การผลิตและคุณลักษณะของโปรตีเนสจากสายพันธุ์ของแบคทีเรียชอบเกลือความเข้มข้นปานกลางในสกุล *VIRGIBACILLUS* ที่คัดแยกจากการหมักน้ำปลา

นายศรชัย สินสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PRODUCTION AND CHARACTERIZATION OF PROTEINASES FROM MODERATELY HALOPHILIC *VIRGIBACILLUS* STRAINS ISOLATED FROM FISH SAUCE FERMENTATION

Sornchai Sinsuwan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Technology

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Academic Year 2006

PRODUCTION AND CHARACTERIZATION OF PROTEINASES FROM MODERATELY HALOPHILIC *VIRGIBACILLUS* STRAINS ISOLATED FROM FISH SAUCE FERMENTATION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

_____

(Asst. Prof. Dr. Piyawan Gasaluck)

Chairperson

_____

(Assoc. Prof. Dr. Jirawat Yongsawatdigul)

Member (Thesis Advisor)

_____

(Asst. Prof. Dr. Sureelak Rodtong)

Member

_____

(Assoc. Prof. Dr. Nongnuch Raksakulthai)

Member

_____ _____

(Assoc. Prof. Dr. Saowanee Rattanaphani) (Asst. Prof. Dr. Suwayd Ningsanond)

Vice Rector for Academic Affairs Dean of Institute of Agricultural Technology

ศรชัย สินสุวรรณ : การผลิตและคุณลักษณะของโปรตีเนสจากสายพันธุ์ของแบคทีเรียชอบเกลือความเข้มข้นปานกลางในสกุล *VIRGIBACILLUS* ที่คัดแยกจากการหมักน้ำปลา (PRODUCTION AND CHARACTERIZATION OF PROTEINASES FROM MODERATELY HALOPHILIC *VIRGIBACILLUS* STRAINS ISOLATED FROM FISH SAUCE FERMENTATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 154 หน้า.

แบคทีเรีย *Virgibacillus* sp. SK37 และ SK33 ซึ่งคัดแยกได้จากตัวอย่างเดือนแรกของการหมักน้ำปลามีความสามารถในการย่อยสลายปลากะตักและเคซีน (Casein) ที่ความเข้มข้นโซเดียมคลอไรด์ร้อยละ 10 และ 25 จึงอาจเป็นสายพันธุ์ที่มีศักยภาพเป็นกล้าเชื้อสำหรับกระบวนการหมักน้ำปลา ซึ่งแนวทางดังกล่าวจะนำไปสู่การพัฒนากระบวนการลดระยะเวลาการหมัก ดังนั้นวัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาคุณลักษณะทางชีวเคมีของโปรตีเนสที่หลั่งนอกเซลล์ (Extracellullar proteinase) และที่ตรึงอยู่กับเซลล์ (Cell-bound proteinase) ที่ผลิตจากแบคทีเรีย *Virgibacillus* sp. SK37 และ SK33

โปรตีเนสที่ตรึงอยู่กับเซลล์และหลั่งนอกเซลล์จาก *Virgibacillus* sp. SK37 สามารถย่อยสลายสารตั้งต้นสังเคราะห์ ซัคซินิล-อะลานีน-อะลานีน-โพรลีน-ฟีนิลอะลานีน-4-เมทิล-7-คลูมารินเอไมด์ (Succinyl(Suc)-Ala-Ala-Pro-Phe-Arg-4-methyl-7-coumarylamides(AMC)) ได้ดี แสดงถึงคุณลักษณะของเอนไซม์สับทิลิซิน (Subtilisin) โปรตีเนสที่หลั่งนอกเซลล์จากแบคทีเรีย *Virgibacillus* sp. SK37 มีสภาวะที่เหมาะสมต่อการเร่งกิจกรรมของเอนไซม์คือที่อุณหภูมิ 65 องศาเซลเซียส และพีเอช 8 กิจกรรมย่อยสลายโปรตีนเพิ่มขึ้นประมาณ 5 เท่าในสภาวะที่มีโซเดียมคลอไรด์เข้มข้นร้อยละ 25 แสดงว่าเป็นโปรตีเนสที่ถูกเร่งกิจกรรมด้วยเกลือโซเดียมคลอไรด์ นอกจากนั้นเมื่อวิเคราะห์กิจกรรมด้วยการปรากฏของแถบใสบนแผ่นอะคริลาไมด์ (Activity staining) ทั้งในสภาวะที่ไม่มีและมีโซเดียมคลอไรด์เข้มข้นร้อยละ 25 พบโปรตีเนสที่มีมวลโมเลกุลขนาดต่าง ๆ คือ 81 67 63 50 38 และ 18 กิโลดาลตัน สำหรับโปรตีเนสที่ตรึงอยู่กับเซลล์จากแบคทีเรีย *Virgibacillus* sp. SK37 สามารถแสดงกิจกรรมสูงสุดที่ 65 องศาเซลเซียส พีเอช 7 และ 9.5 โปรตีเนสนี้สามารถย่อยสลายแอคโตมัยโอซินจากปลากะตักที่โซเดียมคลอไรด์เข้มข้นร้อยละ 5-20 ได้อย่างมีประสิทธิภาพ เอนไซม์มีเสถียรภาพที่ความเข้มข้นโซเดียมคลอไรด์ร้อยละ 25 ที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง

Virgibacillus sp. SK33 สามารถผลิตโปรตีเนสได้สูงสุดในอาหารเหลวสูตรฮาโลแบคทีเรียมดัดแปลง (Modified halobacterium broth) ที่มีแหล่งไนโตรเจนคือสารสกัดจาก

ยีสต์ (Yeast extract) เพปโทน (Peptone) หรือกรดคาซามิโน (Casamino acid) เพียงชนิดใดชนิดหนึ่ง สภาวะเหมาะสมต่อการสร้างโปรตีเนสสำหรับ *Virgibacillus* sp. SK33 คือ ที่โซเดียมคลอไรด์เข้มข้นร้อยละ 5 อุณหภูมิ 40 องศาเซลเซียส สำหรับโปรตีเนสที่หลั่งนอกเซลล์จาก *Virgibacillus* sp. SK33 สามารถย่อยสลายสารตั้งต้นสังเคราะห์ ซัคซินิล-อะลานีน-อะลานีน-โพรลีน-ฟีนิลอะลานีน-4-เมทิล-7-คลูมารินเอไมด์ ได้ดี แสดงถึงคุณลักษณะของเอนไซม์สับทิลิซิน และพบกิจกรรมสูงสุดที่ 50 องศาเซลเซียส และ พีเอช 8 10 และ 11 กิจกรรมการย่อยสลายโปรตีนต่อสารตั้งต้นสังเคราะห์และปลากะตักเพิ่มขึ้นตามความเข้มข้นโซเดียมคลอไรด์จนถึงร้อยละ 25 บ่งชี้ว่าเป็นโปรตีเนสที่ถูกเร่งกิจกรรมด้วยเกลือ สอดคล้องกับผลการวิเคราะห์ด้วยวิธีการการปรากฏของแถบใสบนแผ่นอะคริลาไมด์ ในสภาวะที่มีโซเดียมคลอไรด์เข้มข้นร้อยละ 25 ซึ่งพบโปรตีเนสที่มีมวลโมเลกุลขนาดต่างๆ คือ 56 46 42 32 25 และ 19 กิโลดาลตัน โดยเฉพาะโปรตีเนสขนาด 19 กิโลดาลตันพบแถบใสขนาดใหญ่แสดงถึงกิจกรรมการย่อยสลายโปรตีนในสภาวะความเข้มข้นโซเดียมคลอไรด์สูง จึงเลือกทำบริสุทธิ์เอนไซม์ดังกล่าวด้วยหลักการแยกสารอันตรกิริยาไฮโดรฟอบิก (Hydrophobic interaction chromatography) และด้วยไฮดรอกซีเอพาไทท์ (Hydroxyapatite) พบว่าโปรตีเนสบริสุทธิ์ประกอบด้วย 2 หน่วยย่อย (Dimer) โดยแต่ละหน่วยย่อยมีขนาด 19 กิโลดาลตันเชื่อมติดกันด้วยอันตรกิริยาไฮโดรฟอบิก สภาวะที่เหมาะสมต่อการเร่งกิจกรรมคือที่อุณหภูมิ 55 องศาเซลเซียส และพีเอช 7.5 เอนไซม์บริสุทธิ์มีเสถียรภาพที่อุณหภูมิ 30-60 องศาเซลเซียส และโซเดียมคลอไรด์เข้มข้นร้อยละ 0-25 กิจกรรมย่อยสลายโปรตีนต่อสารตั้งต้นสังเคราะห์และปลากะตักเพิ่มขึ้นเมื่อเพิ่มความเข้มข้นโซเดียมคลอไรด์จนถึงร้อยละ 25 ค่าไอโซอิเลคติค (Isoelectric point) ของเอนไซม์บริสุทธิ์คือ 4.28 บ่งชี้ว่ามีปริมาณกรดอะมิโนที่มีคุณสมบัติเป็นกรด (Acidic amino acids) มาก จึงอาจเป็นเหตุผลให้เอนไซม์สามารถแสดงกิจกรรมภายใต้ความเข้มข้นเกลือสูง เนื่องจากประจุลบบนโครงสร้างเอนไซม์สามารถเกิดอันตรกิริยากับน้ำได้ จึงไม่สูญเสียโครงร่างธรรมชาติ จากงานวิจัยนี้ *Virgibacillus* sp. SK37 และ SK33 เป็นแบคทีเรียที่มีศักยภาพในการสร้างโปรตีเนสที่เร่งกิจกรรมด้วยโซเดียมคลอไรด์ซึ่งเป็นคุณลักษณะที่สำคัญต่อการนำไปประยุกต์ใช้ในการหมักภายใต้สภาวะความเข้มข้นเกลือสูง ทั้งสองสายพันธุ์อาจมีบทบาทสำคัญในการย่อยสลายโปรตีนในการกระบวนการหมักน้ำปลา และมีศักยภาพในการพัฒนาเพื่อใช้เป็นกล้าเชื้อในการหมักน้ำปลาต่อไป

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SORNCHAI SINSUWAN : PRODUCTION AND CHARACTERIZATION OF PROTEINASES FROM MODERATELY HALOPHILIC *VIRGIBACILLUS* STRAINS ISOLATED FROM FISH SAUCE FERMENTATION. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D. 154 PP.

