine proteinase found in muscles of anchovy (*E. japonica*) exhibited optimum catalytic activity at pH 7.5. Activity of these proteinases decreased when NaCl concentration increased over 10%. Heu, Kim, Cho, Godber, and Pyeun (1997) purified cathepsin L-like with optimal pH 6 from muscle of the same anchovy. Furthermore, Choi, Heu, Kim, and Pyeun (2004) suggested that activities of trypsin, chymotrypsin, and cathepsin L purified from anchovy (*E. japonica*) decreased with increasing NaCl concentration. Based on previous studies, many different proteinases were present in fish and might contribute to protein hydrolysis during high salt fermentation. However, endogenous proteinases in Indian anchovy (*Stolephorus* spp.) have not been studied and characterized. The objective of this study was to investigate autolytic activity in Indian anchovy (*Stolephorus* spp.). Additionally, biochemical characteristics of predominant endogenous proteinases were characterized.

3.2 Materials and Methods

3.2.1 Materials and chemicals

Fresh Indian anchovies (*Stolephorus* spp.) caught off the Gulf of Thailand, Chonburi province, were transported to the laboratory at Suranaree University of Technology within 6 h after catch. Fish were kept frozen at –20 ^{o}C and used throughout the experiment. The average fish weight was 2.61 ± 0.47 g with the length

of 7.59 ± 0.34 cm. Casein, hemoglobin (Hb), bovine serum albumin (BSA), L-tyrosine, dimethyl sulfoxide (DMSO), Brij 35, butyloxycarbonyl (Boc)-Gln-Ala-Arg-7-amido-4-methylcoumarin (AMC), Boc-Val-Leu-Lys-AMC, and carbobenzoxy (Z)-Arg-Arg-AMC, phenylmethylsulfonyl fluoride (PMSF), leupeptin, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), bestatin, pepstatin A, *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemicals Co. (St. Louis, Mo., USA). Boc-Asp(oBzl)-Pro-Arg-AMC, succinyl (Suc)-Ala-Ala-Pro-Phe-AMC and Z-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). All other chemicals were of analytical grade.

3.2.2 Autolysis assay

Autolytic activity was measured according to the method of Greene and Babbitt (1990). Whole anchovy was ground and incubated at various temperatures (30-90 ^{o}C). Oligopeptide contents were determined according to the method of Lowry, Rosenbrough, Farr and Randall (1951). The effect of NaCl concentration (0 to 25% w/w) on autolysis of anchovy was also investigated at the optimal temperature. Blanks were prepared by adding 5% trichloroacetic acid (TCA) before incubation at 60 ^{o}C for 20 min. Autolytic activity was expressed as μmole of tyrosine released/min/g mince.

3.2.3 Preparation of crude extracts

Crude extracts were prepared by homogenizing whole anchovy with ice-cold 20 mM phosphate buffer, pH 7.0 at a ratio of 1:2 using a homogenizer (Nihonseiki

Kaisha Ltd., Tokyo, Japan). The homogenate was centrifuged at 17500 × g for 20 min at 4 ^{o}C. The supernatant was filtered through four layers of cheesecloth and Whatman paper No. 4 and used as a crude extract.

3.2.4 Proteinase activity assays

Proteinase activity was determined using either 1% casein or 1% acid denatured hemoglobin (dHb) as a substrate (An, Seymour, Wu, and Morrissey, 1994). The reaction was carried out at 60 ^{o}C for 20 min under a linear range determined from the time course study. TCA-soluble oligopeptides content was determined by Lowry method using tyrosine as a standard (Lowry et al., 1951). Activity was defined as nmole of tyrosine released/min. Specific activity was expressed as the amount of activity per mg protein of crude extract.

3.2.5 Temperature/pH optimum and thermal stability

The activity of crude extract was determined at various temperatures (30-90 ^{o}C) at pH 7.0, as described above. The activity was also assayed at optimal temperature and various pHs ranging from 3-12: pH 3.0-8.0 using McIlvaine's Buffer (0.1 M sodium citrate and 0.2 M sodium phosphate); pH 8.5-9.0 using 0.2 M Tris-HCl; pH 9.5-10, using 0.1 M Glycine-NaOH; pH 12 using 0.1 M $NaHCO_3$-Na_2CO_3.

To study thermal stability, crude extract was incubated at 4, 30, and 60 ^{o}C for up to 8 h. The remaining activity was determined at 60 ^{o}C in 0.1 M glycine-NaOH buffer (pH 9.5) using 1% dHb as a substrate.

3.2.6 Effect of NaCl concentration on proteinase activity

The effect of NaCl on proteolytic activities was determined at various NaCl concentrations (0 to 2.5 M) in 0.2 M Tris-HCl (pH 8.5) and 0.1 M glycine-NaOH (pH 9.5) when casein and dHb was used as a substrate, respectively.

3.2.7 Synthetic substrate specificity

Proteolytic activity was determined using various synthetic substrates including Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Boc-Asp(oBzl)-Pro-Arg-AMC Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC according to the method of Barrett and Kirschke (1981) and Ishida et al. (1995). All synthetic substrates were prepared at either 1 or 10 mM in DMSO. The reaction mixtures containing 0.8 mL of 0.2 M Tris-HCl (pH 8.5), 0.1 mL of 10 μM of substrate solution, and 0.1 mL of diluted crude extracts with 0.1% Brij 35 in a total of 1 mL, were incubated at 60 ^{o}C for 5 min. The reaction was stopped by adding 1.5 mL of the stopping reagent (methanol: n-butanol: distilled deionized water = 35:30:35 (v/v/v)) followed by heating at 95 ^{o}C for 3 min. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured by a spectrofluorophotometer RF-1501 (Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Unit activity was defined as one μmole of AMC released/min. Specific activity was expressed as the amount of unit activity/mg protein of crude extract.

3.2.8 Effect of inhibitors

The effect of various inhibitors (PMSF, TLCK, TPCK, SBTI, bestatin, pepsatatin A, leupeptin and EDTA) and $CaCl_2$ concentration (0-100 mM) on crude proteinases activity was determined using Boc-Asp (oBzl)-Pro-Arg-AMC as a substrate.

3.2.9 Activity staining

Molecular weight was determined by activity staining according to method of Garcia-Carreno, Dimes, and Haard (1993). Crude proteinases were separated on 10% polyacrylamide gel (Laemmli 1970). Subsequently, gel was immerged in 2% casein, 50 mM Tris-HCl (pH 7.5) for 30 min on ice. Caseinolytic activity was initiated by transferring gels in either 0.2 M Tris-HCl (pH 8.5) buffer or the same buffer containing 4.0 M NaCl, and incubated at 60 ^{o}C for 15 min. Subsequently, gels were stained in 0.125% coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 2 h. Destaining was carried out using 25% methanol and 10% acetic acid solution. Clear zone on the blue background indicated the presence of proteinase.

3.3 Results and Discussion

3.3.1 Effects of temperature and salt on autolytic activity

TCA-soluble oligopeptides increased with temperature and reached the maximum at 60 ^{o}C (Figure 3.1). High level of autolytic activity at relatively high temperature indicated the presence of heat stable proteinases. Ishida, Niizeki and Nagayama (1994) showed that the serine proteinase isolated from salted anchovy muscle exhibited maximal activity at 55 ^{o}C. Moreover, proteinases found in threadfin

bream (Kinoshita, Toyohara, and Shimizu, 1990), anchovy (*E. japonica*) (Heu et al., 1995), and Atlantic menhaden (Boye and Lanier 1988) showed maximal activity around 45-65 ^{o}C.

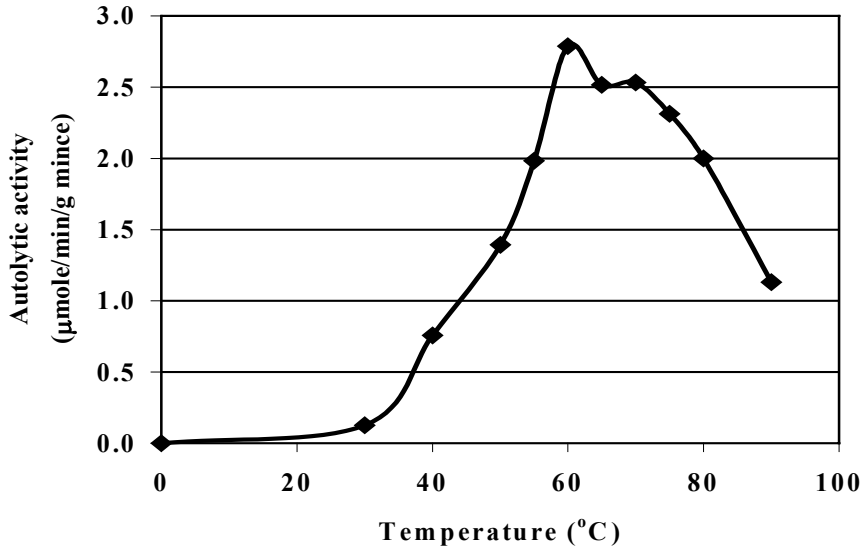

Figure 3.1-Effect of temperature on autolytic activity of Indian anchovy

TCA-soluble oligopeptides decreased with increasing NaCl concentration (Figure 3.2). The autolytic activity at 25% (w/w) NaCl was about 52% of the control (no NaCl), suggesting that endogenous proteinases in Indian anchovy could hydrolyze muscle proteins even at high salt concentration, but to a lesser extent than in the absence of salt. Trypsin-like proteinase activity was observed during the first month of anchovy (*Stolephorus* spp.) fermentation in 22-28% NaCl (Orejana and Liston 1982). Gildberg and Shi (1994) reported the presence of trypsin, chymotrypsin and elastase activity in fish sauce made from cod viscera mixed with 20-25% NaCl. Our results indicated that endogenous proteinases in Indian anchovy could play a major role in protein hydrolysis at high salt content (25-30% NaCl).

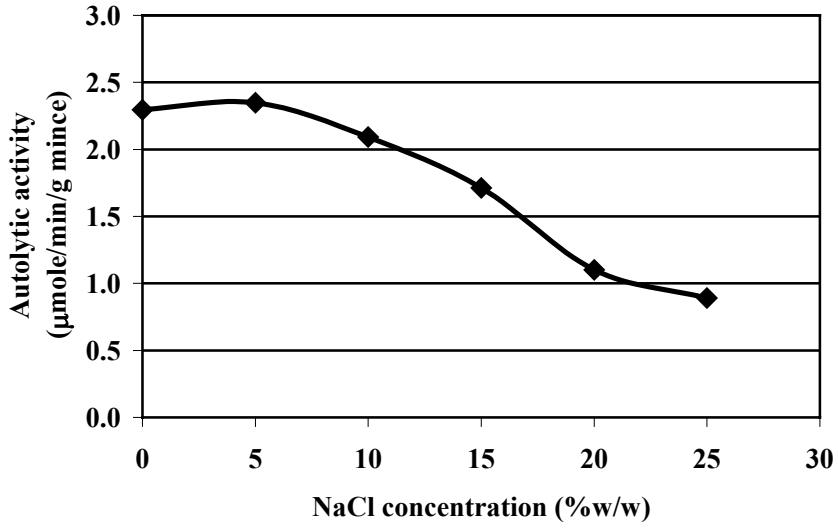

Figure 3.2-Effect of NaCl on autolytic activity of Indain anchovy

3.3.2 Temperature/pH optimum and thermal stability of crude proteinase

Maximum hydrolytic activity of crude proteinase towards casein and dHb was at 60 ^{o}C (Figure 3.3), corresponding to the optimal temperature of autolysis. This implied that majority of proteinases responsible for tissue-softening in Indian anchovy were likely to be extracted by low ionic strength buffer. Optimal pHs of crude proteinases from Indian anchovy were at pH 3.0 and pH 8.5-9.5 (Figure 3.4). Both acid and alkaline proteinases were predominant in Indian anchovy. Alkaline proteinase appeared to be stable at low pH in salted ripened anchovy (Hernandez-Herrero et al., 1999). Additionally, alkaline proteinase exhibited the optimal pH in a broad range of 6-10 depending on the substrate (Kolodziejska and Sikorski, 1996). Typically, fish sauce fermentation is carried out outdoor at temperature of 30-35 ^{o}C with pH ranging from 5.5–5.8, which was far different from the optimum condition of

endogenous proteinases. This would partly explain the limited rate of protein hydrolysis during fish sauce fermentation.

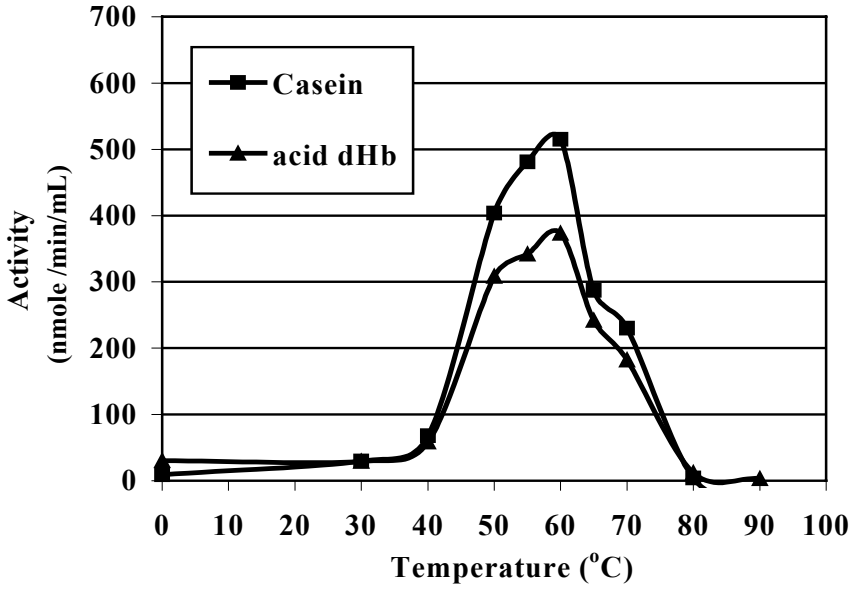

Figure 3.3-Temperature profiles of crude proteinases assayed at pH 7.0

Crude proteinases showed high stability at 4, 30 and 60 ^{o}C (Figure 3.5). Specific activity of crude proteinases incubated at 60 ^{o}C was the highest because heat treatment eliminated contaminant proteins. Crude proteinases were stable up to 8 h at all studied temperatures. High thermal stability of endogenous proteinases at 60 ^{o}C would be advantageous to accelerate protein hydrolysis of Indian anchovy. Incubating whole fish at the optimal temperature (60 ^{o}C) would maximize autolytic without lessening the enzyme activity.

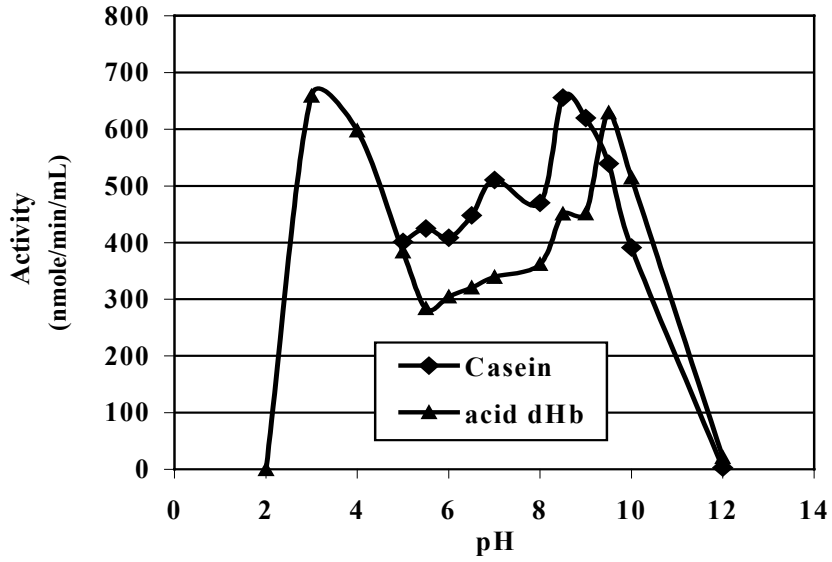

Figure 3.4-pH profiles of crude proteinases assayed at 60 ^{o}C

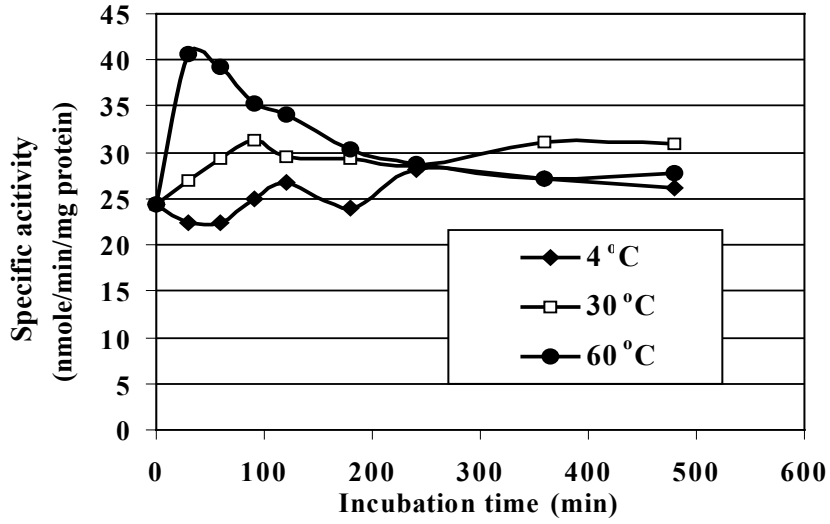

Figure 3.5-Thermal stability of crude extracts incubated at 4, 30, and 60 ^{o}C

3.3.3 Effect of NaCl concentration

Proteolytic activity of crude extracts decreased with increasing NaCl (Figure 3.6). Remaining activity at 2.5 M NaCl was 33% and 20% when using dHb and casein as substrates, respectively. Autolytic activity at 15% NaCl (about 2.5 M NaCl) was approximately 77% of that without NaCl (Figure 3.2). Proteolytic activity of the crude extract appeared to be more sensitive to NaCl than autolytic activity. In autolysis, enzymes were associated with muscle proteins and in the cell matrix, which tended to minimize structural and conformational changes caused by heat and high ionic strength environment. For this reason, proteinases activities were less affected by high salt content in the autolysis study. High salt content (25-30% NaCl) used in fish sauce fermentation is another factor limiting protein hydrolysis. Our results showed that the remaining autolytic activity of Indian anchovy at 10% NaCl was about 90% (Figure 3.2), thus lowering salt content would be a means to increase the rate of protein hydrolysis.

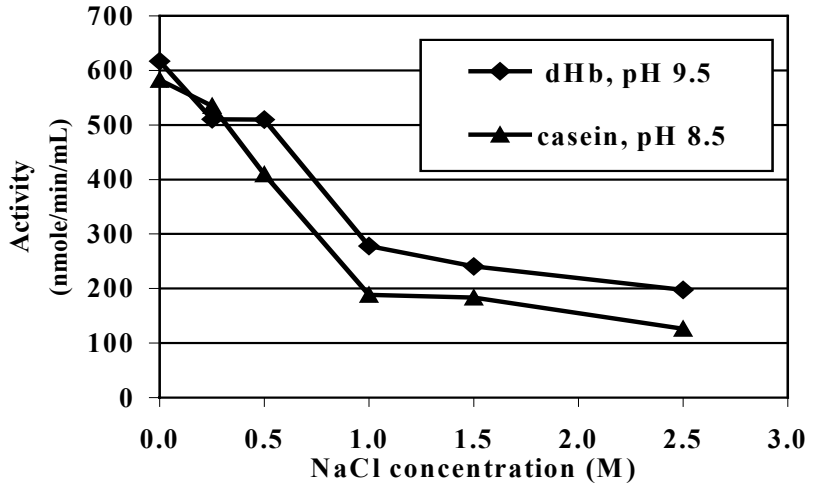

Figure 3.6-Effect of NaCl on proteolytic activity of crude extracts assayed at 60 ^{o}C and either pH 8.5 for casein or pH 9.5 for dHb.

3.3.4 Synthetic substrate specificity

Boc-Asp(oBzl)-Pro-Arg-AMC, a substrate for α-thrombin and trypsin-like proteinase, was strongly hydrolyzed by the crude proteinase from Indian anchovy (Table 3.1). Boc-Val-Leu-Lys-AMC and Boc-Gln-Ala-Arg-AMC, which are substrates for plasmin and trypsin, respectively, were slightly hydrolyzed (Table 3.1). In contrast, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC, which are substrates for chymotrypsin, cathepsin B, and cathepsin L, respectively, were hardly hydrolyzed. Barrett, Rawlings and Woessner (1998) reported that trypsin and trypsin-like enzymes prefer to cleave amide substrate contained Arg and Lys at P_1 position and Pro at P_2 position. Thus, predominant proteinases in the crude extract from Indian anchovy showed a trypsin-like characteristic based on synthetic substrate studies. Ishida et al. (1995) found that neutral serine proteinases type I and II from muscle of salted anchovy (*E. japonica*) exhibited the highest hydrolytic activity towards Boc-Asp(oBzl)-Pro-Arg-AMC and Boc-Gln-Ala-Arg-AMC, respectively. Trypsin-like proteinases were also purified from pyloric caeca of menhaden and rainbow trout, and hydrolyzed the synthetic substrate of trypsin (Pavlisko, Rial, and Coppes, 1999; Kristjansson 1991).

Table 3.1-Hydrolysis activity towards synthetic substrates of crude proteinases at 60°C, pH 8.5.

Substrates	Enzymes	Specific activity (units/mg protein)
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin	0
Boc-Val-Leu-Lys-AMC	Trypsin	4.94 ± 1.34
Boc-Gln-Ala-Arg-AMC	Trypsin	31.80 ± 12.67
Boc-Asp(oBzl)-Pro-Arg-AMC	Trypsin	85.92 ± 5.39
Z-Arg-Arg-AMC	Cathepsin B	0
Z-Phe-Arg-AMC	Cathepsin L	2.24 ± 1.27

3.3.5 Effect of inhibitors

General serine proteinase inhibitor (PMSF, leupeptin) and specific trypsin inhibitor (SBTI and TLCK) effectively inhibited crude proteinases, while E-64, bestatin, EDTA and TPCK showed a slight inhibition (Table 3.2). These results confirmed that predominant proteinases exhibited trypsin characteristics. Typically, trypsin requires moderate $CaCl_2$ concentration (20-50 mM) for activation and conformational stabilization (Barrett et al., 1998). However, Ca^{2+} at concentration up to 100 mM had no effect on proteolytic activity of Indain anchovy (Figure 3.7). It could be speculated that the predominant proteinase was unlikely to be trypsin. Ca^{2+} is required for stability of trypsin, auto-degradation quickly occurs in the absence. Trypsin extracted from pyloric caeca of rainbow trout required Ca^{2+} for stabilization (Kristjansson, 1991). Crude proteinases could play thermal stability without Ca^{2+} (Figure 3.5). Additionally, molecular weight of trypsin is approximately around 25 kDa (Barrett et al., 1998) but predominant proteinases in crude extract exhibited a

molecular weight >30 kDa. Therefore, proteinases in Indian anchovy crude extract appeared to be trypsin-like rather than trypsin.

Table 3.2-Effect of inhibitors on crude proteinases activity

Inhibitors	Final concentration	inhibition (%)
Control	-	0
SBTI	0.023 mg/mL	99.3
TLCK	100 μM	77.7
TPCK	100 μM	3.3
PMSF	1 mM	76.8
Leupeptin	100 μM	97.8
E-64	10 μM	15.8
Pepstatin A	10 μM	0
Bestatin	10 μM	5.1
EDTA	10 mM	27.8

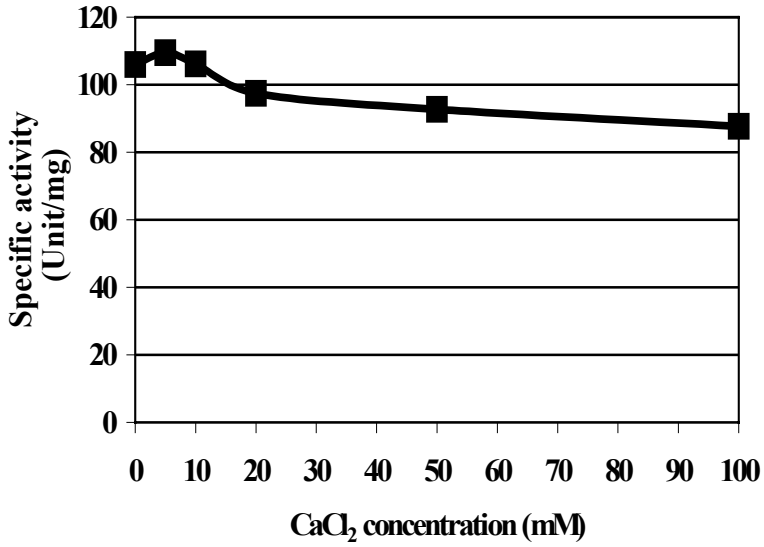

Figure 3.7-Effect of $CaCl_2$ on crude proteinases activity determined at 60°C and pH 8.5.

3.3.6 Activity staining

Molecular weight (MW) of crude proteinases from Indian anchovy was estimated to be 31, 40, 45, 53, and 63 kDa based on activity staining gel electrophoresis (Figure 3.8a, b: lane 1 and 2). Protein bands with caseinolytic activity in the absence of NaCl (Figure 3.8a) was more evident than those observed in the presence of 4.0 M NaCl (Figure 3.8b). Proteolytic activity observed at 4.0 M NaCl confirmed that proteinases in Indian anchovy could hydrolyze protein substrates at high salt content. When crude extracts were partially purified by heat treatment at 60 ^{o}C for 10 min and subsequent precipitation with 30-60% ammonium sulfate (Figure 3.8a, b: lane 3 and 4), more clear bands were observed. The MW of these clear bands was estimated to be 18, 20, 25, 28, 31, 35, 40, 45, 53, 63, 80 and 94 kDa in the absence of NaCl. In the presence of 4.0 M NaCl, protein bands with MW <30 kDa and those with >80 kDa did not show caseinolytic activity. These results indicated that proteinases with MW of 31, 35, 40, 45, 53 and 63 kDa could hydrolyze casein at high salt content. It was likely that these proteinases might play an important role in protein hydrolysis during fish sauce fermentation. Our study was the first to report the activity of proteinases at high salt content (4.0 M NaCl) in Indian anchovy. It should be noted that 2-merceptoethanol (BME) did not affect caseinolytic activity as the pattern of clear bands in the presence of BME was similar to that without BME (Figure 3.8). These results implied that proteinases with caseinolytic activity contained no subunit linked by disulfide bonds. Neutral serine proteinases extracted from salted anchovy had MW about 25 and 37 kDa (Ishida et al., 1995). Since MW of trypsin purified from various fish species was 24-29 kDa (Heu et al., 1995; Martinez et al., 1988; Kristjansson, 1991; and Pavlisko et al., 1999), it was likely that

proteinases exhibiting casinolytic activity at high salt content were trypsin-like proteinases.

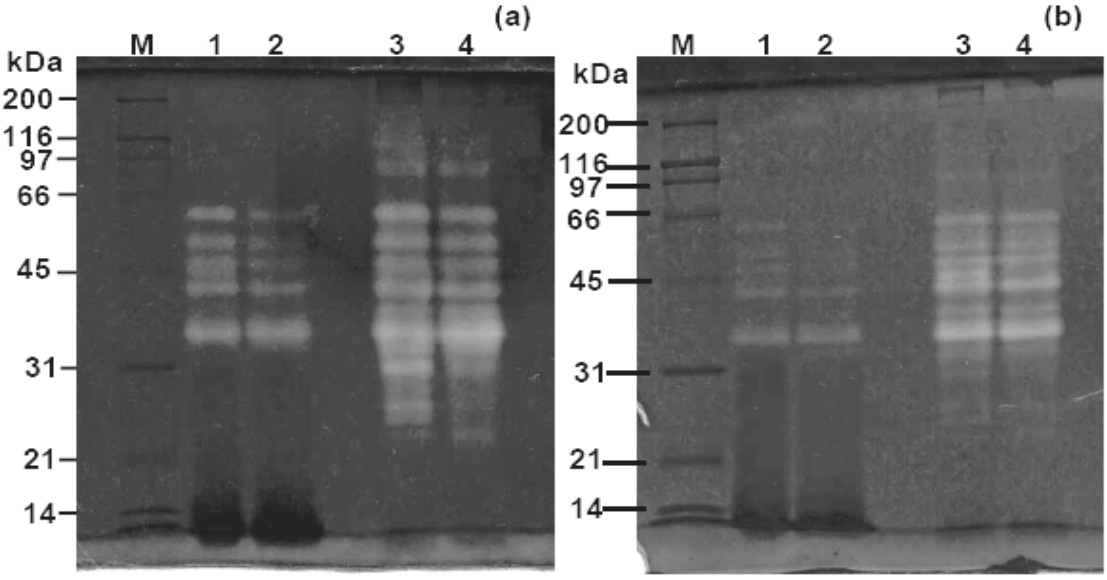

Figure 3.8-Activity staining (SDS- PAGE, 10% acrylamide) of crude proteinases in Indian anchovy incubated at 60 ^{o}C, pH 8.5 in the absence (a), and presence of 4.0 M NaCl (b). Loaded protein content was 30 μg. M: molecular weight marker; lane 1 and lane 2: crude extract; lane 3 and 4: partially purified proteinases by heat treatment and precipitation with 30-60% ammonium sulfate. Lane 1,3 and lane 2,4 were samples without and with 2-mercaptoethanol, respectively.