VIRGIBACILLUS STRAINS/BACTERIAL PROTEINASE/NaCl-ACTIVATED PROTEINASE/FISH SAUCE

Virgibacillus sp. SK37 and SK 33 isolated from fish sauce fermented for 1 month showed high proteolytic activity towards anchovy and casein substrates at 10% and 25% NaCl. They could be the potential strains for starter culture development aiming at reducing fermentation time. Overall objective of this study was to elucidate biochemical characteristics of extracellular and cell-bound proteinases produced by these strains.

Extracellular and cell-bound proteinases from *Virgibacillus* sp. SK37 preferably hydrolyzed succinyl (Suc)-Ala-Ala-Pro-Phe-4-methyl-7-coumarylamides (AMC), suggesting a subtilisin-like characteristic. Extracellular proteinase from *Virgibacillus* sp. SK37 showed optimum condition at 65 °C and pH 8. Proteolytic activity was increased about 5 times in the presence of 20% NaCl, indicating the characteristic of NaCl-activated proteinase. Based on activity staining, several proteinases with molecular mass of 81, 67, 63, 50, 38 and 18 kDa were observed both in the absence and presence of 25% NaCl. Cell-bound proteinase from *Virgibacillus* sp. SK37 showed the maximum activity at 65 °C, pH 7 and 9.5. The proteinase

effectively hydrolyzed anchovy actomyosin at 5-20% NaCl. In addition, the enzyme remained stable at 30 °C, 25% NaCl for 24 h.

Virgibacillus sp. SK33 showed maximum proteinase production in the modified halobacterium broth containing only a single nitrogen source of either yeast extract, peptone or casamino acid. Optimal proteinase production for *Virgibacillus* sp. SK33 was at 5% NaCl, 40 °C. Extracellular proteinase of *Virgibacillus* sp. SK33 efficiently hydrolyzed Suc-Ala-Ala-Pro-Phe-AMC, suggesting a subtilisin-like characteristic. The proteinase exhibited optimum activity at 50 °C and pH 8, 10 and 11. Proteolytic activity towards a synthetic substrate and anchovy increased with NaCl concentration up to 25%, indicating the characteristic of NaCl-activated proteinase. Several proteinases with molecular weight (MW) of 56, 46, 42, 32, 25 and 19 kDa showed caseinolytic activity at 25% NaCl. Proteinase with MW of 19 kDa exhibited large proteolytic band indicating high activity at high NaCl concentration. Therefore, it was further purified to electrophoretic homogeneity using hydrophobic interaction chromatography and hydroxyapatite. The enzyme had a dimer with a monomer of 19 kDa. The dimer was stabilized by hydrophobic interactions. Temperature and pH optimum of the purified proteinase was at 55 °C and pH 7.5. The enzyme was stable at 30-60 °C and 0-25% NaCl. Proteinase activity towards both synthetic and anchovy substrates was activated by NaCl up to 25%. Isolectric point (pI) of the purified proteinase was 4.28, indicating that it contained high acidic amino acids. It should be postulated that negative charges on external structure interacting to hydrate NaCl resulted in halophilic enzyme characteristic. Based on these results, *Virgibacillus* sp. SK37 and SK33 were potential sources of NaCl-activated proteinases. They would play an important role in protein hydrolysis

during fish sauce fermentation and might be applicable for starter culture development.

School of Food Technology Student's Signature _____

Academic Year 2006 Advisor's Signature _____

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CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

A_{450}	Absorbance at 450 nm
Ala	Alanine
AM	Actomyosin
AMC	4-Methyl-7-coumarylamides
ANOVA	Analysis of variance
Arg	Arginine
Asp	Aspartic acid
β-ME	2-Mercaptoethanol
Boc	*t*-Butyloxycarbonyl
BPN’	Bacterial proteinase Nagase
Bzl	Benzyl
°C	Degree Celsius
CFU/mL	Colonies forming unit per milliliter
cm	Centimeter
Cm	Casamino acid broth
CM	Carboxymethyl
CRD	Completely randomized design
Cys	Cysteine
Da	Dalton
DEAE	Diethylaminoethyl
DMRT	Duncan Multiple Range Test

LIST OF ABBREVIATIONS (Continued)

DTT	Dithiothreitol
E-64	*Trans*-epoxysuccinyl-L-leucylamido-(4-guanidine)-butane
EDTA	Ethylenediaminetetraacetic acid
FB	Fish broth
g	Gram
×g	Relative centrifugal fields
Gln	Glutamine
h	Hour
HA	Hydroxyapatite
HIC	Hydrophobic interaction chromatography
His	Histidine
HL	Halobacterium broth
HL-C	Halobacterium broth without casamino acid
HL-P	Halobacterium broth without peptone
HL-Y	Halobacterium broth without yeast extract
IAA	Iodoacetic acid
IEF	Isoelectric focusing
kDa	Kilodalton
K_m	Michaelis constant
L	Liter
Leu	Leucine
Lys	Lysine
M	Molar

LIST OF ABBREVIATIONS (Continued)

mA	Milliampere
mg	Milligram
min	Minute
mL	Mililiter
mM	Millimolar
MW	Molecular weight
MWCO	Molecular weight cut-off
N	Normality
native-PAGE	Native-polyacrylamide gel electrophoresis
NCBI	The National Center for Biotechnology Information
NEO	Neopeptone broth
NEM	*N*-Ethylmaleimide
nm	Nanometer
nmole	Nanomole
PAGE	Polyacrylamide gel electrophoresis
PCA	Plate count agar
Phe	Phenylalanine
pI	Isoelectric point
Pm	Peptone broth
%	Percent
PMSF	Phenylmethanesulfonyl fluoride
*p*NA	*p* Nitroanilide
Pro	Proline

LIST OF ABBREVIATIONS (Continued)

Q	Quaternary ammonium
R_f	Relative migration value
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
s	Second
S	Protein standard marker
SAS	Statistical Analysis System
SDS	Sodiumdodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
SKS	Skim milk salt broth
SP	Sulfopropyl
Suc	Succinyl
TCA	Trichloroacetic acid
TLCK	*N*-Tosyl-L-lysine chloromethyl ketone
TPCK	*N*-Tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris(hydroxymethyl)aminomethane
μL	Microliter
μm	Micrometer
U	Unit activity
V	Volt
Val	Valine
V_e	Elution volume

LIST OF ABBREVIATIONS (Continued)

Vh	Volthours
V_{max}	Maximum velocity
V_o	Void volume
W	Watt
Ym	Yeast extract broth
Z	Carbobenzoxy

CHAPTER I

INTRODUCTION

1.1 Introduction

Fish sauce is a condiment consumed in Southeast Asia and is an important source of nutrients (Sikorski and Ruiter, 1994). The production of fish sauce involved the addition of salt to uneviscerated fish at a ratio of approximately 1:3. It usually takes at least 6 months for fish to liquefy and another 1-3 months to ripen for the full flavor and aroma development (Jay, 2000). Therefore, this traditional fermentation is a very slow process required about 1-1.5 years, which is a major limitation for the growth of fish sauce industry. Moreover, the traditional fermentation required large area and the fermentation process is difficult to control. Therefore, acceleration of natural fermentation process will reduce the capital cost and improve business profitabilities.

High salt content reduces the water activity to prevent putrefactive microorganisms from spoilage. It simultaneously allows halophilic bacteria to proliferate. Proteinases from halophilic bacteria or fish viscera/muscle convert protein to amino acids and peptides (Beddows, 1998). These compounds contributed to an unique characteristic of fish sauce. Many reports have indicated the characteristics of proteinases produced by extremely and moderately halophilic bacteria. Proteinase from *Halobacterium halobium* showed maximum activity at 3 M NaCl (17.5%) (Izotova et al., 1983), whereas arhaebacterium *Natrialba asiatica* 172 P1 proteinase showed chymotrypsin-like characteristic with optimal activity at 30%

NaCl (Kamekura and Seno, 1990). Activity of serine proteinase from *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-3 isolated from fish sauce fermentation increased with NaCl concentration (Hiraga et al., 2005; Namwong et al., 2006). *Halobacillus thailandensis* sp. produced serine proteinases with molecular weight (MW) of 100 and 17 kDa, and metalloproteinase with MW of 42 kDa (Chaiyanan et al., 1999). Therefore, there are several proteinase-producing bacteria found during fish sauce fermentation. These bacteria could be used to develop starter culture to accelerate fish sauce fermentation.

Besides extracellular proteinases, cell-bound proteinases have been extensively studied in lactic acid bacteria. These enzymes play an important role in cell growth. *Lactococcus lactis* SK11 without a gene of cell-bound proteinase (*prtP* gene) were not able to grow in milk medium (Vos et al., 1989). This is due to a lack of peptides and amino acids. In addition, these compounds also contribute to flavor development during cheese ripening. Thus far, cell-bound proteinases have never been studied in moderately halophilic bacteria isolated from fish sauce.

Secretion of proteinase from bacteria is dependent on available nutrients, media composition and physical factors, such as NaCl concentration, temperature and incubation time. In order to maximize bacterial proteinase production, such factors must be determined. Casein supplemented in a medium induced maximum proteinase production of *Bacillus clausii*, while the supplementation of whey protein, malt extract, tryptone, peptone, soytone and yeast extract lower its proteinase production (Kumar, Joo, Koo, Paik, and Chang, 2004). Yeast extract was the best nitrogen source for proteinase production of *Bacillus stearothermophilus* (Razak et al., 1994). These results indicated that composition of culture media greatly affected proteinase

production of microorganisms. In addition, amount of nitrogen source in the medium is also a key parameter. Proteinase production of *Bacillus* sp. JB-99 was repressed by the excessive amount of yeast extract, peptone, beef extract and gelatin (Johnvesly and Naik, 2001). Therefore, parameters affecting proteinase production should be studied to obtain the maximum yield and activity. Few studies have reported the optimal proteinase production of moderately halophilic bacteria, particularly those isolated for fish sauce.