3.4 Conclusions

Autolytic activity of Indian anchovy showed the optimal temperature at 60 ^{o}C. Crude extract exhibited an optimum pH at 8.5-9.5. Trypsin-like proteinases were the predominant proteinase in the crude extract. Several proteinases with different molecular weight were observed in activity staining at 4.0 M NaCl, suggesting that proteinases from Indian anchovy could participate in protein hydrolysis during fish sauce fermentation.

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CHAPTER IV

SOURCE OF PROTEOLYTIC ENZYMES IN INDIAN ANCHOVY (*Stolephorus* spp.) AND CHANGES OF PROTEOLYTIC ACTIVIES DURING FISH SAUCE FERMENTATION

Abstract

Trypsin-like proteinase and trypsin exhibited the highest activity in viscera of Indian anchovy (*Stolephorus* spp.) while only activity of trypsin-like proteinases was predominant in the muscle. Molecular weight (MW) of proteinases in viscera was estimated to be 31, 35, 44, 49, and 57 kDa by activity staining in the presence of 4.0 M NaCl. MW of proteinase extracted from muscle was 56 kDa. Trypsin-like, trypsin, chymotrypsin, and cathepsin L were found in commercial fish sauce samples throughout 12 months of fermentation. In contrast, leucine-aminopeptidase activity was detected only at the first month. MW of proteinases found in fish sauce at various fermentation periods was estimated to be 37, 47 and 53 kDa based on activity staining at 4.0 M NaCl.

Keywords: Proteolytic enzyme, Indian anchovy, fish sauce, viscera, muscle.

4.1 Introduction

Fish sauce is a popular condiment in Southeast Asia and gradually gains popularity worldwide. Fish sauce is produced by mixing 25-30% salt with small fish and fermented for 8-12 months. Indian anchovy (*Stolephorus* spp.), a major raw material for fish sauce production, is susceptible to proteolytic degradation similar to anchovy (*Engraulis encrasicholus*) (Martinez and Gildberg, 1988). Important proteinases found in viscera included pepsin, trypsin, chymotrypsin, carboxypeptidase and aminopeptidase, whereas those found in muscle tissue were cathepsins, calpain, neutral trypsin-like and alkaline proteinases (Haard, 1994). Thus, many proteolytic enzymes were presented in fish.

Proteolytic enzymes that are prevalent during fish sauce fermentation have been investigated. Orejana and Liston (1982) reported that trypsin-like proteinase was found during the first month of fish sauce (patis) fermentation made from anchovy (*Stolephorus* spp.). Activity of aminopeptidase was found during the first two months of fish sauce fermentation prepared from sardine (Vo Van, Kusakabe, and Murakumi, 1984). Alkaline proteinase was stable for three months after fermentation of sardine fish sauce (Noda, Vo Van, Kusukabe, and Murakami, 1982). Cathepsin B and cathepsin L were found during the first month of fish sauce made from Pacific whiting (Tungkawachara, Park, and Choi, 2003). Yatsunami and Takenaka (2000) reported activities of trypsin-like, chymotrypsin-like, and aminopeptidase-like in the brine fraction of the fermented sardine (*Etrumeus micropus*) with rice bran. Moreover, trypsin, chymotrypsin, cathepsin L and aminopeptidase were typically found in anchovy (*E. japonica* and *E. encrasicholus*) (Martinez and Serra, 1989; Heu, Kim, and Pyeun, 1995; Heu, Kim, Cho, Godber, and Pyeun, 1997). Our previous

study demonstrated that trypsin-like proteinases extracted from the whole Indian anchovy appeared to play an important role in protein hydrolysis at high salt content (25%) (Siringan, Raksakulthai, and Yongsawatdigul, 2005). However, source of these enzymes has not been identified. In addition, proteolytic activity changes during fish sauce fermentation have not been studied. Changes of proteolytic activity during fish sauce fermentation may lead to a better understanding of protein hydrolysis. Therefore, the objective of this study was to investigate proteolytic activities in viscera and muscle of Indian anchovy. In addition, changes of various proteolytic activities, namely trypsin-like, trypsin, chymotrypsin, cathepsin L, and aminopeptidase, during fermentation were elucidated.

4.2 Materials and Methods

4.2.1 Materials and chemicals

Indian anchovies (*Stolephorus* spp.) were caught off the Gulf of Thailand, Chonburi province and transported to the laboratory at Suranaree University of Technology within 6 h after catch. Upon arrival, viscera and muscle were separated from the whole fish and kept at –20 ^{o}C. Average fish weight was 2.61 ± 0.47 g with the length of 7.59 ± 0.34 cm. Fish sauce samples at various fermentation time of 1-12 months were obtained from three different plants in Rayong province, Thailand. Chemicals purchased from Sigma Chemical Co. (St. Louis, MO, USA) were bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Brij 35, butyloxycarbonyl (Boc)-Gln-Ala-Arg-7-amido-4-methylcoumarin (AMC), Boc-Val-Leu-Lys-AMC, and carbobenzoxy (Z)-Arg-Arg-AMC, 4-nitroaniline, leucyl-4-nitroaniline, 2-mercaptoethanol. Acrylamide and Tris-base were purchased from Promega

Corporation (Madison, WI, USA). Standard molecular weight broad range and Bis (N, N'-methylane-bis-acrylamide) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Boc-Asp(oBzl)-Pro-Arg-AMC, succinyl (Suc)-Ala-Ala-Pro-Phe-AMC and Z-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). All other chemicals were of analytical grade.

4.2.2 Crude extract from whole, viscera and muscle of Indian anchovy

Twenty grams of viscera, muscle, and whole fish were homogenized with ice-cold 20 mM phosphate buffer, pH 7.0 at a ratio 1:2. The homogenate was centrifuged at 17500 ×g for 20 min at 4 ^{o}C (An, Seymour, Wu, and Morrissey, 1994). The supernatant was collected and filtered through four layers of cheesecloth and Whatman paper No. 4 and used as crude extracts.

4.2.3 Preparation of fish sauce samples

The collected fish sauces samples at various fermentation times (1, 3, 5, 7, 9, and 12 month) were filtered through Whatman paper No. 1. Subsequently, these samples were dialyzed against 100 volumes of 20 mM phosphate buffer, pH 7 for 24 h at 4 ^{o}C using SnakeSkin™ pleated dialysis tubing with molecular weight cutoff (MWCO) 10000 (Pierce Chemical Company, Rockford, IL, USA). Dialysates were concentrated using Centricon® YM10 (Millipore Corporation, Bedford, MA, USA), and used for determination of proteolytic activity.

4.2.4 Proteolytic activity at 60 ^{o}C and pH 8.5

Proteolytic activities of the crude extract from whole fish, muscle, and viscera were determined using Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Boc-Asp(oBzl)-Pro-Arg-AMC Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC according to the method of Barrett and Kirschke (1981) and Ishida, Sugiyama, Sato and Nagayama (1995). Proteolytic activities of viscera, muscle, and fish sauce samples towards various synthetic substrates were determined at 60 ^{o}C and pH 8.5. All synthetic substrates were prepared at either 1 or 10 mM in DMSO. The reaction mixture containing 0.8 mL of 0.2 M Tris-HCl (pH 8.5), 0.1 mL of 10 μM of substrate solution, and 0.1 mL of diluted crude extracts with 0.1% Brij 35 in a total of 1 mL, were incubated at 60 ^{o}C for 5 min. The reaction was added 1.5 mL of the stopping reagent (methanol: n-butanol: distilled deionized water = 35:30:35 (v/v/v)) followed by heating at 95 ^{o}C for 3 min. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured by a spectrofluorophotometer RF-1501 (Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Unit activity was defined as one μmole of AMC released/min. Specific activity was expressed as the amount of unit activity per mg protein of crude extract.

4.2.5 Proteolytic activities in crude extract and fish sauce

Trypsin activity was followed according to the method of Ishida et al. (1995) and Martinez and Serra (1989). The reaction mixture contained 10 μM Boc-Asp (oBzl)-Pro-Arg-AMC as a substrate, 0.05 M Tris-HCl containing 20 mM $CaCl_2$, pH

8.2 and diluted crude extract in a total volume of 1 mL. Reaction was carried out at 37 ^{o}C for 5 min.

Chymotrypsin activity was followed according to the method of Ishida et al. (1995) and Martinez and Serra (1989). The reaction mixture containing 0.1 mL of 10 μM Suc-Ala-Ala-Pro-Phe-AMC, 0.8 mL of 0.1 M Tris-HCl containing 100 mM $CaCl_2$, pH 7.8 and 0.1 mL of diluted crude extract in a total volume of 1 mL was incubated at 37 ^{o}C for 5 min.

Cathepsin L activity was determined according to the method of Barrett and Kirschke (1981). The reaction mixture containing 0.1 mL of 10 μM Z-Phe-Arg-AMC as a substrate, 0.8 mL of 0.34 M sodium acetate, 4 mM EDTA, 8 mM dithiothreitol (DTT), pH 5.5 and 0.1 mL of crude extract in a total volume of 1 mL was incubated at 37 ^{o}C for 5 min.

Cathepsin B activity was determined according to the method of Barrett and Kirschke (1981). The reaction mixture containing 0.1 mL of 10 μM Z-Arg-Arg-AMC, 0.8 mL of 88 mM KH_2PO_4, 12 mM Na_2HPO_4, 1.33 mM EDTA, 8 mM DTT, pH 6.0 and 0.1 mL of diluted crude extract in a total volume of 1 mL was incubated at 37 ^{o}C for 5 min.

Leu-aminopeptidase (LAP) activity was determined at 50 ^{o}C using 20 mM L-leucyl-4-nitroaniline (4-NA) as the substrate. Reaction mixture contained 0.1 mL of substrate solution, 0.85 mL of 0.2 M Tris-HCl, pH 8.0, and 0.05 mL of enzyme extract. The reaction was stopped by adding 0.5 mL of 80% acetic acid after incubated for 30 min. The release of nitroaniline was measured at 405 nm (Hebert, Raya, and De Giori, 2000). One unit of LAP activity was defined as the amount of enzyme hydrolyzed 1 nmole of 4-nitroaniline/min.

4.2.6 Activity staining

Activity staining was determined according to method of Garcia-Carreno, Dimes, and Haard (1993). Crude proteinases from fish and fish sauce samples (1, 3, 5, 7, 9, and 12 months) were separated on 12.5% and 10% polyacrylamide gel, respectively (Laemmli 1970). Subsequently, gel was immerged in 2% casein, 50 mM Tris-HCl (pH 7.5) for 30 min on ice. Caseinolytic activity was initiated by incubating gels in 0.2 M Tris-HCl (pH 8.5) buffer containing 4.0 M NaCl, and incubated at 60 ^{o}C for 15-30 min. Subsequently, gels were stained in 0.125% coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 2 h. Destaining was carried out using 25% methanol and 10% acetic acid solution. Clear zone on the blue background indicated the presence of proteinase.

4.3 Results and discussion

4.3.1 Proteolytic activity towards synthetic substrates at 60 ^{o}C and pH 8.5

From our previous study, crude extract from whole Indian anchovy exhibited the highest activity at 60 ^{o}C and pH 8.5 (Siringan et al., 2005). Thus, this condition was used to determine proteolytic activity of crude proteinases from viscera and muscle. Boc-Asp(oBzl)-Pro-Arg-AMC was mostly hydrolyzed by the crude extract prepared from viscera and whole fish (Table 4.1). The crude extract from muscle strongly hydrolyzed Boc-Gln-Ala-Arg-AMC and Boc-Asp(oBzl)-Pro-Arg-AMC. These results indicated that the main source of proteinases was mainly derived from viscera. Martinez and Gildberg (1988) also suggested that viscera was an important source of proteinases responsible for autolytic activity of small pelagic fish. In addition, Yatsunami and Takenaka (2000) reported that proteinase in brine of sardine

fermentation with rice bran showed similar biochemical characteristics to proteinase in viscera. Proteinases in fish sauce could hydrolyze synthetic substrates containing Arg at P_1 position similar to those obtained from whole Indian anchovy (Table 4.1). These results implied that trypsin-like proteinases in crude anchovy were stable at high salt content (25% NaCl) and remained in fish sauce at least up to 1 month.

Table 4.1-Proteolytic activities towards synthetic substrates of crude anchovy extracts from various sources

Substrates	Activity (Units/mg protein)			Activity (Unit/mL)
	Whole	Viscera	Muscle	Fish sauce *
Boc-Asp(oBzl)-Pro-Arg-AMC	68.46 ± 0.66	102.51 ± 0.66	14.86 ± 0.39	888.07 ± 46.89
Boc-Val-Leu-Lys-AMC	4.01 ± 1.65	9.25 ± 1.65	2.62 ± 0.28	105.17 ± 7.16
Boc-Gln-Ala-Arg-AMC	21.86 ± 10.20	21.68 ± 0.12	17.21 ± 8.69	265.18 ± 84.09
Suc-Ala-Ala-Pro-Phe-AMC	0	3.02 ± 1.56	2.99 ± 1.91	57.90 ± 20.49
Z-Phe-Arg-AMC	1.28 ± 1.08	7.96 ± 0.81	2.30 ± 0.27	169.58 ± 11.12
Z-Arg-Arg-AMC	0	2.75 ± 0.05	1.08 ± 0.01	68.87 ± 0.83

* Fish sauce sample was fermented for 1 month.