Recently, proteinase-producing bacteria from fish sauce fermentation have been isolated and identified to be *Virgibacillus* sp. according to 16S rRNA gene sequence. These strains (SK37 and SK33) showed high proteolytic activity towards anchovy substrate and could be promising strains for starter culture development for fish sauce fermentation. To successfully utilize these strains as starter cultures, optimum proteinase production and biochemical characteristics of these proteinases must be determined.

1.2 Research objectives

The objectives of this research were:

(1) To biochemically characterize cell-bound and extracellular proteinases from *Virgibacillus* sp. SK37 and SK33 isolated from fish sauce.

(2) To study the effect of medium composition, NaCl concentration and temperature on proteinase production of *Virgibacillus* sp. SK33, a potential source of NaCl-activated proteinase.

(3) To purify the major proteinase of *Virgibacillus* sp. SK33 responsible for anchovy hydrolysis at high NaCl concentration.

1.3 Research hypothesis

Extremely and moderately halophilic bacteria contribute a significant role in protein hydrolysis during fish sauce fermentation. They can grow and produce proteinase, resulting in protein hydrolysis under high NaCl concentration. Therefore, these bacteria could be used as a starter culture to increase the degree of protein hydrolysis during fish sauce production.

1.4 Expected results

Results from this research will lead to more understandings in the biochemical nature of proteinase produced from moderately halophilic bacteria. Factors affecting proteinase production of *Virgibacillus* sp. SK33 is an important information required to optimize the efficient enzyme production. Knowledge gained from this study could be applied to develop starter cultures for further use in fish sauce fermentation process.

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CHAPTER II

LITERATURE REVIEWS

2.1 Fish sauce

2.1.1 Fish sauce production

Fish sauce is prepared from whole, ungutted anchovy (*Stolephorus* spp.) by mixing fish with solar salt at a ratio 3:1 and stored in a concrete tank for about 12-18 months. Fish sauce fermentation process is shown in Figure 2.1. During fermentation, the soluble proteins are leached out by osmotic effect. In addition, endogenous proteinases are liberated within cells and hydrolyze muscle to soluble proteins (Beddows, 1998). Endogenous proteinases were observed throughout 12 months of fish sauce fermentation (Siringan, Raksakulthai, and Yongsawatdigul, 2006b). Halophilic bacteria found in fish sauce also produced proteolytic enzymes (Thongthai, McGenity, Suninanalert, and Grant, 1992). Thus, endogenous and halophlic bacterial proteinases play an important role in protein hydrolysis of fish sauce fermentation.

2.1.2 Endogenous proteinases during fish sauce fermentation

Endogenous proteinases play a major role in protein hydrolysis of fish sauce (Haard and Simpson, 1994), while the enzymes from microorganisms may assist during fermentation process (Gildberg, 1993; Saisithi, 1994; Beddows, 1998).

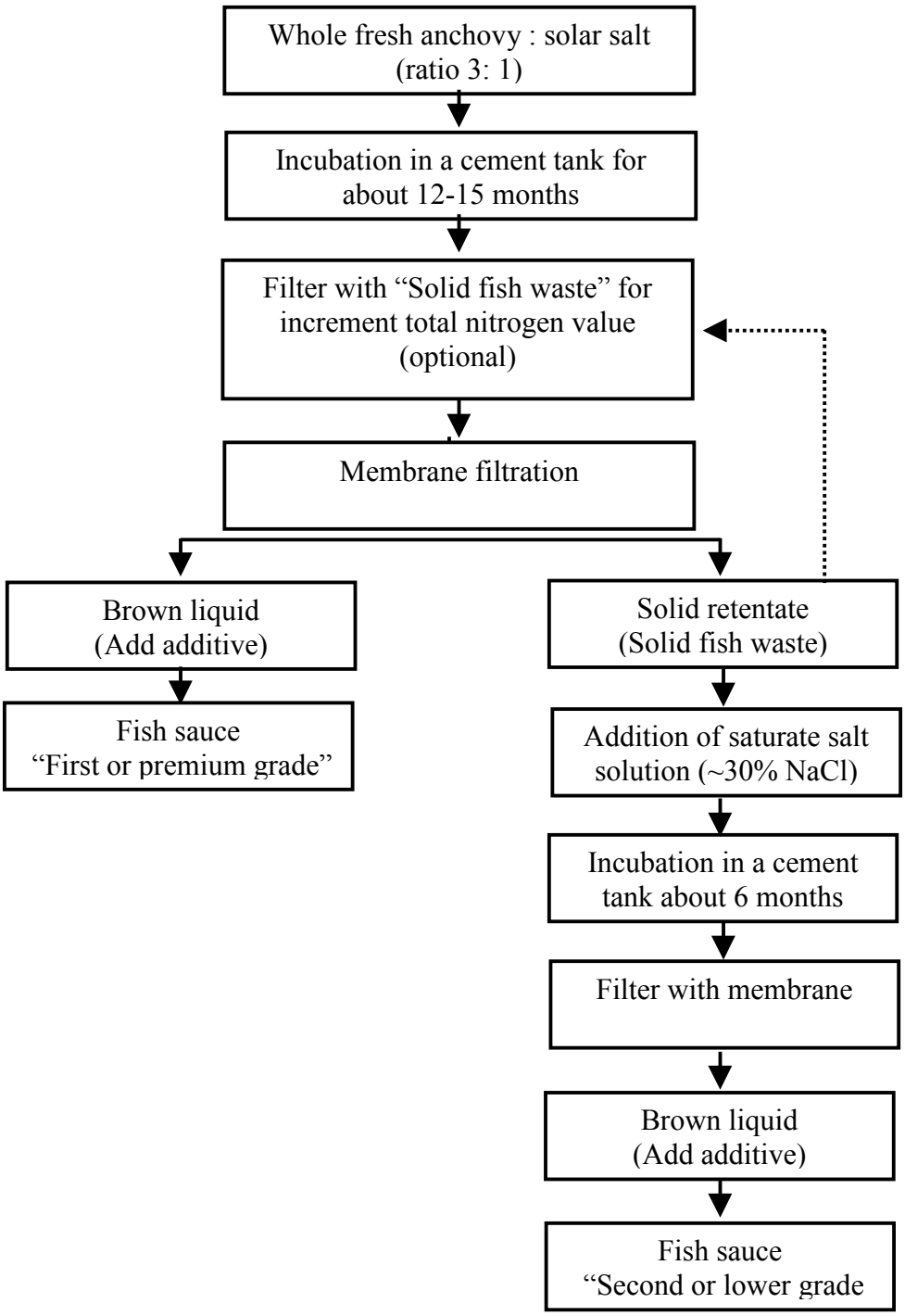

Figure 2.1 Fish sauce fermentation process

Noda, Vo-Van, Kusakabe, and Murakami (1982) showed that alkaline proteinase III from the pyloric caeca and acid proteinase II from stomach of sardine were stable for three months during fermentation at 22% salt at 30 ºC. Gildberg and Shi (1994) reported that chymotrypsin was the most important proteinase during fermentation of Atlantic cod (*Gadus morhua*) fish sauce, whereas the elastase activity decreased rapidly at the initial stage of fermentation. Vo-Van, Kusakabe, and Murakami (1984) showed that the aminopeptidase activity from fresh sardine (*Sardinops melanosticta*) intestinal organs was high for the first 2 months and decreased thereafter. Orejana and Liston (1981) found that endogenous trypsin-like proteinase mainly involved in the first month of patis (fish sauce produced in the Philippines) fermentation. Although endogenous proteinase was found during fish sauce fermentation, the activity was minimum. Endogenous proteinases from Indian anchovy (*Stolephorus spp.*) showed optimal temperature at 60 °C and pH 3.0 and 8.5-9.5 (Siringan, Raksakulthai, and Yongsawatdigul, 2006a). Heu, Pyeum, Kim, Cho, and Godber (1991) reported that the optimum activity of anchovy (*Engravlis japonica*) viscera was at 48 °C and pH 9.5. Moreover, high level of salt reduces the proteolytic activities. Noda et al. (1982) reported that alkaline proteinase activity decreased with an increased salt content. Typically, fish sauce fermentation takes place at 30-35 °C, 25-30% NaCl and pH 5.5-6, which is not an optimal condition of endogenous proteinases. Thereby, fish sauce fermentation is extremely long fermentation process.

2.1.3 Bacteria in fish sauce fermentation

The action of endogenous proteinases results in amino acids and peptides, which are nutrients for bacterial growth during fermentation (Saisithi, 1994). Saisithi,

Kasemsarn, Liston, and Dollar (1966) isolated *Bacillus, Streptococcus, Micrococcus, Staphylococcus* and coryneforms from nampla fermented for 9 months. These bacteria produced volatile fatty acids when grown in culture medium. Lopetcharat and Park (2002) found species of *Staphylococcus, Bacillus,* and *Micrococcus* in Pacific whiting (*Merluccius productus*) fish sauce. Beddows and Ardeshir (1979b) claimed that bacteria were involved in aroma development of budu fermentation. Budu with addition of antimicrobial agents did not possess typical sensory characteristics of fish sauce. Bacteria found in fish sauce fermentation play a major role, not only in aroma development but also involvement of protein hydrolysis. Numerous studies showed that bacteria can produce proteinase. Norberg and Hofsten (1969) showed that halobacterium strains isolated from sea salt produced an extracellular proteinase which could hydrolyze gelatin and casein. Strains of *Bacillus subtilis* JM-3 isolated from fish sauce fermentation produced acid proteinase (Kim and Kim, 2005). *Filobacillus* sp. RF2-5, *Bacillus subtilis* CN2 and *Halobacillus* sp. SR5-3 produced serine proteinase (Uchida et al., 2004; Hiraga et al., 2005; Namwong et al., 2006). Chaiyanan et al. (1999) reported that strains of *Halobacillus* spp. secreted serine and metalloproteinases. These studies indicated that extremely and moderately halophilic bacteria might contribute to protein hydrolysis during fish sauce fermentation.

2.1.4 Acceleration of fish sauce fermentation

Several methods have been proposed to accelerate fish sauce fermentation process. Raising fermentation temperature to about 45 ºC for a couple of week at low salt concentration reduced fermentation time from 1 year to 2 months (Gildberg,

1993). In addition, the fermentation time was reduced to 2 months by adjusting pH to 11 at low salt concentration to activate the alkaline digestive proteinases and inactivate endogenous trypsin and chymotrypsin inhibitors. Flavor of the finished product was comparable to that of the traditional fish sauce (Díaz-López and García-Carreño, 2000). Beddows and Ardeshir (1979b) produced fish sauce using hydrochloric acid, however, the acidified fish sauce had very little aroma and flavor. Gildberg, Hermes, and Orejana (1984) accelerated autolysis at the initial stage by acidifying anchovies (pH 4) (*Stolephorus spp.*) in the presence of 5% NaCl at 40 °C for 5 days and it was found that flavor of the product was acceptable after incubation for 2 months. In addition, several researchers have studied the addition of exogenous enzymes for protein hydrolysis. Quaglia and Orban (1987) reported that Alcalase and commercial papain were suitable for protein hydrolysis of sardine (*Sardina pilchardus*), resulting in high solubility and nitrogen recovery. Rebeca, Peña-Vera, and Díaz-Castañeda (1991) used bacterial proteinases to hydrolyze fish (*Mugil cephalus*) proteins and suggested that proteins were solubilized faster with Pescalase 560 (*Bacillus* proteinase) than HT-200 (*Bacillus* proteinase) and Proteinase N (*Bacillus* proteinase). Beddows and Ardeshir (1979a) used bromelain to obtain fish sauce within 18 to 21 days. The accelerated product contained comparable nitrogen compounds to the traditional fish sauce, but had very little aroma. Male capelin (*Mallotus villosus*) fish sauce was produced rapidly by adding squid hepatopancreas tissue (Raksakulthai, Lee, and Haard, 1986). Squid hepatopancreas added fish sauce showed higher acceptance than commercial product from the Philippines. However, these approaches have never been successfully adopted by the industry due to the high cost of enzyme.

2.2 Halophilic extracellular proteinases

Bacteria that can grow in saline environment are divided into two types. Firstly, halophilic archaea accumulate salt within their cytoplasms to maintain an osmotic balance against hypersaline environment. Intracellular and extracellular enzymes function in the presence of salts. Secondly, halophilic or halotolerant eubacteria maintain an osmotic balance within cytoplasm by accumulating high concentration of various organic osmotic solutes (e.g. sugar, amino acids, and ectoines). Consequently, salt content within intracellular is low and intracellular enzyme is inactive at high salt content (Margesin and Schinner, 2001). For this reason, proteinases from halophilic and nonhalophilic bacteria showed different structural aspects and biochemical characteristics.

2.2.1 Catalytic mechanism

Most halophilic proteinases have been identified as serine proteinase. Amino acid sequences of halophilic proteinase were homologous with subtilisin-type serine proteinase (Völkl, Markiewicz, Stetter, and Miller, 1994). The proteinase showed the conserved catalytic regions, especially catalytic triad of Ser221, His64 and Asp32. It might conclude that evolution of halophilic proteinase related to eubacterial subtilisin (Stepanov et al., 1992). Therefore, catalytic mechanism of halophilic proteinase could be similar to that of subtilisin. In subtilisin, Ser221 attacks a peptide bond to form tetrahedral intermediate. The tetrahedral intermediate decomposes to release the polypeptide chain on the amino side of peptide bond (C-terminal segment). The acyl-enzyme intermediate is rapidly deacrylated to form a new tetrahedral intermediate.

Subsequently, carboxyl group (as new C-terminal of product) of substrate dissociates from enzyme, resulting in N-terminal segment (Voet and Voet, 2004).

2.2.2 Structural feature under salt conditions

Nonhalophilic proteinase was usually inactive under high salt concentration. NaCl, an antichaotropic salt, tends to stabilize enzyme structure (Arakawa and Timasheff, 1982). NaCl increases the intramolecular hydrophobic interaction and surface tension of water lending to folded state. Surface tension of water increases with NaCl, resulting in exclusion of water around protein surface and protein aggregation. Degree of aggregation is correlated with an increase of NaCl concentration (Arakawa and Timasheff, 1982). Hence, active site of nonhalophilic proteinase would be disrupted by aggregation, resulting in reduction of catalytic activity in the presence of high NaCl concentration.

Proteinases from halophilic bacteria showed different characteristics in the presence of NaCl. Typically, they required 1-4 M NaCl (5.8-23.3%) for catalytic activity and stability. The enzymes are irreversibly inactivated at lower salt concentrations. Halophilic proteinases usually contained more acidic amino acids than nonhalophilic proteinases. *Halobacterium halobium* proteinase contained ~23.1% aspartic, glutamic residues, and their amides per molecule, whereas subtilisin BPN' from *Bacillus amyloliquefaciens* contained only ~15.6% per molecule (Stepanov et al., 1992). Glu243Arg mutant halophilic malate dehydrogenase required higher NaCl for enzyme stability than did wild-type, indicating that acidic amino acid would play an important role in halophilic protein characteristics (Madern, Pfister, and Zaccai, 1995). Excess acidic residues of halophilic proteinase are normally found

on the surface (Rao and Argos, 1981). This attracted the hydrated salts and water molecules into its surface (Rao and Argos, 1981; Madern et al., 1995). Thereby, halophilic proteinase is active at high NaCl content.

Generally, nonhalophilic protein showed high stability in the absence of salt because of hydrophobic effect. Nonpolar groups decrease the entropy of water creating hydrophobic interaction of enzyme structure. Halophilic proteins at low salt content underwent denaturation with loss of secondary and tertiary structure (Madern et al., 1995). This is because halophilic protein had a weak hydrophobic core compared to nonhalophilic protein (Lanyi, 1974). In the absence of salt, nonpolar interior of halophilic protein was exposed lending to loss stability. These suggested that hydrophobic core was insufficient to stabilize the native protein, thereby salting out agent was required to interact with protein surface resulting in an increase in hydrophobicity of core structure. Thus, halophilic proteinases required high NaCl concentration for their catalytic activity.

In addition, the two cysteine residues of halophilic proteinase from *Haloferax mediterranei* R4 (halolysin R4) located on C-terminal region which was not found in subtilisin showed a significant role in catalytic activity. The activity of both mutated halolysin R4 cysteine 316 substituted with serine (Cys316Ser) and two cysteines 316/352 substituted with serine (Cys316Ser/Cys352Ser) proteinases decreased to ~30% of the original activity at 25% NaCl, whereas wild-type proteinase remained active (Kamekura, Seno, and Dyall-Smith, 1996). Therefore, cysteine residues on C-terminal region of halophile proteinase might play an important role in reinforcing conformation stability in the presence of NaCl.

2.2.3 Biochemical characteristics

Biochemical properties of halophilic proteinase are summarized in Table 2.1. Normally, optimal activity of halophilic proteinases was in alkaline range. Molecular weight (MW) of halophilic proteinase was larger than subtilisin (~27 kDa) (Ballinger and Wells, 1998).

Table 2.1 Biochemical characteristics of extracellular serine halophilic proteinases

Species	kDa	pI	Preferred residue at P_1	Optimum activity		References
				T (^oC)	pH	
Desulfurococcus mucosus	52	8.7	Tyr, Ala, Ser, Arg	100	7.2	Cowan et al. (1987)
Haloferax mediterranei R4	41	4.08	Leu, Tyr, Phe	-	-	Kamekura and Seno (1993, 1996)
Halobaterium mediterranei	41	7.5	Leu	55	8-8.5	Stepanov et al. (1992)
Halobacterium halobium S9	71	-	Phe	40	8.7	Capiralla et al. (2002)
Halobacterium halobium	41	-	Leu	-	8-9	Izotova et al. (1983)
Halobacterium sp. PB407	-	-	-	40	8	Kanlayakrit et al. (2004)
Pyrobaculum aerofilum	33	6.57	-	>100	Neutral to alkaline	Völkl et al. (1994)
Pyrococcus abyssi 549	150+ 105+60	--	Leu, Phe	95	9	Dib et al. (1998)
Natrialba magadii	45	-	Phe, Tyr, Leu	60	8-10	Giménez et al. (2000)

2.3 Cell-bound proteinases

Cell-bound proteinase is an enzyme which associated with cell envelope through a membrane anchor and cell wall-spanning region (Reid and Coolbear, 1998). This type of proteinase has been founded in *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

(Siezen and Leunissen, 1997; Reid and Coolbear, 1998). Cell-bound proteinase was different among strains. Cell-bound proteinase from *Lactobacillus halveticus* showed different activities including casein hydrolase, aminopeptidase, dipeptidase and esterase (Ezzat, El Soda, and El Shafei, 1993), whereas only casein hydrolase was reported in *Lactobacillus casei* cell-bound proteinase (El Soda, Desmazeaud, Le Bars, and Zevaco, 1986). Studies of cell-bound proteinases have been focused in lactic acid bacteria which are starter cultures for cheese production and other fermented milk products. This proteinase is a key enzyme in the initial stage of casein degradation to provide peptides for translocation into the cell and to contribute flavor development of cheese (Ezzat et al., 1993; Bockelmann, 1995; Reid and Coolbear, 1998).