4.3.2 Proteolytic activities in crude extract from Indian anchovy

Trypsin-like proteinases exhibited the highest activity in the whole fish, viscera and muscle extract (Table 4.2). Moreover, trypsin showed higher activity than chymotrypsin and cathepsin L in all samples. Leucine-aminopeptidase (LAP) was mainly found in viscera. Predominant proteinases derived from anchovy viscera included trypsin-like, trypsin, and LAP activity. Martinez and Serra (1989) showed that high level of trypsin, chymotrypsin, elastase, and aminopeptidase activities were found in pyloric caeca and intestine of anchovy (*E. encrasicholus*). Furthermore, Heu

et al. (1995) purified trypsin and chymotrypsin from viscera of anchovy (*E. japonica*), which might contribute to protein hydrolysis. However, Choi, Heu, Kim, and Pyeun (2004) reported that chymotrypsin and cathepsin L-like induced higher degree of hydrolysis in myofibrillar proteins of anchovy (*E. japonica*) than did trypsin. Although, cathepsin L activities are typically found in fish muscle, its activity was insignificant in Indian anchovy. These results confirmed that major proteinases in whole fish were mainly derived from viscera. It was in agreement with Martinez and Gildberg (1988) who reported that belly burst of anchovy was caused by proteolytic degradation of abdominal tissue by the leakage of proteolytic enzymes from digestive tract. Two trypsin-like proteinases purified from digestive tract of anchovy (*E. encrasicholus*) that contributed to myofibrillar protein degradation (Martinez, Olsen, and Serra, 1988).

Table 4.2-Various proteolytic activities of crude extract from whole fish, viscera, and muscle of Indain anchovy

Enzymes	Activity (Units/mg protein)		
	Whole	Viscera	Muscle
Typsin-like proteinases*	57.77 ± 0.88	66.99 ± 4.13	14.06 ± 0.65
Trypsin*	21.61 ± 0.42	30.53 ± 7.30	4.43 ± 1.15
Chymotrypin*	0.05 ± 0.01	1.19 ± 0.26	0.94 ± 0.08
Cathepsin L*	0.09 ± 0.01	1.39 ± 0.19	0.89 ± 0. 03
Leu-aminopeptidase (LAP)**	4.56 ± 0.38	6.67 ± 0.14	2.07 ± 0.08

*Unit activity was defined as 1 μmole of AMC released/min
** Unit activity was defined as 1 nmole of 4-nitroaniline liberated/min

Proteinases in muscle might also play an important role in proteolysis during fish sauce fermentation. Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguoz-Jerez, and Mora-Ventura (1999) reported that alkaline proteinase (pH 9.0) was predominantly found in anchovy muscle (*E. encrasicholus*) and brine of salted anchovies. Ishida et al. (1995) reported that two neutral serine proteinases, which involved muscle degradation during ripening of salted anchovy, were found in muscle of anchovy (*E. japonica*). However, these results indicated that muscle proteinases exhibited lower activity than viscera proteinases.

4.3.3 Proteolytic activities in fish sauce

Fish sauce obtained from three different plants contained high activity of trypsin-like and trypsin (Figure 4.1a). These results indicated that trypsin-like proteinases exhibited stability at high salt content (25-30% NaCl) for up to 12 months. Orejana and Liston (1982) reported that trypsin-like activity in fish sauce decreased at the fifth month of fermentation. However, our study demonstrated that trypsin-like activity remained up to 12 months of fermentation (Figure 4.1a). Therefore, trypsin-like proteinases in Indian anchovy might contribute to protein hydrolysis during fish sauce fermentation.

Activity of cathepsin L gradually decreased as fermentation progressed, whereas chymotrypsin activity was low throughout 12 months (Figure 4.1b). However, cathepsin L showed higher activity than chymotrypsin, but lower than trypsin-like proteinases activity. Cathepsin L is lysosomal proteinase that was involved was muscle degradation of various fish species, such as Pacific whiting, mackerel etc. (An, Weerasinghe, Seymour, and Morrissey, 1994; Aoki and Ueno,

1997). Tungkawachara et al. (2003) reported that cathepsin L exhibited high activity in fish sauce prepared from Pacific whiting at first month of fermentation. However, cathepsin L did not appeared to be a predominant proteinase in either muscle or viscera of Indian anchovy. It should be noted that cathepsin L activity was not detected in some samples reflecting its variation of proteinase activity. Additionally, all proteinases activities in fish sauce samples from three different plants were varied of proteolytic activities among plants. This might be due to different raw material used in fish sauce fermentation.

Aminopeptidases play an important role in contributing to free amino acids in fish sauce. LAP activity decreased with fermentation time (Figure 4.1c). LAP exhibited the highest activity at the first month and decreased afterward. This was in agreement with the changes of aminopeptidase activity in sardine fish sauce (Vo Van et al., 1984). Thus, aminopeptidase displayed an activity only at the initial stage of fish sauces fermentation. LAP activity might be inhibited by the end products, such as amino acids and small peptides (Orejana and Liston, 1982).

4.3.4 Activity staining

According to activity staining at 4.0 M NaCl, molecular weight (MW) of proteinases in viscera extract was estimated to be 31, 35, 44, 49, and 57 kDa similar to those of whole extract, while proteinase found in muscle showed MW of 56 kDa (Figure 4.2). These results indicated that several proteinases in viscera of Indian anchovy could participate in protein hydrolysis at high salts content. Proteinases extracted from muscle showed less activity at 4.0 M NaCl.

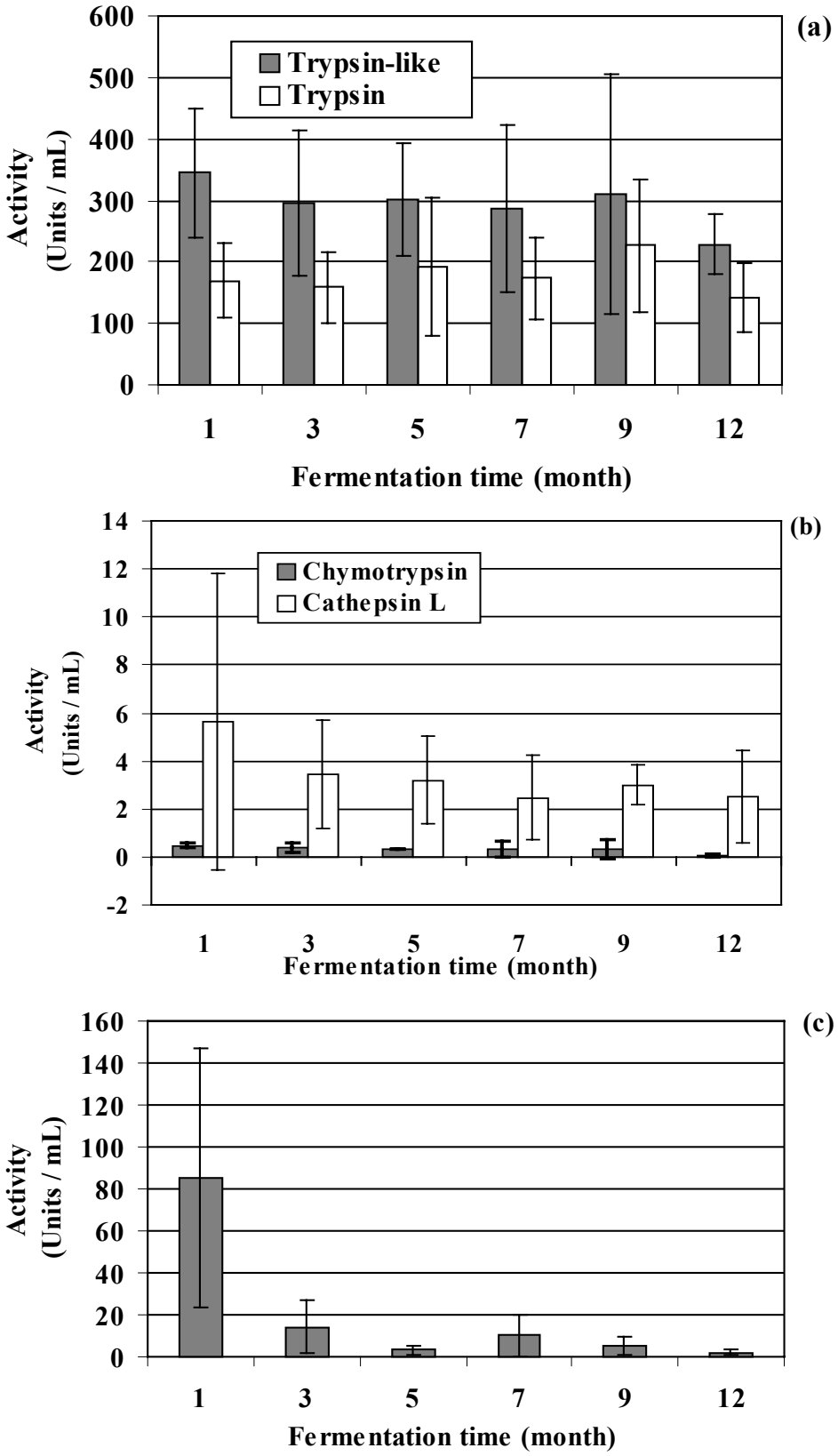

Figure 4.1-Proteolytic activities of commercial fish sauce samples at various fermentation periods: trypsin-like proteinase and trypsin (a); chymotrypsin and cathepsin L (b); and LAP (c).

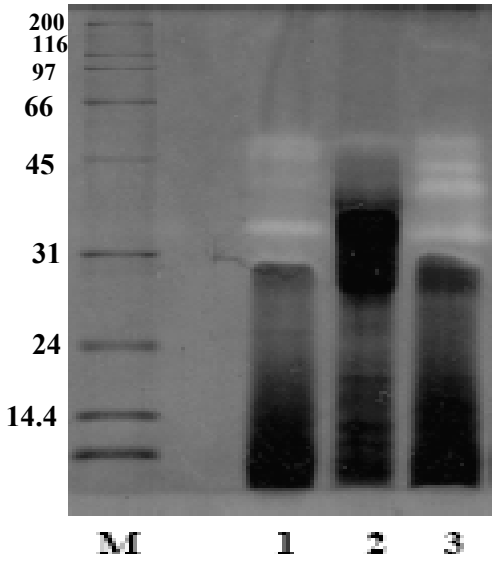

Figure 4.2-Activity staining (SDS-PAGE 12.5% acrylamide) of crude extract from whole fish, viscera, and muscle of Indian anchovy, incubated at 60^{o}C for 30 min, 0.2 M Tris-HCl pH 8.5 containing 4.0 M NaCl. M: standard marker; Lane 1: whole fish extract; 2: muscle extract; 3: viscera extract. Loaded protein was 35 μg.

MW of proteinases found in fish sauce at 1, 3, 5, 7, 9, and 12 months was estimated to be 37, 47, and 53 kDa (Figure 4.3). While partially purified proteinases from whole Indain anchovy showed MW of 30, 33, 37, 44, 47, 49, 54, and 68 kDa. Three predominant proteinases found in fish sauce exhibited the same MW to those partially purified proteinases based on activity staining at 4.0 M NaCl. It was likely that proteinases from anchovy were responsible for protein hydrolysis during fish sauce fermentation. Gildberg and Shi (1994) reported that trypsin, chymotrypsin, and elastase was recovered from fish sauce made from cod viscera. Another source of proteinases was from *Halobacterium* sp., which was found in solar salts and raw material (Norberg and Hofsten, 1967). Biochemical characteristics of halophilic

proteinase have been studied. Stepanov, Rudenskaya, Revina, Gryznova, Lysogorskaya, Filippova, and Ivanova (1992) reported that halophilic proteinase from *Halobacterium mediterranei* exhibited optimal pH and temperature at 8-8.5 and 55 ^{o}C, respectively with MW of 41 kDa. MW of halophilic proteinase from *H. halobium* was estimated to be 66 kDa based on SDS-PAGE (Ryu, Kim, and Dordick, 1994). Izotava, Strongin, Chekulaeva, Sterkin, Dsfoslavskaya, Lyublinskaya, Timokhina, and Stepanov (1983) reported that halophilic proteinase produced by *H. halobium* showed MW of 56 kDa based on SDS-PAGE and pH optimum of 8-9. Some halophilic proteinase was stable and active at high salt content but these enzymes were rapidly inactivated below 2.0 M NaCl. However, proteinase activity from halophilic bacteria was likely to be diminished when NaCl concentration was below 4.0 M due to conformational changes at low salt (Ryu et al., 1994). In our study, crude extract from fish sauce was obtained by dialysis to eliminate NaCl. Thus, three proteinases observed in fish sauce might not be related to those produced by halophilic bacteria. In addition, extracellular proteinases produced from non-halobacteria were not stable at high salt content. Our results indicated that endogenous proteinases in Indian anchovy were stable at high salt content. Moreover, smear band around 200 kDa on the top of the gel was observed in fish sauce samples at 1, 5, and 7 months but not in the partially purified proteinases. Ohkubo, Miyagaya, Osatomi, Hara, Nozaki, and Ishihara (2004) reported that serine proteinase from skeletal muscle of lizardfish had a MW of 230 kDa based on gel filtration and consisted of two subunits with 110 and 100 kDa under reducing condition based on SDS-PAGE. Therefore, it was hypothesized that higher molecular proteinase might be endogenous proteinase in muscle.

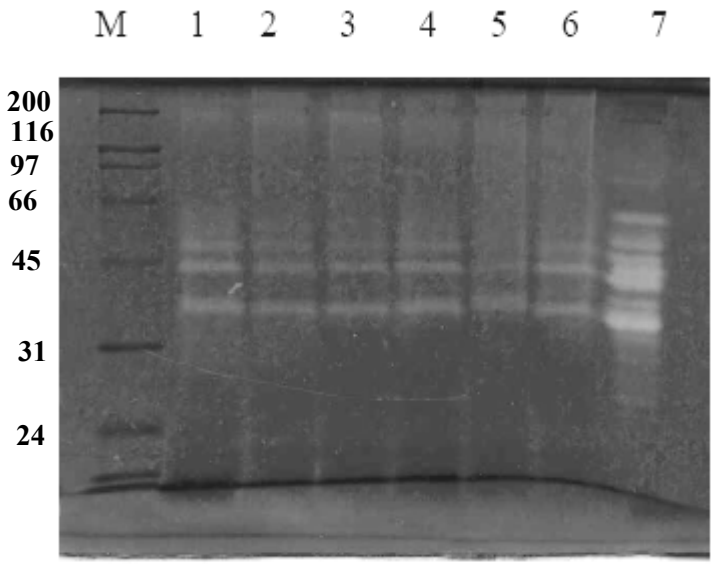

Figure 4.3-Activity staining (SDS-PAGE 10% acrylamide) of proteinases found in fish sauce (1-12 mo.) incubated at 60 ^{o}C, pH 8.5 in the presence of 4.0 M NaCl for 15 min. M: Molecular weight marker; lane 1-6: fish sauce fermented at 1, 3, 5, 7, 9, and 12 mo., respectively; lane 7: partially purified proteinase by heat treatment at 60 ^{o}C for 10 min and 30-60% ammonium sulfate precipitation. Loaded protein was 15 μg.