2.3.1 Location of cell-bound proteinases

Cell envelope is a part that separates cell from environment. Cell membrane directly surrounds the cytoplasm of cell, while cell wall envelops the membrane (McKane and Kandel, 1985). Lipid bilayer of cell membrane is amphipatic lipids. C-terminal of polypeptide chain of cell-bound enzyme might attach to the surface of membrane or embed in the lipid layer. C-terminal region embedding in an integral part of membrane could be abundant non-polar amino acids, which can form hydrophobic interaction with lipids. On the other hand, C-terminus binding to peripheral part of membrane might be rich in polar amino acids, which can form electrostatic or hydrogen bond with polar head of lipid or integral protein (Palmer, 1991). C-terminal region was an important part in anchoring cell-bound proteinase to cell envelope. Deletion of C-terminus from cell-bound proteinase resulted in a liberation of the enzyme (Haandrikman et al., 1989; Vos, van Asseldonk, et al., 1989).

Cell-bound proteinase without C-terminus did not change the activity and substrate specificity as compared to the intact cell-bound proteinase (Kok, Leenhouts, Haandrikman, Ledeboer, and Venema 1988).

Cell-bound proteinase is encoded by gene, for example, gene from *Lactoccoccus lactis*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lb. helveticus* is referred to *prtP*, *prtB*, *prtS* and *prtH*, respectively. C-terminus of cell-bound proteinase encoded by *prtP* and *prtS* genes contained two regions of α-helix part and hydrophilic tail. α-Helix region of cell-bound proteinase from *Lactococcus lactis* SK11 contained 18-20 hydrophobic amino acids anchoring in the cell membrane (Vos, Simons, Siezen, and de Vos, 1989). Hydrophilic tail contained positively charge residues which interacted with negatively charged phospholipids of cell membrane (Piard et al., 1997). In addition, C-terminus had proline, glycine, threonine and serine rich region that bound to peptidoglycan (Pancholi and Fischetti, 1988; Rathsam and Jacques, 1998). Thus, cell-bound proteinases encoding by *prtP* and *prtS* genes embedded into lipid bilayer membrane and cell wall. However, α-helix part and hydrophilic tail were not observed in cell-bound proteinase from *prtB* and *prtH* genes encoding (Germond, Delley, Gilbert, and Atlan, 2003). C-terminus of this proteinase contained lysine rich region, which formed interaction with negative charges of teichoic acids of cell wall (Gilbert et al., 1996; Germond et al., 2003). Therefore, PrtB (expressed from *prtB*) and PrtH (expressed from *prtH*) cell-bound proteinases might only attach to the cell wall.

2.3.2 Isolation of cell-bound proteinases

Typically, low ionic strength, sonication, freezing and thawing have been accomplished to liberate cell-bound enzymes which attached to polar groups of membrane (Penefsky and Tzagoloff, 1971). Moreover, detergent and organic solvent have successfully been used to solubilize cell-bound enzyme which embedded to lipid bilayer (Penefsky and Tzagoloff, 1971). Cell-bound proteinase from *Lactobacillus helveticus* L89 was extracted by Triton X-100 and Nonidet P40 (Martín-Hernández, Alting, and Exterkate, 1994). However, removal of cell-bound proteinase from *Streptococcus thermophilus* CNRZ 385 required culture in a medium containing glycine because glycine inhibited peptidoglycan formation by loosening cell wall structure (Fernandez-Espla, Garault, Monnet, and Rul, 2000).

Occasionally, autolysis and proteolytic enzyme (e.g. trypsin treated) have been applied to remove cell-bound proteinase from membrane (Wilk, 2001). Cell-bound proteinase from *Lactococcus lactis* sups. *cremoris*, *Brevibacterium linens*, *Propionibacterium acidipropionici*, *Pediococcus* sp., *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Bifidobacterium infantis*, *Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Streptococcus lactis* and *Streptococcus cremoris* have been successfully removed from cell membrane by autoproteolytic process (Thomas, Jarvis, and Skipper, 1974; Mills and Thomas, 1978; El Soda et al., 1986; Laan and Konings, 1989; Kojic, Fira, Banina, and Topisirovic, 1991; Ezzat et al., 1993). Cell-bound proteinase in the presence of Ca^{2+} showed stable conformation and destabilized by self-digestion at C-terminal after Ca^{2+} removal. Thus, autolytic process liberated free cell-bound proteinase and C-terminal region attaching at cell envelope (Laan and Konings, 1989; Exterkate and Alting, 1999). Interestingly, free cell-bound proteinase

still possessed proteolytic activity. Therefore, MW of free cell-bound proteinase was smaller than that of cell-bound proteinase. For instance, cell-bound proteinase of *Lactococcus lactis* SK11 had a MW of 187 kDa, but that of soluble form resulted from autolysis was 135 kDa (Vos, Simons, et al., 1989). In addition, cell-bound proteinase from *Streptococcus cremoris* HP was easily removed by stirring intact cell with buffer (pH 6.5) at 25 °C for 30 min (Exterkate and de Veer, 1985). Since C-terminus of cell-bound proteinase associated to membrane by a Ca^{2+} bridge, diffusion of Ca^{2+} dissociated cell-bound proteinase to a soluble form (Exterkate and de Veer, 1985; Coolbear, Reid, and Pritchard, 1992).

2.3.3 Conversion of inactive form to active cell-bound proteinases

Lactococcal cell-bound proteinase was initially synthesized as an inactive form that was called pre-pro-proteinase (Gilbert et al., 1996). During or after translocation pre-pro-proteinase across cell membrane, N-terminal signal peptide of the pre-pro-proteinase was removed to convert into an inactive pro-proteinase, which bound to cell envelope. N-terminal region of inactive pro-proteinase contained PrtM region and prosequence (Haandrikman et al., 1991). PrtM region induced self-digestion for removal prosequence, leading to a conversion of the inactive form to an active proteinase (Vos, van Asseldonk, et al., 1989; Haandrikman et al., 1991). N-terminus (PrtM and prosequence region) was removed, thereby MW of active proteinase was less than the inactive pro-proteinase about 20 kDa (Haandrikman et al., 1991; Laan and Konings, 1991). MW of inactive proteinase from *Lactococcus lactis* SK11 and *Lactococcus lactis* subsp. *lactis* showed 206 and ~185 kDa, whereas active proteinase showed 187 and ~165 kDa, respectively (Vos, Simons, et al., 1989).

2.3.4 Biochemical characterization of cell-bound proteinase

Cell-bound proteinases from various species including *Lactobacillus helveticus* CNRZ 303, *Lactobacillus casei* NH14, *Lactobacillus casei* NCDO 151 and *Pediococcus* sp. LR were serine proteinase (Ezzat, El Soda, and El Shafei, 1988; Kojic et al., 1991; Ezzat et al., 1993). Cell-bound proteinase from *Lactococcus* sp., *Streptococcus* sp. and *Lactobacillus* sp. was a member of subtilisin family (Fernandez-Espla et al., 2000; Siezen and Leunissen, 1997). Their structures might contain at least three Ca^{2+}-binding loops, which reinforced conformation stability (Siezen and Leunissen, 1997; Reid and Coolbear, 1998; Exterkate and Alting, 1999). Structure of streptococcal cell-bound proteinase lacked Ca^{2+} binding sites (Fernandez-Espla et al., 2000). However, activity of cell-bound proteinase from *Streptococcus thermophilus* CNRZ 385 was increased ~6.5 times at 5 mM Ca^{2+} (Shahbal, Hemme, and Renault, 1993). Typically, Ca^{2+} can activate and stabilize soluble form of cell-bound proteinase at low concentration (~1-5 mM), but higher concentration of Ca^{2+} showed inhibitory effect (Reid and Coolbear, 1998). Activity of cell-bound proteinase from *Lactococcus lactis* SK11 decreased in concomitant with thermal stability when Ca^{2+} was removed (Exterkate and Alting, 1999). Proteinase from *Streptococcus cremoris* AC1 was rapidly inactivated at >1 mM Ca^{2+} (Geis, Bockelmann, and Teuber, 1985). In contrast to these findings, Ca^{2+} was ineffective to stabilize conformation for cell-bound proteinase from *Lactobacillus casei* subsp. *casei* IFPL 731 (Fernández de Palencia, Peláez, Romero, and Martín-Hernández, 1997). In addition, activity of cell-bound proteinase from *Lactobacillus helveticus* L89 was slightly inhibited by Ca^{2+} up to 10 mM (Martín-Hernández et al., 1994).

MW of cell-bound proteinases in a soluble form ranged from 45 to 180 kDa (Table 2.2). These were larger than subtilisin having MW of ~27 kDa (Ballinger and Wells, 1998). Most of cell-bound proteinases showed optimum pH in acidic to neutral range (Table 2.2). Isoelectric point (pI) of cell-bound proteinases was in acidic range. Cell-bound proteinase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397, *L. casei* NCDO 11 and *Streptococcus cremoris* AC1 showed pI of 4.5-4.75, 4.8 and 4.6 respectively (Geis et al., 1985; Laloi, Atlan, Blanc, Gilbert, and Portalier, 1991; Næs, Chrzanowska, and Blom, 1991). Casein specificity of cell-bound proteinase varied with strains. Cell-bound proteinase from *Lactobacillus casei* NH14, *L. lactis* subsp. *lactis* NCDO 763, *Streptococcuscremoris* HP, *S. cremoris* C_{13}, *S. cremoris* E_8 and *S. cremoris* AC1 preferentially hydrolyzed β-casein (Exterkate and de Veer, 1985; Geis et al., 1985; Visser, Exterkate, Slangen, de Veer, 1986; Monnet, Bockelmann, Gripon, and Teuber, 1989; Kojic et al., 1991), whereas *Streptococcus cremoris* AM1 and *S. cremoris* SK11 showed proteolytic activity towards α_{s-1}-, κ-, and β-caseins (Visser et al., 1986).

Table 2.2 Biochemical characteristics of cell-bound proteinase from various bacteria.

Species	Classification	Optimum activity		kDa	Casein hydrolysis	References
		T (^{o}C)	pH			
Lactobacillus casei NCDO	Serine	40	7	-	-	Ezzat et al. (1988)
	Serine-matallo	45	7.5	-	-	
L. casei subsp. casei IFPL 731	Serine	40	6	150	$\beta > \alpha_{s1}$	Fernández de Palencia et al. (1997)
L. casei NCDO 151	Serine	35-37	4.8[a] and 5.6[b]	36×4	-	Næs et al. (1991)
L. bulgaricus NCDO1489	Metallo	45-50	5.2-5.8	-	-	Argyle et al. (1976)

[a] determined with hemoglobin and [b] casein as a substrate

Table 2.2 (Continued)

Species	Classification	Optimum activity		kDa	Casein hydrolysis	References
		T (°C)	pH			
L. delbrueckii subsp. *lactic* ACA-DC 178	Serine	40	6	-	β > α and κ	Tsakalidou et al. (1999)
L. delbrueckii subsp. *bulgaricus* CNRZ 397	Cysteine	42-45	5.5-6	170	β > α	Laloi et al. (1991)
L. helveticus L89	Serine	50	7	180	α_{s-1} and β	Martín-Hernández et al. (1994)
Streptococcus thermophilus CNRZ 385	Serine	37	7	153	β > α_{s1} > whole	Shanbal et al. (1993); Fernandez-Espla et al. (2000)
and CNRZ 703	Serine	45	7	-	β > α_{s1} > whole	
S. cremoris AC1	Serine	40	5.5-6	145	β	Geis et al. (1985)
S. lactis NCDO 763	Serine	32-38	4.8[a] and 6-6.5[b]	80	-	Monnet et al.(1987)

[a] determined with hemoglobin and [b] casein as a substrate

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CHAPTER III

NaCl-ACTIVATED EXTRACELLULAR PROTEINASE FROM *VIRGIBACILLUS* SP. SK37 ISOLATED FROM FISH SAUCE FERMENTATION

3.1 Abstract

Virgibacillus sp. SK37 showed large clear zone on skim milk agar containing 25% NaCl and exhibited high extracellular proteolytic activity in skim milk broth containing 10% NaCl. Optimum conditions of the crude proteinase were at pH 8.0 and 65 °C. The proteinase was strongly inhibited by phenylmethanesulfonyl fluoride (PMSF) and preferably hydrolyzed Suc-Ala-Ala-Pro-Phe-AMC, suggesting the serine proteinase with a subtilisin-like characteristic. Proteolytic activity increased with NaCl concentration up to 20%. Ca^{2+} activated the enzyme activity but reduced enzyme stability at 65 °C. Several proteinases with dominant molecular mass (MW) of 81, 67, 63, 50, 38 and 18 kDa were detected on native-polyacrylamide gel electrophoresis (native-PAGE) activity staining in the absence and presence of 25% NaCl. These results demonstrated that *Virgibacillus* sp. SK37 produced salt-activated extracellular proteinases.

Key word: *Virgibacillus* sp., bacterial proteinase, NaCl-activated proteinase, fish sauce

3.2 Introduction

Moderately halophilic bacteria can grow well at relatively high salt concentration (3-14.5% NaCl), and show a reduced growth rate at higher NaCl (Kushner, 1992). These bacteria along with thermophilic and alkaliphilic are extremophile microorganisms which have numerous potential for biotechnology application (Margesin and Schinner, 2001). In addition, halophilic bacteria are a valuable source of proteinase for enzymology studies (De Castro, Maupin-Furlow, Giménez, Herrera Seitz, and Sánchez, 2006). Several proteinases from moderate halophiles have been characterized. Activity of chymotrypsin-like proteinase from marine psychrophile PA-43 was activated by NaCl (Irwin, Alfredsson, Lanzetti, Gudmundsson, and Engel, 2001), whereas activity of serine proteinase from *Salinivibrio costicola* 18AG decreased above 2% NaCl (Lama, Romano, Calandrelli, Nicolus, and Gambacorta, 2005). Serine metalloproteinase was characterized from *Pseudoalteromonas* sp. CP76 (Sánchez-Porro, Mellado, Bertoldo, Antranikian, and Ventosa, 2003), while *Salinivibrio* sp. AF-2004 produced a zinc-metalloproteinase (Karbalaei-Heidari, Ziaee, Schaller, and Amoozegar, 2007). Based on these reports, moderate halophiles secreted different proteinases. Although numerous proteinases from moderately halophilic bacteria isolated from hypersaline lake, salt spring, salterns soils and saline soils have been characterized, proteinases from bacteria isolated from fish sauce fermentation have been rarely studied.

Fish sauce is a popular condiment consumed in Southeast Asia. It is produced by a natural fermentation involving addition of salt to uneviscerated fish at a ratio of approximately 1:3. Since fermentation takes place under high salt content (~30% NaCl), proteolytic activity derived from fish muscle and digestive tracts inevitably

decreased. Therefore, an increase in total nitrogen and amino acid content was extremely slow requiring about 12-18 months for the complete fermentation. Acceleration of protein hydrolysis of fish sauce fermentation has been a challenge. A variety of moderately halophilic bacteria was found during fish sauce fermentation, such as *Filobacillus* sp., *Lentibacillus salicampi*, *L. juripiscarius* and *Halobacillus* sp. (Hiraga et al., 2005; Namwong et al., 2005; Namwong et al., 2006). These bacteria showed proteolytic activity. Therefore, starter culture to increase the degree of protein hydrolysis during fish sauce production might be a method of choice to reduce fermentation period.

Strain SK37 isolated from fish sauce fermented for 1 month showed the highest proteinase activity among 165 strains tested. It was identified to be *Virgibacillus* sp. SK37 according to 16S rRNA gene sequence. *Virgibacillus* sp. SK37 was a Gram-positive/variable, long rod of 0.6-0.7 x 3.0-6.6 μm, non-motility and terminal or subterminal ellipsoidal spores. It can grow at wide pH range of 4-11 and 20-45 °C. Preliminary results showed that *Virgibacillus* sp. SK37 grew and hydrolyzed anchovy at 25% NaCl. The objective of this study was to characterize extracellular proteinase from *Virgibacillus* sp. SK37.

3.3 Materials and methods

3.3.1 Chemicals

t-Butyloxycarbonyl(Boc)-Asp(oBzl)-Pro-Arg-4-methyl-7-coumarylamides (AMC), succinyl (Suc)-Ala-Ala-Pro-Phe-AMC and carbobenzoxy (Z)-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Z-Arg-Arg-AMC, leupeptin, trypsin inhibitor I

(soybean), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), bestatin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidine)-butane (E-64), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), dithiothreitol (DTT), 2-mercaptoethanol (β-ME), bovine serum albumin, L-tyrosine and casein were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Ethylenediaminetetraacetic acid (EDTA), L-cysteine and L-histidine were purchased from Fluka (Buchs, Switzerland).

3.3.2 Preparation of crude proteinase

Virgibacillus sp. SK37 was cultivated in neopeptone broth (NEO, 0.5% neopeptone, 1% $MgSO_4 \bullet 7H_2O$, 0.2% KNO_3, 0.0005% ferric citrate and 1% glycerol) containing 10% NaCl at 35 °C, 100 rpm, for 3 days. Crude extracellular proteinase was collected by centrifuging the cultivated broth at 8,000×g for 30 min, at 4 °C (RC 28S, Sorvall Co., Newtown, Conn., USA). Crude proteinase was dialyzed against 20 mM Tris-maleate (pH 7.0) using dialysis bag with molecular weight cut-off (MWCO) of 10 kDa (Pierce Chemical Company, Rockford, IL USA).

3.3.3 Proteinase activity assay

Proteinase activity was assayed using azocasein (Sigma Chemical Co.; St. Louis, Mo., USA) as a substrate according to the method of An, Seymour, Wu, and Morrissey (1994). Crude proteinase (500 μL) was added to the pre-incubated reaction mixture containing 2 mg azocasein and 200 mM Tris-HCl (pH 8.0), in a final volume of 1 mL. The reaction was incubated at the optimal temperature for 30 min, then

stopped by adding 500 μL cold 50% trichloroacetic acid (TCA). Unhydrolyzed protein was precipitated at 4°C for 15 min and centrifuged at 10,000×g for 10 min (Eppendorf AG 22331, Hamburg, Germany). The supernatant, 1 mL, was added to 100 μL of 10 N NaOH and the absorbance was determined at 450 nm (GBC UV/VIS 916, GBC Scientific Equipment PTY, LTD., Australia). Blank determinations were conducted in the same manner except that the enzyme solution was added after TCA solution. Proteolytic activity was calculated as the difference in absorbance at 450 nm between sample and blank (ΔA_{450}).

3.3.4 Temperature and pH optimum

Effect of temperature on proteinase activity was carried out at 4 to 80 °C in 50 mM Tris-maleate (pH 7.0) by the method of An et al. (1994), using azocasein as substrate as described above. pH profile was measured at 65 °C at various pHs: pH 5, 5.5, 6 using 100 mM sodium acetate; pH 6.5, 7 using 50 mM Tris-maleate; pH 7.5, 8, 8.5, 9 using 200 mM Tris-HCl; and pH 9.5, 10, 11 using 200 mM glycine-NaOH.

3.3.5 Effect of inhibitors and ions on proteinase activity

The effect of inhibitors including leupeptin, trypsin inhibitor I (soybean), TLCK, TPCK, PMSF, EDTA, L-histidine, bestatin, pepstatin A, E-64, NEM, IAA and reducing agents (DTT, β-ME and L-cysteine) on proteinase activity was determined. The effect of metal ions, Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Hg^{2+} and Zn^{2+} was also investigated. The remaining activity was calculated using the activity of the control (without inhibitor) as 100%.

3.3.6 Substrate specificity

Proteinase activity was assayed by the modified method of Barrett and Kirschke (1981) using various synthetic substrates at 1 μM, including Boc-Asp(oBzl)-Pro-Arg-AMC, Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC. The reaction mixture (1 mL) contained 200 μL crude proteinase, 1 μM synthetic substrate and 200 mM Tris-HCl (pH 8.0), and incubated at 65 °C for 10 min. The reaction was stopped by adding 1.5 mL of the stopping solution (30% butanol, 35% methanol and 35% deionized water). Fluorescence intensity was measured at excitation wavelength of 380 nm and emission wavelength of 460 nm (RF-1501, Shimadzu Co., Kyoto, Japan).