4. 4 Conclusions

Trypsin-like proteinases, which were mainly found in whole fish extract, were derived from viscera of Indian anchovy. These enzymes were also observed in fish sauce throughout 12 months of fermentation. MW of proteinases in fish sauce was estimated to be 40, 51, and 58 kDa similar to that of partially purified proteinases based on activity staining at the optimum condition of crude extract at 4.0 M NaCl. These results indicated that endogenous proteinases in Indian anchovy could participate in protein hydrolysis during fish sauce fermentation. Thus, activity of trypsin-like proteinases might contribute to protein hydrolysis at high salt content.

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CHAPTER V

PATIAL PURIFICATION AND CHARACTERIZATION OF TRYPSIN-LIKE PROTEINASES IN INDIAN ANCHOVY (*Stolephorus* spp.)

Abstract

Four fractions (P111, P21, P31, and P4) of proteinases were obtained from various purification steps including heat treatment (60 ^{o}C, 10 min), 30-60% ammonium sulfate precipitation, anion exchange, hydrophobic interaction, and gel filtration chromatography. Optimal temperature and pH of all fractions were 50-60 ^{o}C and 8.5, respectively. All partially purified proteinases preferably hydrolyzed substrates containing Arg at P_1 position. All proteinases were inhibited by soybean trypsin inhibtor, leupeptin, and *N*-tosyl-L-lysine chloromethyl ketone. All fractions were Ca^{2+}independent except for P111. Partially purified proteinases were stable at 35 ^{o}C up to 12 h. However, their activity decreased about 40% when incubated at the optimal temperature (50-55 ^{o}C) for 2 h. Only P111 was stable at its optimal temperature (60 ^{o}C) up to 12 h. Molecular weight (MW) of P111, P21, and P31 was estimated to be 27, 33, 37, 43, 48, 55, 60, and 65 kDa, while MW of P4 was 39 kDa based on activity staining. All partially purified proteinases hydrolyzed washed anchovy mince at 4.0 M NaCl, pH8.5 at 35 ^{o}C and at their optimal temperatures (50-60 ^{o}C).

Keyword: Indian anchovy (*Stolephorus* spp.), trypsin-like proteinase, partial purification, and characterization

5.1 Introduction

Indian anchovy (*Stolephorus* spp.) is a pelagic small fish, which contain high proteinase activity and popularly used as a raw material for fish sauce production in Southeast Asia. Fish sauce is a traditional condiment prepared by mixing anchovies with 25-30% salt and incubated at ambient temperature (30-40 ^{o}C). Protein solubilization occurred during fermentation by the action of proteolytic activity to produce amino acids and small peptides. Proteinases involved in muscle degradation have been studied. Martinez and Gildberg (1988) reported that proteolytic enzymes from digestive tract of anchovy (*Engraulis encrasicholus*) degraded abdominal tissue. Heu, Pyeun, Kim, and Godber (1991) reported that two alkaline proteinases isolated from viscera of anchovy (*E. japonica*) were identified to be chymotrypsin-like serine proteinases. Two trypsin-like enzymes isolated from digestive tract of anchovy were important for muscle degradation (Martinez, Olsen, and Serra, 1988). Two neutral serine proteinases, which involved muscle degradation during ripening of salted anchovy (*E. japonica*), were purified (Ishida, Sugiyama, Sato, and Nagaya, 1995). However, proteinases from Indian anchovy have never been purified and characterized.

Trypsin-like proteinase was observed in the first two months of fish sauce (patis) fermentation (Orejana and Liston, 1982). Proteinases responsible for protein hydrolysis during fish sauce fermentation might be active even at high NaCl concentration. In our previous study, trypsin-like proteinases were predominantly

found in Indian anchovy (*Stolephorus* spp.) and also hydrolyzed muscle proteins at high salt content (4.0 M NaCl) (Siringan, Raksakulthai, and Yongsawatdigul, 2005). Thus, these proteinases might play an important role in protein hydrolysis during fish sauce fermentation. Furthermore, activities of these enzymes were also found in fish sauce throughout 12 months of fermentation. Thus, the objective of this study was to partially purify and characterize trypsin-like proteinases in Indian anchovy.

5.2 Materials and Methods

5.2.1 Materials and chemicals

Indian anchovies (*Stolephorus* spp.) were caught off the Gulf of Thailand, Chonburi province and subsequently transported to the laboratory at Suranaree University of Technology within 6 h after catch. The average fish weight were 2.61 ± 0.47 g with the length of 7.59 ± 0.34 cm. Chemicals purchased from Sigma Chemical Co. (St. Louis, MO, USA) were bovine serum albumin (BSA), L-tyrosine, dimethyl sulfoxide (DMSO), Brij 35, butyloxycarbonyl (Boc)-Gln-Ala-Arg-7-amido-4-methylcoumarin (AMC), Boc-Val-Leu-Lys-AMC, and carbobenzoxy (Z)-Arg-Arg-AMC, 2-mercaptoethanol (BME) and proteinase inhibitors. Acrylamide, ammonium persulfate and Tris-base were purchased from Promega Corporation (Madison, WI, USA). Standard molecular weight broad range, sodium dodecylsulfate (SDS), coomassie blue, and Bis (N, N'-methylane-bis-acrylamide) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Boc-Asp(oBzl)-Pro-Arg-AMC, succinyl (Suc)-Ala-Ala-Pro-Phe-AMC and Z-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). Diethylaminoethyl (DEAE) Sephacel, Sephacryl S-

300, and Phenyl Sepharose high performance were purchased from Amersham Biosciences (Uppsala, Sweden). All other chemicals were of analytical grade.

5.2.2 Purification of proteinases

Crude extract was prepared by homogenizing whole anchovy with ice-cold 20 mM phosphate buffer, pH 7.0 at a ratio of 1:2 using a homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan). The homogenate was centrifuged at 17500 × g for 20 min at 4 ^{o}C. The supernatant was filtered through four layers of cheesecloth and Whatman paper No. 4 and used as a crude extract. The crude extract was precipitated at 30-60% saturation of ammonium sulfate. The precipitates were dissolved in 50 mM Tris-HCl, pH 8.0 and subsequently dialyzed against 100 volumes of the same buffer for 24 h using SnakeSkin™ pleated dialysis tubing with 10000 molecular weight cutoff (MWCO) (Pierce Chemical Co., Rockford, IL, USA). The dialysate was centrifuged at 5000 × g for 5 min. The supernatant was loaded onto a DEAE Sephacel chromatography column (1.5 × 30 cm) equilibrated with 50 mM Tris-HCl, pH 8.0 and eluted with a linear gradient of 0-1.0 M NaCl, 50 mM Tris-HCl, pH 8.0 at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected. Fractions containing proteinase activity were pooled and applied onto a Phenyl Sepharose chromatography column (1.5 × 30 cm) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 1.5 M ammonium sulfate. After the column was washed with 3 bed volumes of 50 mM Tris-HCl, pH 8.0 containing 1.5 M ammonium sulfate, a linear gradient from 1.1 to 0 M ammonium sulfate was performed at a flow rate of 0.5 mL/min, and a fraction of 5 mL were collected. Fractions containing the proteinase activity were pooled and concentrated using 30000 MWCO ultrafiltration membrane (Vivascience A.G.,

Hannover, Germany). Concentrated sample was applied to Sephacryl S-300 column (1.5 × 80 cm) equilibrated with 50 mM Tris-HCl, pH 8.0. The elution was carried out with the same buffer at a flow rate of 0.5 mL/min. The profile of protein elution was measured at 280 nm and proteinase activities were determined as described below. Protein content was measured as described by Lowry, Rosenbrough, Farr, and Randall (1951).

5.2.3 Proteinase activity assay

Proteolytic activity was determined according to the method of Barrett and Kirschke (1981) and Ishida et al. (1995). The reaction mixture containing 0.8 mL of 0.2 M Tris-HCl (pH 8.5), 0.1 mL of 10 μM of Boc-Asp(oBzl)-Pro-Arg-AMC and 0.1 mL of diluted enzyme solution with 0.1% Brij 35 in a total of 1 mL, was incubated at 60 ^{o}C for 5 min. The reaction was stopped by adding 1.5 mL of the stopping reagent (methanol: n-butanol: distilled deionized water = 35:30:35 (v/v/v)) followed by heating at 95 ^{o}C for 3 min. Fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured with a spectrofluorophotometer RF-1501 (Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Unit activity was defined as one μmole of AMC released/min. Specific activity was expressed as the amount of unit activity per mg protein.

5.2.4 Temperature and pH optimum

Activities of partially purified proteinases were determined at various temperatures (30-65 ^{o}C) at pH 8.5. The activity was also assayed at the optimal temperature and various pHs ranging from 5.5-11: pH 5.5-7.0, using McIlvaine's

Buffer (0.1 M sodium citrate and 0.2 M sodium phosphate); pH 8.0-9.0, using 0.2 M Tris-HCl; pH 9.5-11, using 0.1 M Glycine-NaOH.

5.2.5 Substrate specificity

Various synthetic substrates, including Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Boc-Asp(oBzl)-Pro-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC, and Z-Phe-Arg-AMC were used for determining substrate specificity of partially purified proteinases. Proteolytic activity was determined at the optimal pH and temperature.

5.2.6 Thermal stability

Partially purified proteinases were incubated at 35 ^{o}C and at their respective optimal temperatures for up to 12 h. The remaining activity was determined at respective optimal temperature and pH at each time interval. Remaining activity was expressed as the percentage of activity remained after incubation at each time interval considering the activity measured in the sample without incubation as 100%.

5.2.7 Effect of proteinase inhibitors and $CaCl_2$

The effect of various inhibitors, including phenylmethylsulfonyl fluoride (PMSF), leupeptin, bestatin, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid, soybean trypsin inhibitor (SBTI), SDS, dithiothreitol (DTT) was determined. The effect of $CaCl_2$ concentration at 0-30 mM on activity was also investigated.

5.2.8 Activity staining

Molecular weight was estimated using activity staining according to the method of Garcia-Carreno, Dimes, and Haard (1993). Partially purified proteinases were separated on 10% polyacrylamide gel (Laemmli, 1970). Gels were soaked in 2% casein, 50 mM Tris-HCl (pH 7.5) and shaken for 1 h on ice. Caseinolytic activity was initiated by transferring gels to 0.2 M Tris-HCl (pH 8.5) buffer containing 4.0 M NaCl, and incubated at 60 ^{o}C for 10 min. Subsequently, gels were stained in 0.125% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 2 h. Destaining was carried out using 25% methanol and 10% acetic acid solution. Clear zone on the blue background indicated the presence of proteinase.

5.2.9 Hydrolysis of washed anchovy muscle at 4.0 M NaCl

Dorsal muscle of Indian anchovy was homogenized in 50 mM NaCl, 10 mM phosphate buffer, pH 7.0 at a ratio of meat to buffer of 1: 5. Subsequently, the homogenate was centrifuged at 10000 × g for 5 min at 4 ^{o}C. Precipitates were dissolved in the same buffer and then centrifuged at 10000 × g for 5 min, 4 ^{o}C twice. The precipitates were designated as washed mince. Washed mince (6 g.) was homogenized in 100 mL of 4.0 M NaCl in 0.2 M Tris-HCl, pH 8.5 and subsequently centrifuged at 5000 × g for 10 min at 4 ^{o}C. The supernatant was used as a substrate. Protein content of soluble washed mince protein was estimated to be 1 mg/mL by Lowry method using BSA as a standard.

Hydrolysis of washed mince solution at 4.0 M NaCl, pH 8.5 was determined. Reaction mixture contained 1.1 mL of washed mince solution and 0.1 mL of partially purified proteinases (500 Unit/mL). The mixture was incubated either at a respective

optimal temperature for 1 h or at 35 ^{o}C for 3 h. Reaction was stopped by adding 0.8 mL of 12.5% trichloroacetic acid (TCA). Oligopeptide contents were determined by Lowry method using tyrosine as a standard. Blanks were added 0.8 mL of 12.5% TCA before adding the enzyme. The control was carried out in the absence of proteinases.

Proteolytic activity of partially purified proteinases towards washed mince of anchovy was also investigated. One gram of washed mince was homogenized with 2 mL of 4.0 M NaCl in 0.2 M Tris-HCl, pH 8.5. The reaction was started by adding 0.2 mL of partially purified proteinase, equivalent to 500 Units, and incubated for 1 h. The reaction was terminated by adding 8 mL of 5% SDS and heating at 90 ^{o}C for 30 min. All samples were centrifuged at 10000 × g for 5 min to collect supernatant. Controls at both temperatures were carried out in the absence of partially purified proteinases. Degradation patterns of washed mince were evaluated using SDS-polyacylamide gel electrophoresis (PAGE).

5.3 Results and discussion

5.3.1 Purification of proteinases

Proteinases in whole Indian anchovy (*Stolephorus* spp.) were separated to four peaks by DEAE Sephacel (Figure 5.1). Each peak (P1-P3) exhibited several clear bands on SDS-PAGE activity staining (Figure 5.2). There were several proteinases in each fraction with different MW. Only one distinct clear band was observed in P4. All fractions except for P4 were collected for further purification. P1, P2, and P3 fractions were applied onto hydrophobic interaction chromatography (Figure 5.3a, b, c). Fraction P1 was separated to 2 peaks, P11 and P12 (Figure 5.3a). P11 was eluted

during washing the column, suggesting that P111 weakly interacted with hydrophobic media. Only one peak showing protolytic activity was obtained from P2 and P3, noted as P21 and P31, respectively (Figure 5.3b, 5.3c). Hydrophobic interaction chromatography (HIC) appeared to effectively separate proteinases from other protein components. The purification fold of all fractions increased after HIC (Table 5.1). P11 and P12 fractions were further applied to Sephacryl S-300 column (Figure 5.4). Only one peak (P111) with high activity was obtained from P11 (Figure 5.4a). P12 fraction was separated into three activity peaks, P121, P122, and P123 (Figure 5.4b). P121 and P122 showed similar molecular weight, optimal pH and temperature to that of P21 fraction (data not shown). P123 exhibited MW of ~80 kDa with the least activity at high salt when tested on activity staining (data not shown). Therefore, only P111, P21, P31, and P4 were collected for further characterization.