3.3.7 Effect of $CaCl_2$ and NaCl on proteinase activity

The effect of $CaCl_2$ (10-100 mM) on proteinase activity was determined by the modified method of Barrett and Kirschke (1981) as described above, using Suc-Ala-Ala-Pro-Phe-AMC as a substrate.

Effect of NaCl on proteinase activity was determined by incubating the proteinase in 200 mM Tris-HCl (pH 8.0) containing various NaCl concentrations (0-20%). In the presence of NaCl, deionized water was used instead of butanol to avoid phase separation. Subsequently, sample was heated at 90 °C for 5 min to terminate the activity.

3.3.8 Activity staining

Activity staining was determined using native polyacrylamide gel electrophoresis activity staining according to García-Carreño, Dimes, and Haard

(1993). Sample containing crude proteinase 0.02 U (nmole of AMC/min), 10% glycerol, 0.063 mM Tris-HCl (pH 6.8), 0.05% bromophenol blue and either 1 mM PMSF, 10 mM EDTA or 10 mM L-histidine was loaded into polyacrylamide gel (12.5% T) and ran at 100 V. Subsequently, it was immersed in 2% casein, 100 mM Tris-HCl, pH 8.0 at 4 °C for 30 min and it was washed twice with 100 mM Tris-HCl (pH 8.0). Proteolytic reaction was carried out in 100 mM Tris-HCl, pH 8.0, containing either no salt or 25% NaCl and incubated at 65 °C for 30 min. Gel was stained in 0.1% Coomassie brilliant blue R-250, 40% methanol and 10% acetic acid for 1 h and it was destained in 25% ethanol and 10% acetic acid. Clear zone indicated the caseinolytic activity.

3.3.9 Effect of $CaCl_2$ on proteinase stability

Effect of $CaCl_2$ on proteinase stability was investigated by pre-incubating the crude enzyme in 200 mM Tris-HCl (pH 8.0) containing various $CaCl_2$ concentrations (0-100 mM) at 65°C for 2 h. When incubation time was reached, samples were immediately cooled in iced water. The relative activity was calculated using activity of sample without incubation at each $CaCl_2$ concentration as 100%.

Proteinase stability was monitored by pre-incubating the crude proteinase in 200 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ or in the absence of $CaCl_2$. Samples were incubated at either 30 °C for 24 h or 65 °C for 4 h. Samples were rapidly cooled and assayed by the modified method of Barrett and Kirschke (1981) as described above.

3.3.10 Molecular weight estimation

Molecular weight (MW) of crude proteinase was estimated using native-PAGE activity staining (García-Carreño et al., 1993) and Ferguson plot analysis (Bollag, Rozycki, and Edelstein, 1996). A semilogarithmic plot between the relative migration value (R_f) and acrylamide concentrations (6, 7, 8, 9, 10 and 12.5% T) was constructed and the regression was analyzed. The negative slopes were plotted against the MWs value of protein standard (Sigma Chemical Co., St. Louis, Mo., USA). The MW of crude proteinase was estimated from a linear relationship with MW of protein standard.

3.4 Results and discussion

3.4.1 Temperature and pH optimum

Proteinase activity of crude proteinase from *Virgibacillus* sp. SK37 increased with temperature and reached maximum activity at 65 °C (Figure 3.1), indicating a characteristic of heat-activated proteinase. Proteinase from moderately halophilic bacteria, *Pseudoalteromonas* sp. CP76, *Salinivibrio costicala* 18AG, marine psychrophile PA-43 and *Salinivibrio* sp. AF-2004 showed optimum temperature at 55-65 °C (Irwin et al., 2001; Sánchez-Porro et al., 2003; Lama et al., 2005; Karbalaei-Heidari et al., 2007). Optimum pH of crude proteinase from *Virgibacillus* sp. SK37 was found at pH 6 and 8 (Figure 3.2). Proteinase from *Pseudoalteromonas* sp. CP76, *Salinivibrio costicala* 18AG, marine psychrophile PA-43 and *Salinivibrio* sp. AF-2004 showed optimum pH of 8-8.5 (Irwin et al., 2001; Sánchez-Porro et al., 2003; Lama et al., 2005; Karbalaei-Heidari et al., 2007). Different optimal pHs suggested that crude enzyme might contain several proteinases. Proteinase from *Bacillus* sp.

Wai 21a also exhibited acid proteinase characteristics with MW of 45 kDa (Prescott, Peek, and Daniel, 1995), while *Bacillus subtilis* JM-3 proteinase showed maximum activity at pH 5.5 with MW of 17.1 kDa (Kim and Kim, 2005).

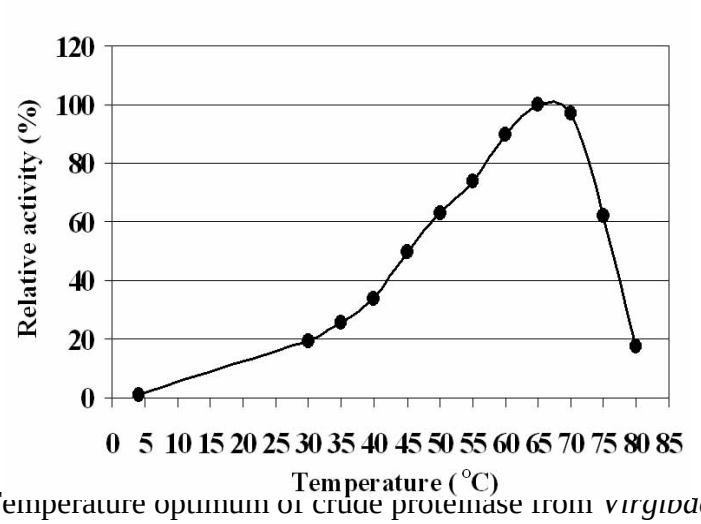

Figure 3.1 Temperature optimum of crude proteinase from *Virgibacillus* sp. SK37

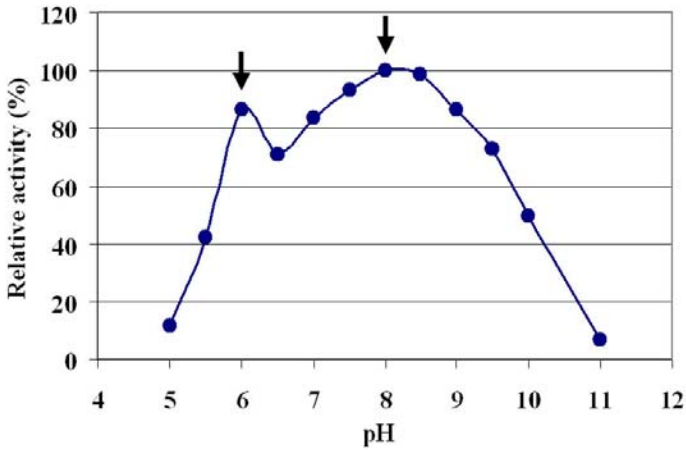

Figure 3.2 pH optimum of crude proteinase from *Virgibacillus* sp. SK37

3.4.2 Effect of inhibitors and ions on proteinase activity

Proteinase activity of *Virgibacillus* sp. SK37 was strongly inhibited by PMSF, a serine proteinase inhibitor (Table 3.1). A chymotrypsin-like proteinase inhibitor, TPCK, did not inhibit proteolytic activity. Trypsin-like proteinase inhibitors (leupeptin, soybean inhibitor and TLCK) and cysteine inhibitors (E-64, NEM and IAA) did not affect proteinase activity. Metalloproteinase inhibitors showed

moderate inhibition. Based on these results, crude proteinase from *Virgibacillus* sp. SK37 was serine proteinase. Bacteria isolated from fish sauce fermentation secreted various proteinases including serine proteinase from *Bacillus subtilis* CN2, *Halobacillus* sp. SR5-3 and *Filobacillus* sp. RF2-5, acid proteinase from *Bacillus subtilis* JM-3 and metalloproteinase from *Halobacillus thailandensis* sp. (Chaiyanan et al., 1999; Uchida et al., 2004; Hiraga et al., 2005; Kim and Kim, 2005; Namwong et al., 2006).

Activity of *Virgibacillus* sp. SK37 proteinase was not affected by various metal ions, except Hg^{2+}, which showed a slight inhibitory effect (Table 3.1). Proteinase from γ-Proteobacterium DGII and *Brevibacterium linens* ATCC 9174 was inhibited by Cu^{2+}, Co^{2+}, Mn^{2+}, Hg^{2+} and Zn^{2+} (Rattray, Bockelmann, and Fox, 1995; Sana, Ghosh, Saha, and Mukherjee, 2006), whereas *Salinivibrio costicala* 18AG proteinase was activated by Cu^{2+} and Mn^{2+} (Lama et al., 2005). Monovalent cations (Li^{+}, Na^{+} and K^{+}) did not reduce proteinase activity, but divalent cations, Mg^{2+} and Ca^{2+}, showed moderate activation (Table 3.1). Proteinase activity of *Bacillus pumilus*, *Bacillus* sp. NCDC 180 and *Brevibacterium linens* ATCC 9174 also slightly increased with Ca^{2+} and Mg^{2+} concentration (Rattray et al., 1995; Kumar, Tiwari, and Jany, 1999; Huang, Peng, Li, Wang, and Zhang, 2003), whereas activity of *Bacillus* sp. Ve1 proteinase decreased at 5 mM Ca^{2+} and Mg^{2+} (Gupta et al., 2005). Activity of *Bacillus mojavensis* and *B. licheniformis* AP-1 proteinases were not affected by Mg^{2+} and Ca^{2+} (Beg and Gupta, 2003; Tang et al., 2004). Typically, efficiency of catalytic activity depends upon conformational flexibility (Daniel, 1996). Divalent salt might induce structural changes leading to more flexibility of substrate-binding flaps (Szeltner and Polgár, 1996). However, hydrated salts would form specific

electrostatic interaction on protein surface and active site of the enzyme (Inouye, Lee, Nambu, and Tonomura, 1997). Therefore, salts might increase or decrease catalytic activity.