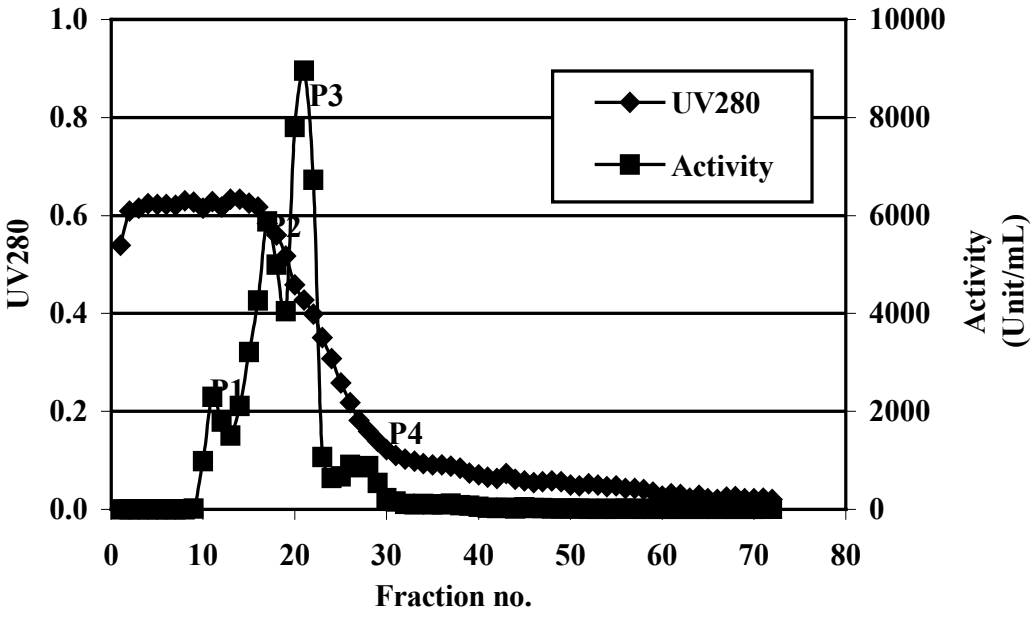

Figure 5. 1-Chromatogram of proteinases obtained from DEAE Sephacel.

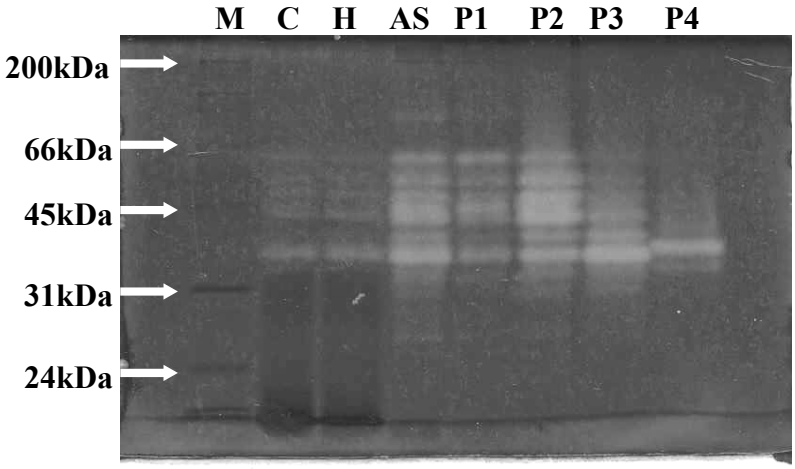

Figure 5.2-Activity staining of proteinases obtained from various purification steps and incubated at 60 ^{o}C, 4.0 M NaCl, pH 8.5. M; molecular weight marker, C: crude extraction; H: heated fraction; AS: 30-60 % ammonium sulfate precipitation; P1, P2, P3, and P4: fractions from DEAE-Sephacel, respectively. Loaded protein was 30 µg in lane 1-3, 3 µg in lane 4-6, and 1.5 µg in lane 7.

Table 5.1-Purification table of partially purified proteinase from Indian anchovy

Steps	Total activity (x 10^3 Units)	Specific Activity (x 10^3 Units/mg)	Purity (Fold)	Yield (%)
Crude extract	1107.66	0.08	1.00	100.00
Heat treatment (60 ^{o}C, 10 min)	1653.13	0.24	2.86	149.24
30-60% $(NH_4)_2SO_4$ precipitation	1261.78	2.12	25.13	113.91
DEAE Sephacel (P1)	32.79	0.44	5.21	2.96
> Phenyl Sepharose (P11)	7.23	0.33	3.91	0.65
□ Sephacryl S-300 (P111)	2.48	1.31	15.52	0.22
> Phenyl Sepharose (P12)	33.15	24.56	291.56	2.99
□ Sephacryl S-300 (P121)	0.04	0.42	4.97	0.00
□ Sephacryl S-300 (P122)	1.45	11.15	132.44	0.13
□ Sephacryl S-300 (P123)	2.00	7.59	90.14	0.18
DEAE Sephacel (P2)	125.82	5.91	70.14	11.36
> Phenyl Sepharose (P21)	253.19	187.55	2226.91	22.86
DEAE Sephacel (P3)	216.72	21.89	259.92	19.57
> Phenyl Sepharose (P31)	358.99	217.57	2583.36	32.41
DEAE sephacel 4 (P4)	53.12	15.40	182.83	4.80

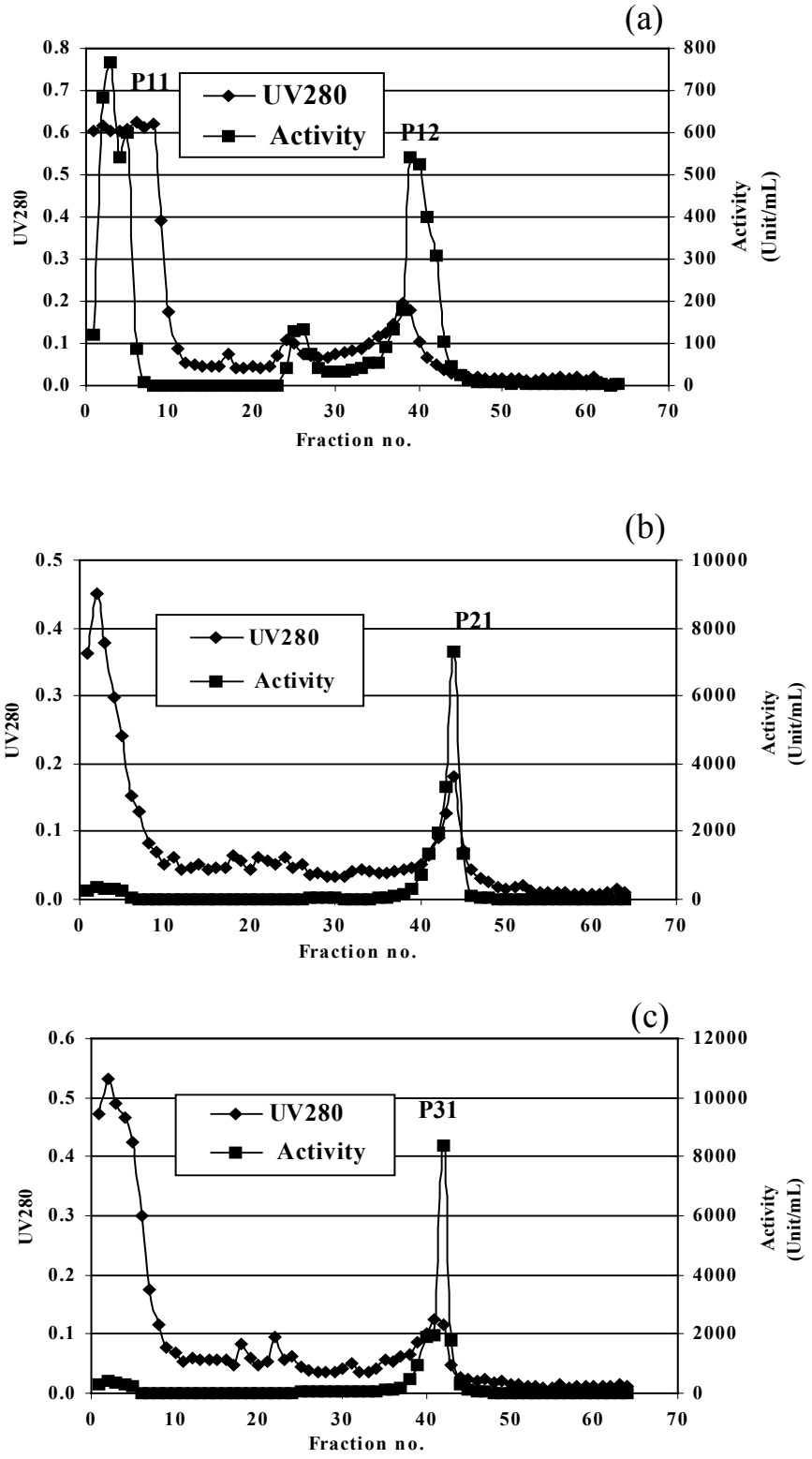

Figure 5.3-Chromatogram of proteinases P1(a), P2(b), P3(c) loaded onto Phenyl-Sepharose.

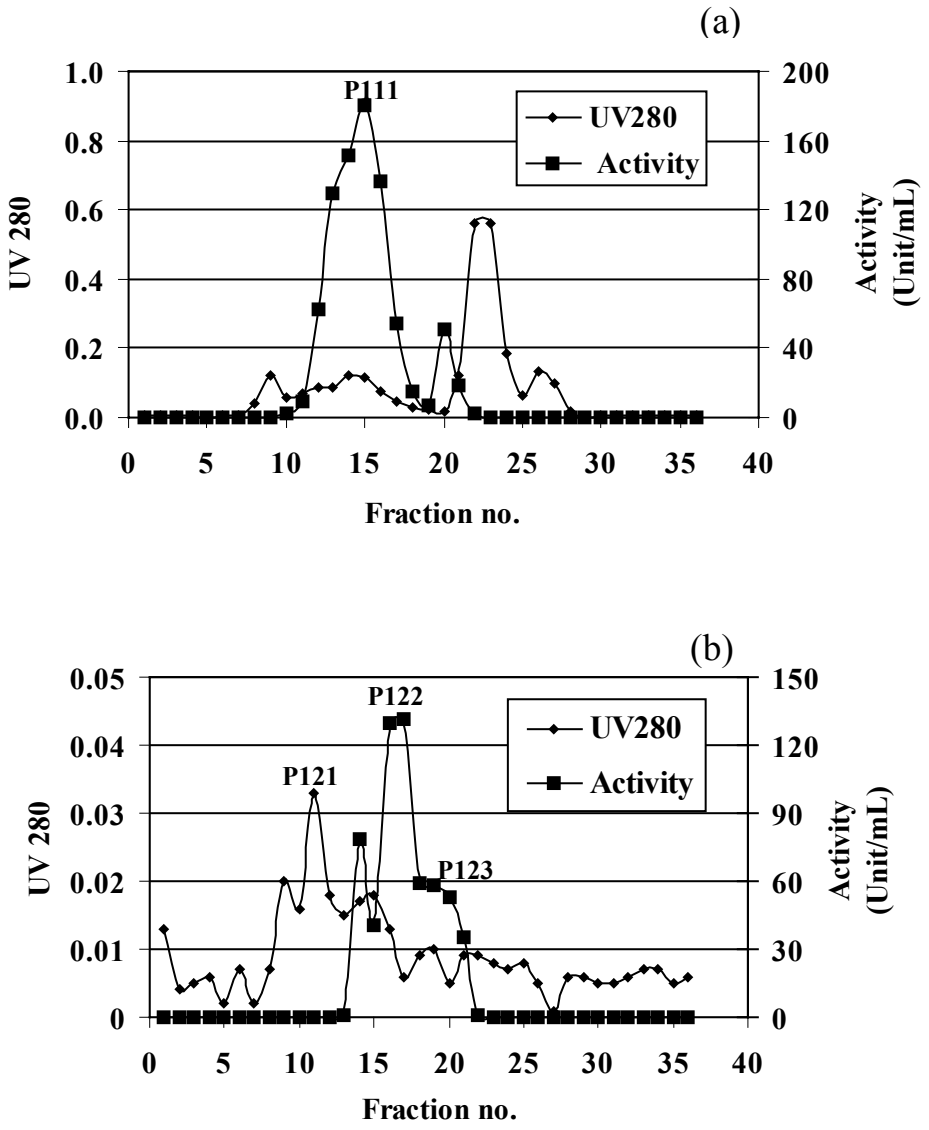

Figure 5.4-Chromatogram of proteinases, P11(a) and P12(b) loaded onto gel filtration (Sephacryl S-300)

5.3.2 Temperature and pH optimum

Activity of P111 sharply increased at 60 ^{o}C (Figure 5.5a). Optimal temperature of P31 and P4 was 55 ^{o}C, while that of P21 was 50 ^{o}C (Figure 5.5b, c, and d). P21 and P31 exhibited the highest activity among partially purified proteinase

obtained. Optimal temperature of these enzymes was similar to that of crude proteinase and autolytic activity of whole fish (Siringan et al., 2005).

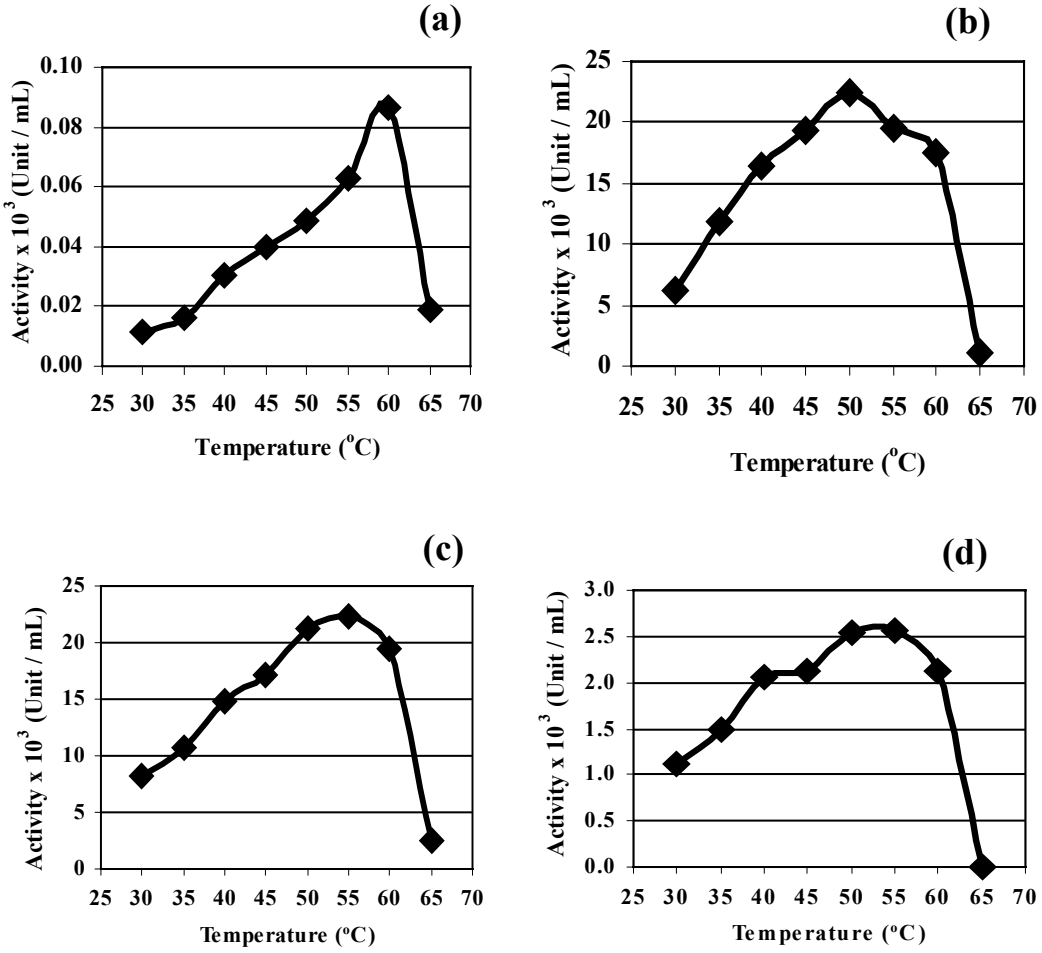

Figure 5.5-Temperature profiles of partially purified proteinases, using Boc-Asp(oBzl)-Pro-Arg-AMC as a substrat assayed at pH 8.5, P111(a); P21(b); P31(c); P4(d).

Optimal pH of all fractions was 8.5 (Figure 5.6). Thus should be noted that activities of these enzymes were minimal at pH 5.5-6.0 (Figure 5.6). It could be hypothesized that protein hydrolysis by these enzymes during fish sauce fermentation would be minimal. These might explain a long fermentation period of fish sauce. Based on these results, all partially purified proteinases exhibited maximum activity at high temperature and alkaline pH. Alkaline proteinase in anchovy (*E. encrasicholus*) which was a predominant enzyme in salted anchovy showed the optimal temperature and pH at 50 ^{o}C and 8.0, respectively (Hernandez-Herrero et al., 1999). Two trypsin-like proteinases purified from the digestive tract of anchovy (*E. encrasicholus*) exhibited pH optimal at 8.0-9.0. In our previous study, trypsin-like proteinases, which were predominantly found in whole fish extract, were stable during fish sauce fermentation up to 12 months (Siringan et al., 2005). Therefore, acceleration of protein hydrolysis by fish endogenous protoeinase could be achieved when optimal temperature and pH of proteinases are attained. Gildberg (2000) reported that rate of protein hydrolysis of fish sauce from male capelin and cod intestine increased when pH of fermentation was initially adjusted to pH 8.0, an optimal pH of cod trypsin.

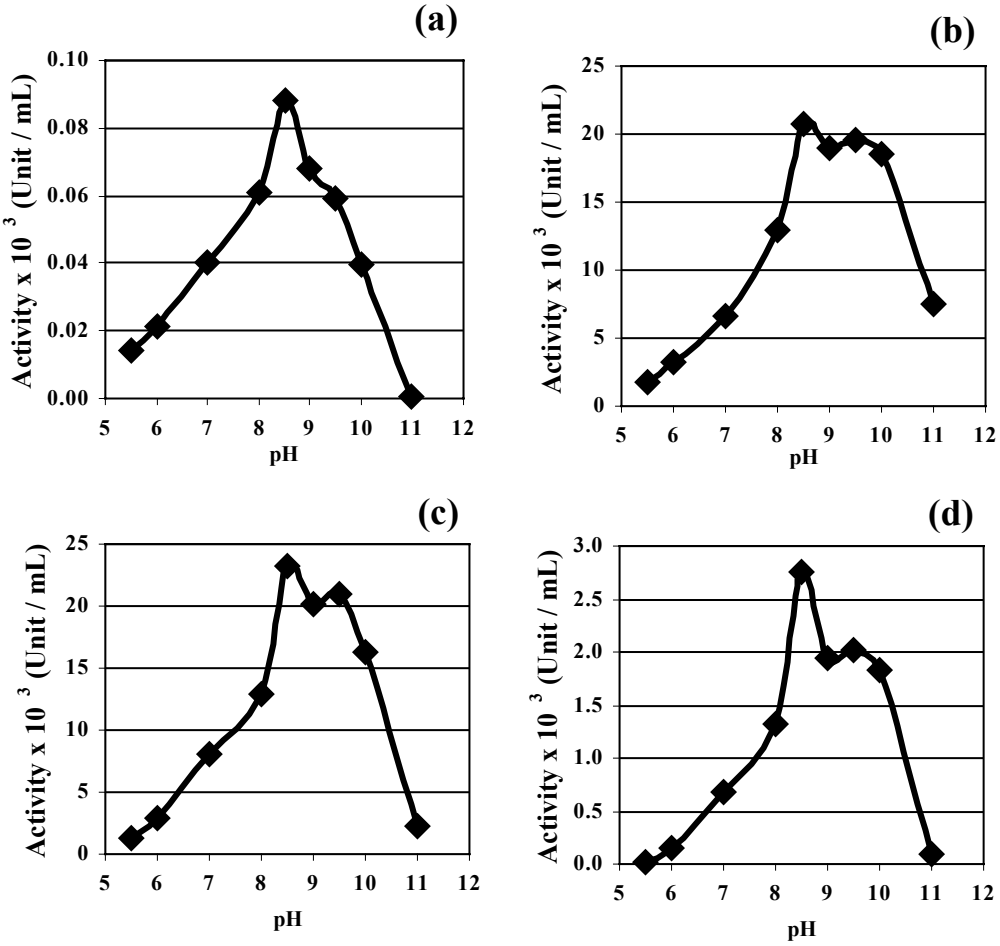

Figure 5.6- pH profiles of partially purified proteinases, using Boc-Asp(oBzl)-Pro-Arg-AMC as a substrat assayed at each respective optimal temperature, P111(a); P21(b); P31(c); P4(d).

5.3.3 Substrate specificity

All fractions efficiently hydrolyzed Boc-Asp(oBzl)-Pro-Arg-AMC and Boc-Gln-Ala-Arg-AMC (Table 5.2), which are substrates of trypsin, while Boc-Val-Leu-Lys-AMC and Z-Phe-Arg-AMC were slightly hydrolyzed. Suc-Ala-Ala-Pro-Phe-AMC and Z-Arg-Arg-AMC, which are substrates of chymotrpsin and cathepsin B, respectively, were hardly hydrolyzed. These results indicated that patially purified

proteinases were trypsin-like due to the preferential cleavage of Arg at P_1 position. Trypsin-like proteinases purified from pyloric caeca of menhaden and rainbow trout hydrolyzed the synthetic substrate of trypsin, which contained Arg at P_1 position (Pavlisko, Rial, and Coppes, 1999; Kristjansson 1991). Ishida et al. (1995) found that neutral serine proteinases type I and II from muscle of salted anchovy (*E. japonica*) exhibited the highest hydrolytic activity towards Boc-Asp(oBzl)-Pro-Arg-AMC and Boc-Gln-Ala-Arg-AMC, respectively. It should be mentioned that proteolytic activities of P21 and P31 were the higher than others. P31 and P21 appeared to be predominant proteinases purified from Indian anchovy.

Table 5.2-Hydrolytic activity towards various synthetic substrates of partially purified proteinases at its optimal temperature and pH.

Substrates	**Activity (Unit/mL)**			
	P111	**P21**	**P31**	**P4**
Boc-Asp(oBzl)-Pro-Arg-AMC	82.25	18,890	21,120	3,140
Boc-Val-Leu-Lys-AMC	3.07	1,300	360	120
Boc-Gln-Ala-Arg-AMC	29.06	9,210	7,950	1,340
Suc-Ala-Ala-Pro-Phe-AMC	0	250	0	0
Z-Arg-Arg-AMC	0	250	0	0
Z-Phe-Arg-AMC	2.96	1,370	300	20

5.3.4 Thermal stability

All proteinase fractions were stable at 35 °C, an average temperature of fish sauce fermentation (Figure 5.7). At their optimal temperature, activity of P21, P31, and P4 decreased about 40% after 2 h incubation and remained stable afterward throughout 12 h (Figure 5.7 b, c, and d). Only P111 was stable throughout 12 h at its optimal temperature (Figure 5.7a). In our previous study, autolytic activity of Indian anchovy exhibited maximum at 60 °C, corresponding to optimal temperature of P111 (Siringan et al., 2005). Thus, P111 would contribute to autolysis in *situ* of anchovy.

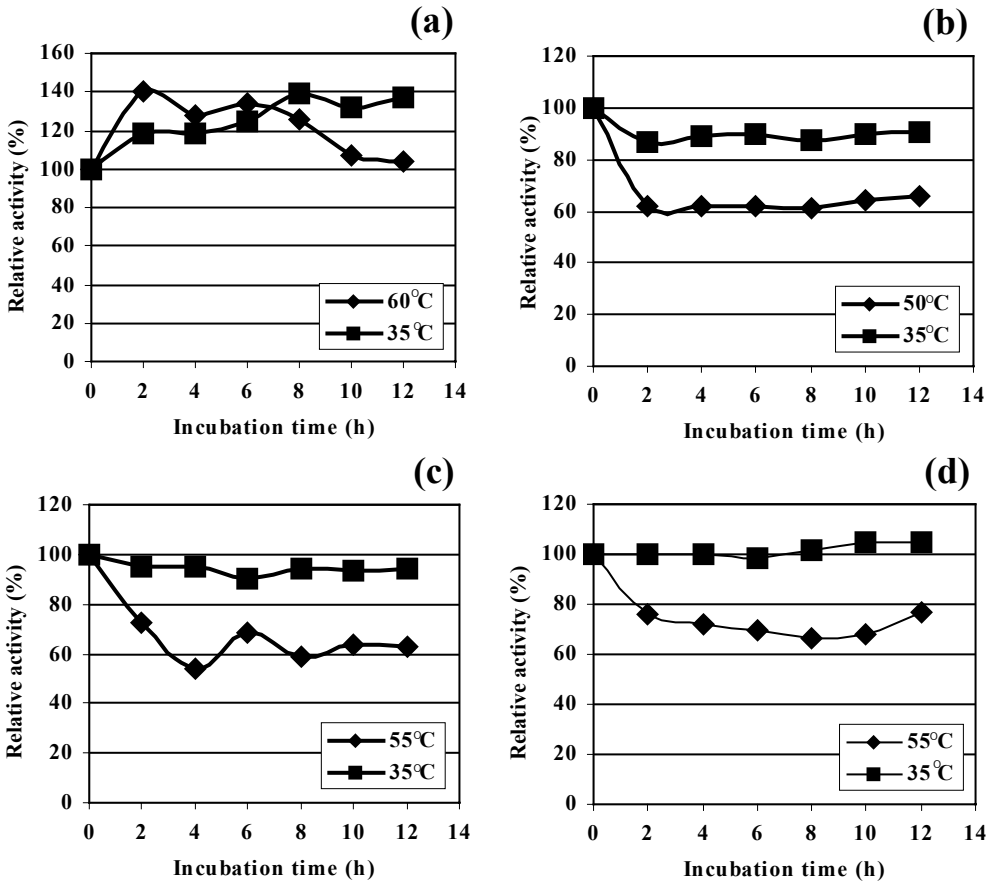

Figure 5.7-Thermal stability of partially purified proteinases at 35 °C and each respective temperature, P111(a); P21(b); P31(c); P4(d).

5.3.5 Effect of proteinase inhibitors and $CaCl_2$

Activity of all fractions was greatly suppressed by SBTI, leupeptin, and SDS (Table 5.3). TLCK and PMSF moderately inhibited proteolytic activity of all fractions. While E-64 and iodoacetic acid which are cysteine proteinase inhibitors, slightly inhibited proteinase activity. P111 was moderately inhibited by EDTA, while P21, P31, and P4 were slightly inhibited. Bestatin, a metallo poteinase inhibitor specific for amionopeptidase, slightly inhibited activities of all proteinases. DTT inhibited activity of all partially purified proteinase to varied degree. This maybe because DTT disrupted disulfide bonds which stabilized conformation of native enzymes.

Table 5.3-Effect of proteinase inhibitors on proteolytic activity

Inhibitors	Classification	Final concentration	Inhibition (%)			
			P111	P2	P3	P4
EDTA	Metallo proteinase	10 mM	43.00	10.64	23.41	25.42
PMSF	Serine proteinase	10 mM	42.66	53.76	43.83	50.93
Bestatin	Metallo proteinase	10 μM	0.00	18.04	2.72	0.26
Iodoacetic acid	Cysteine proteinase	1 mM	0.00	13.94	7.49	0.00
E-64	Cysteine proteinase	10 μM	24.54	63.76	55.39	41.38
Leupeptin	Serine and cysteine proteinase	100 μM	101.71	99.60	103.68	103.42
TLCK	Serine proteinase	100 μM	15.54	87.98	51.86	69.33
SBTI	Serine proteinase	0.02 mg/mL	101.24	98.66	103.17	103.12
SDS	Detergent	2%(w/v)	101.86	99.28	103.71	103.42
DTT	Reducing reagent	10 mM	79.42	75.45	71.52	55.86
Control	-	-	0.00	0.00	0.00	0.00

Activities of P21, P31, and P4 decreased with increasing $CaCl_2$ concentration (Figure 5.8). Hjelmeland and Raa (1982) also reported that Ca^{2+} had no effect on activity of trypsin from capelin. P111 appeared to be slightly activated by 5 mM $CaCl_2$. Ca^{2+} had a stabilizing effect on trypsin-like proteinases from anchovy (Martinez et al., 1988). Ca^{2+} also prevented conformational changes of trypsin, resulting is a more compact structure and is more resistant to autolysis (Walsh, 1970).

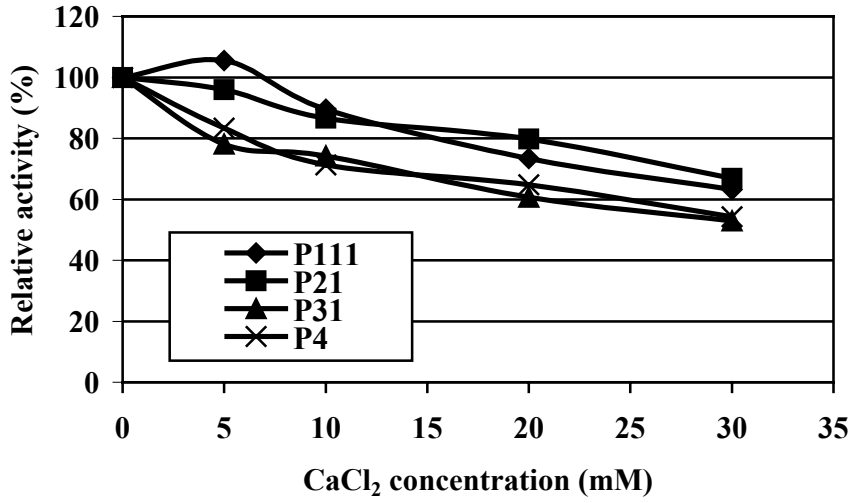

Figure 5.8-Effect of $CaCl_2$ on activity of various patially purified proteinases

5.3.6 Activity staining

Molecular weight (MW) of P111 fraction was estimated to be 27, 38, 47, 58, 60, and 65 kDa, while P21 showed MW of 37, 43, 48, 58, 60, and 65 kDa (Figure 5.9). P31 showed MW of 27, 33, 37, 43, and 50 kDa. Only one distinct caseinolytic activity band at 39 kDa was observed in P4 (Figure 5.9). Proteinases with various molecular masses that exhibited activity at 4.0 M NaCl were purified from anchovy. MW of trypsin isolated from carp, anchovy, rainbow trout was estimated to be 25-30 kDa (Cao, Osatomi, Sizuki, Hara, Tachibana, and Ishihara, 2000; Martinez et al,

1988; Kristjansson, 1991). Several proteinases were found in Indian anchovy and hydrolyzed protein at high salt content. In addition, several proeinases with caseinolytic activity on Native-PAGE showed similar to patterns to those obtained from SDS-PAGE (data not shown). These results suggested that partially purified proteinases might be a single polypeptide without subunit.

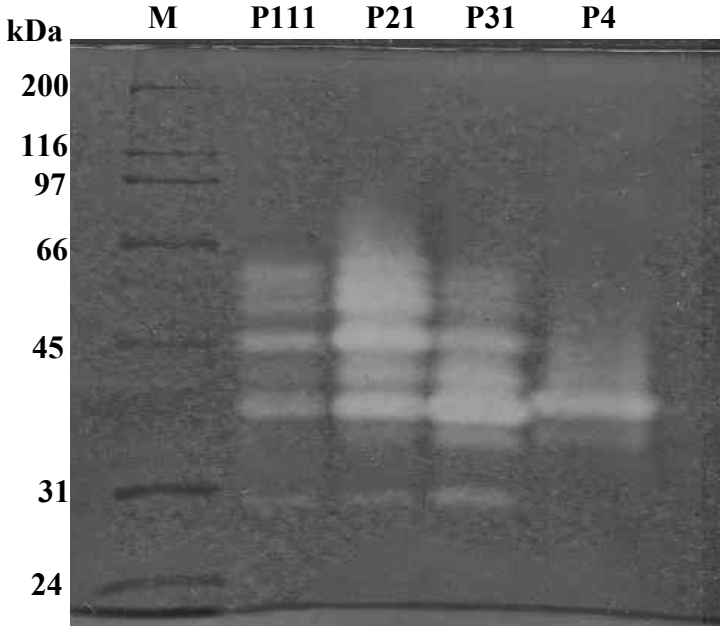

Figure 5.9-Activity staining of partially purified proteinases in Indian anchovy incubated at 60 ^{o}C, pH 8.5, 4.0 M NaCl. M; Molecular weight marker. Loaded protein was 3 µg. P111, P21, P31, and P4 denoted for proteinase fractions.

5.3.7 Hydrolysis of washed anchovy muscle at 4.0 M NaCl

All partially purified proteinases hydrolyzed washed anchovy mince at 4.0 M NaCl (pH 8.5) at 35 ^{o}C and at an optimal temperature of each fraction (Figure 5.10). P111 showed higher activity than others at the optimal temperature due to its higher thermal stability at the optimal temperature. However, activities of all fractions at 35 ^{o}C were lower than those at the optimal temperature. It should be noted that all

fractions did not hydrolyze washed anchovy mince at 4.0 M NaCl, pH 5.5 (data not shown). Normally, fermentation pH and temperature during fish sauce production ranged from 5.5 to 5.8 and at 25 to 35 ^{o}C, respectively. Under such conditions, proteinases activities from Indian anchovy appeared to be minimal. However, trypsin-like proteinases in Indian anchovy could be activated by increasing incubation temperature to about 50 to 60 ^{o}C and adjusting pH to 8.5 so that proteolytic activites would be enhanced.

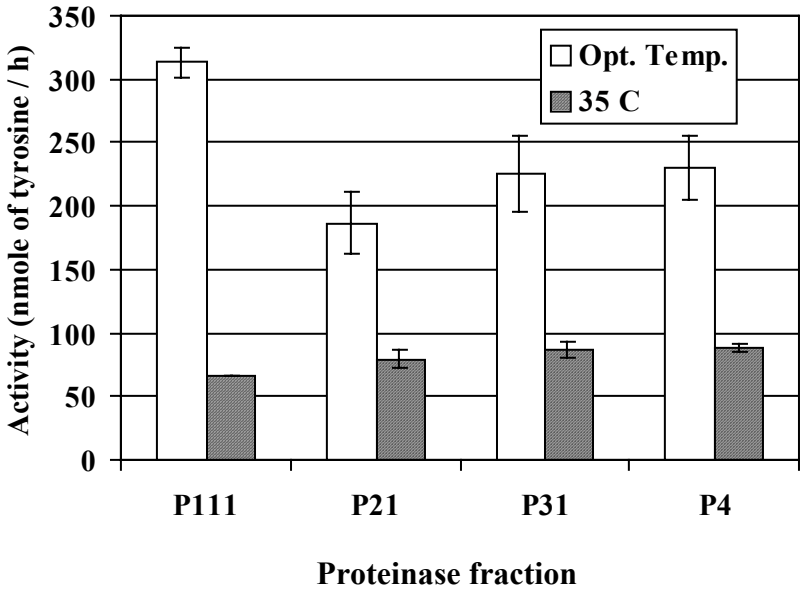

Figure 5.10-Proteolytic activity of partially purified proteinases towards washed mince solubilzed in 4.0 M NaCl, 0.2 M Tris-HCl, pH 8.5.

Myosin heavy chain (MHC) disappeared in all samples incubated at 35, 50, 55, and 60 ^{o}C regardless of addition of partially purified proteinases (Figure 5.11a,b). It was evident that substantial proteinase activities were present in the washed mince. These proteinases could be myofibril-bound enzymes because washed mince was

prepared by extensive washing and dewatering. These myofibril-bound proteinases appeared to participate in MHC-degradation at 35 and 50-60 ^{o}C. Addition of P111 and P31 induced proteolysis at 60 and 55 ^{o}C, respectively (Figure 5.11a). Actin (Ac) was also greatly degraded when partially purified proteinases (P111 and P31) was added (Figure 5.11a). P21 and P4 slightly degraded actin. Proteolysis of actin was minimal at 35 ^{o}C (Figure 5.11b). These results indicated that endogenous proteinases in anchovy muscle might be active even at 4.0 M NaCl at their temperature and pH optimum. Myofibril-bound serine proteinase (MBSP) purified from various fish species exhibited optimal activity at 55-60 ^{o}C and pH 8.0-9.0 (Osatomi, Sasai, Cao, Hara, and Ishihara, 1997; Cao, Osatomi, Hara, and Ishihara, 2000; Ohkubo, Miyagawa, Osatomi, Hara, Nozaki, Ishihara, 2004). MBSP extensively degraded MHC at 55-60 ^{o}C (Cao et al 2000; Ohkubo et al., 2004). It was likely that MBSP in anchovy muscle might be activated 55 and 60 ^{o}C, pH 8.5. Moreover, P111 appeared to extensively hydrolyze washed anchovy mince at 4.0 M NaCl, pH 8.5, despite that its activity towards synthetic substrates was lower than P21 and P31 (Table 5.2). The activity of partially purified proteinases varied depending on type of substrate. In the previous study, activity of crude proteinases decreased at 2.5 M NaCl using casein and hemoglobin as substrates, while autolytic activity remained about 50% at 25% NaCl (w/w) (Siringan et al., 2005). Kinoshita, Toyohara, and Shimuzu (1990) reported that serine proteinases purified from skeletal muscle of threadfin bream hydrolyzed MHC to a greater extent than casein. However, Choi, Heu, Kim, and Pyeun (2004) reported that trypsin and chymotrypsin showed similar hydrolytic activity towards casein and myofibrillar proteins. These indicated that different proteinases exhibited difference of preferential substrate.

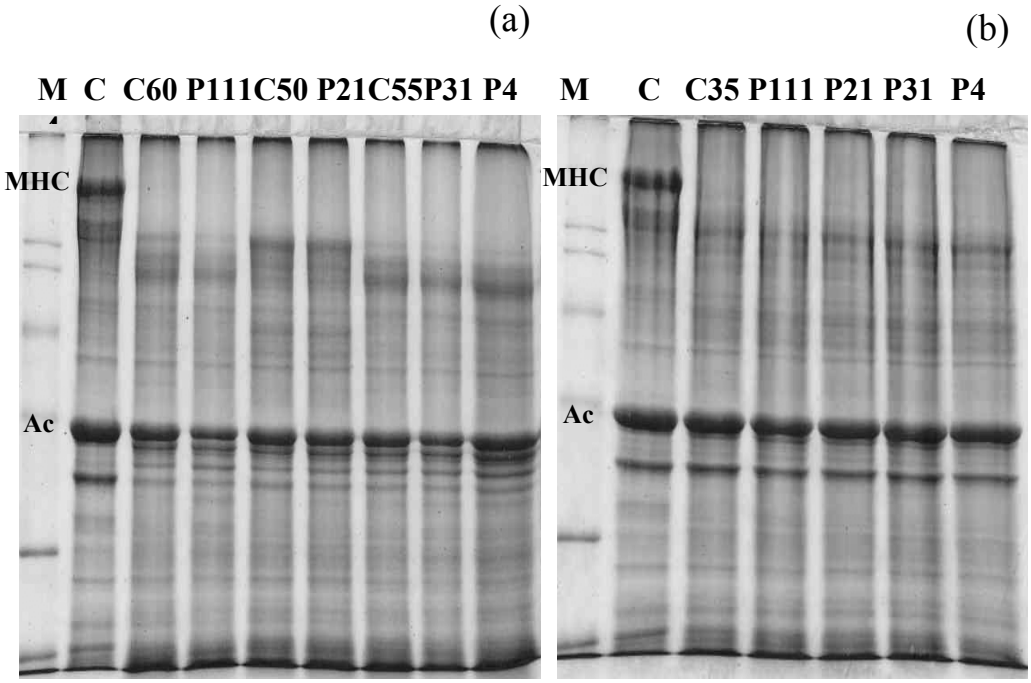

Figure 5.11-SDS-PAGE (10% acrylamide) patterns of washed mince degradation in 4.0 M NaCl, 0.2 M Tris-HCl, incubated at optimum temperature (a), incubated at 35 ^{o}C (b). C: control without added proteinase, C60: 60 ^{o}C, C50: 50 ^{o}C, C55: 55 ^{o}C, and C35: 35^{o}C, P: proteinases fraction 111, 21, 31, and 4. Loaded sample was 5µL.

5.4 Conclusions

Partially purified proteinases exhibited the optimal temperature and pH at 50-60 ^{o}C and 8.5, respectively. Several trypsin-like proteinases were observed in activity staining at 4.0 M NaCl. All partially purified proteinases also hydrolyzed washed anchovy mince at 4.0 M NaCl, pH 8.5. Adjusting pH and temperature of fish sauce fermentation to optimal conditions of proteinasess could be a means to accelerate fish sauce fermentation.

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CHAPTER VI

SUMMARY

Autolytic activity of Indian anchovy showed the optimal temperature at 60 ^{o}C. Crude extract exhibited an optimum pH at 8.5-9.5. Trypsin-like proteinases were the predominant proteinase in the crude extract. Trypsin-like proteinases, which were mainly found in whole fish extract, were derived from viscera of Indian anchovy. Several proteinases with different molecular weights (28-68 kDa) were observed in activity staining at 4.0 M NaCl, suggesting that endogenous proteinases from Indian anchovy could participate in protein hydrolysis at high salt concentration. Furthermore, trypsin-like proteinases were also observed throughout 12 months of fish sauce fermentation. Molecular weight of proteinases in fish sauce at various fermentation time was estimated to be 40, 51, and 58 kDa, which was similar to that of partially purified proteinases based on activity staining. Several partially purified trypsin-like proteinases exhibited caseinolytic activity at 4.0 M NaCl, pH 8.5. Optimal temperature and pH of partially purified proteinases were 50-60 ^{o}C and pH 8.5, respectively. In addition, these enzymes extensively hydrolyzed washed anchovy muscle at 4.0 M NaCl at their optimal temperature and pH 8.5. Endogenous proteinases with MW of 40, 51, and 58 kDa also exhibited stability throughout 12 months of fish sauce fermentation. Thus, activation of endogenous proteinases by adjusting pH and temperature to their optimal conditions would be alternative to accelerate fish sauce fermentation.

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