Table 3.1 Effect of various inhibitors and ions on enzyme activity of the crude extracellular proteinase from *Virgibacillus* sp. SK37

Substances	Targeted enzyme	Final concentration	Relative activity (%)
Leupeptin	Trypsin-like and some cysteine proteinases	100 μM	80
Trypsin inhibitor I (soybean)	Trypsin-like proteinase	0.02 mg/mL	78
TLCK	Trypsin-like proteinase	100 μM	89
TPCK	Chymotrypsin-like proteinase	100 μM	87
PMSF	Serine proteinase	1 mM	13
EDTA	Metallo proteinase	10 mM	76
L-Histidine	Metallo proteinase	10 mM	87
Bestatin	Aminopeptidase	10 μM	109
Pepstatin A	Acid proteinase	10 μM	100
E-64	Cysteine proteinase	10 μM	100
N-Ethylmaleimide	Cysteine proteinase	1 mM	100
Iodoacetic acid	Cysteine proteinase	1 mM	103
Dithiothreitol		10 mM	90
2-Mercaptoethanol		10 mM	100
L-Cysteine		10 mM	96
Mono- and di-valent cations			
Li^+		10 mM	104
Na^+		10 mM	96
K^+		10 mM	93
Mg^{2+}		10 mM	116
Ca^{2+}		10 mM	118
Metal ions			
Cu^{2+}		1 mM	93
Cd^{2+}		1 mM	113
Co^{2+}		1 mM	95
Mn^{2+}		1 mM	105
Hg^{2+}		1 mM	72
Zn^{2+}		1 mM	92

3.4.3 Substrate specificity

Crude proteinase of *Virgibacillus* sp. SK37 only cleaved Suc-Ala-Ala-Pro-Phe-AMC, suggesting that P_1 of the proteinase preferred aromatic amino acid residue (Table 3.2). Suc-Ala-Ala-Pro-Phe-chromogenic is a typical substrate for subtilisin (Graycar, Ballinger, and Wells, 2004). According to the effect of inhibitor and substrate specificity, the crude proteinase from *Virgibacillus* sp. SK37 showed subtilisin-like characteristics. *Filobacillus* sp. RF2-5 proteinase preferably cleaved phenylalanine, methionine and threonine at P_1 (Hiraga et al., 2005), while *Halobacillus* sp. SR5-3 proteinase efficiently hydrolyzed leucine glutamine, and alanine at P_1 (Namwong et al., 2006). Proteinase from *Bacillus licheniformis* SMI 4.C.1 efficiently hydrolyzed substrate containing phenylalanine and leucine at P_1 (Manachini and Fortina, 1998), while proteinase from *Bacillus thermoruber* was found to hydrolyze Z-Ala-Ala-Leu- *p* nitroanilide (*p*NA), indicating a subtilisin-like characteristic (Manachini, Fortina, and Parini, 1988). In addition, *Bacillus* sp. PS719 proteinase cleaved arginine at P_1, suggesting a trypsin-like proteinase (Hutadilok-Towatana, Painupong, and Suntinanalert, 1999).

Table 3.2 Substrate specificity of crude proteinase from *Virgibacillus* sp. SK37

Synthetic substrates	Specificity	Relative activity (%)
Boc-Asp(oBzl)-Pro-Arg-AMC	Trypsin-like, α-thrombin-like	2
Boc-Gln-Ala-Arg-AMC	Trypsin-like	0
Boc-Val-Leu-Lys-AMC	Plasmin-like	5
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin-like, subtilisin-like	100
Z-Phe-Arg-AMC	Cathepsin L-like	5
Z-Arg-Arg-AMC	Cathepsin B-like	0

3.4.4 Effect of $CaCl_2$ on proteinase activity and stability

Proteinase activity from *Virgibacillus* sp. SK37 increased with Ca^{2+} concentration (Figure 3.3). Activity of *Bacillus* sp. PS719 proteinase increased with Ca^{2+} up to 2 mM (Hutadilok-Towatana et al., 1999). Proteinase from *Salinivibrio costicala* 18AG was increased 2.4 times in the presence of 2 mM Ca^{2+} (Lama et al., 2005), while activity of *Halobacterium halobium* S9 proteinase increased 2 times at 1 mM Ca^{2+} (Capiralla, Hiroi, Hirokawa, and Maeda, 2002). However, proteinase from moderately halophilic bacteria, *Pseudoalteromonas* sp. CP76, was slightly inhibited by 5 and 10 mM Ca^{2+} (Sánchez-Porro et al., 2003). Activity of *Salinivibrio* sp. AF-2004 and *Bacillus subtilis* Y-108 proteinases was not affected by Ca^{2+} (Yang, Shih, Tzeng, and Wang, 2000; Karbalaei-Heidari et al., 2007).

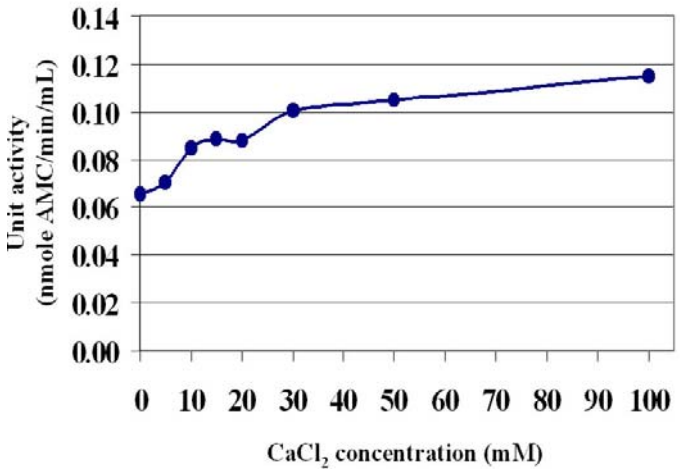

Figure 3.3 Effect of $CaCl_2$ concentration on the crude proteinase activity of *Virgibacillus* sp. SK37

Ca^{2+} decreased thermal stability of *Virgibacillus* sp. SK37 proteinase at higher concentrations (Figure 3.4). The activity of crude proteinase at 100 mM Ca^{2+} was only 30% of the control when incubated at 65 °C for 2 h, whereas 70% residual activity was noticed in the absence of Ca^{2+} (Figure 3.4). In addition, proteinase from

Virgibacillus sp. SK37 was stable at 30 °C in the absence of Ca^{2+} and 10 mM Ca^{2+}, but the enzyme was more unstable at its optimum temperature (65 °C) in the presence of 10 mM Ca^{2+} (Figure 3.5). At 65 °C, activity gradually decreased to 61% of the original at 4 h in the absence of Ca^{2+}, whereas 45% activity was retained in the presence of 10 mM Ca^{2+}. Proteinase activity and stability from marine psychrophile PA-43 was unaffected by Ca^{2+} (Irwin et al., 2001), whereas activity and stability of *Bacillus brevis* proteinase increased with Ca^{2+} (Banerjee, Sani, Azmi, and Soni, 1999). Proteinase activity from *Bacillus mojavensis* was not affected by Ca^{2+}, but thermal stability increased with Ca^{2+} up to 10 mM (Beg and Gupta, 2003). Thermal stability of proteinase from *Bacillus stearothermophilus* F1 increased with Ca^{2+} concentration up to 20 mM (Rahman et al., 1994). Alkaline-stable proteinase from *Bacillus* sp. GX6638 increased the half-life about 10 times when added 2 mM Ca^{2+} (Durham, Stewart, and Stellwag, 1987). Stability of proteinase from *Bacillus* sp. KSM-K16 was increased between 50-70 °C at 5 mM Ca^{2+} (Kobayashi, Hakamada, Hitomi, Koike, and Ito, 1996). *Bacillus cereus* BG1 and *Bacillus* sp. AH-101 proteinases required Ca^{2+} for stability when incubated above 40 and 60 °C, respectively (Takami, Akiba, and Horikoshi, 1989; Ghorbel, Sellami-Kamoun, and Nasri, 2003).

Ca^{2+} plays a major role in enzyme stabilization because it maintains the active conformation of the enzyme at high temperature (Kumar et al., 1999). However, our study revealed that Ca^{2+} activated the activity of *Virgibacillus* sp. SK37 proteinase, but decreased thermal stability at the optimum temperature (65 °C). Ca^{2+} is a chaotropic agent that promotes protein unfolding (Arakawa and Timasheff, 1982).

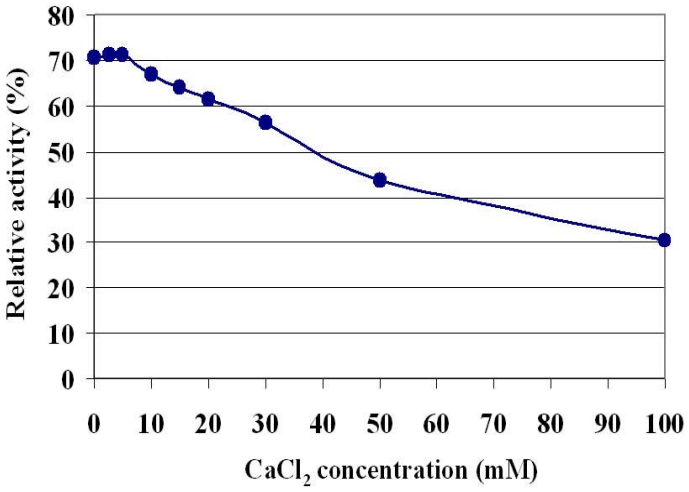

Figure 3.4 Effect of $CaCl_2$ concentration on stability of *Virgibacillus* sp. SK37 proteinase incubated 65 °C for 2 h

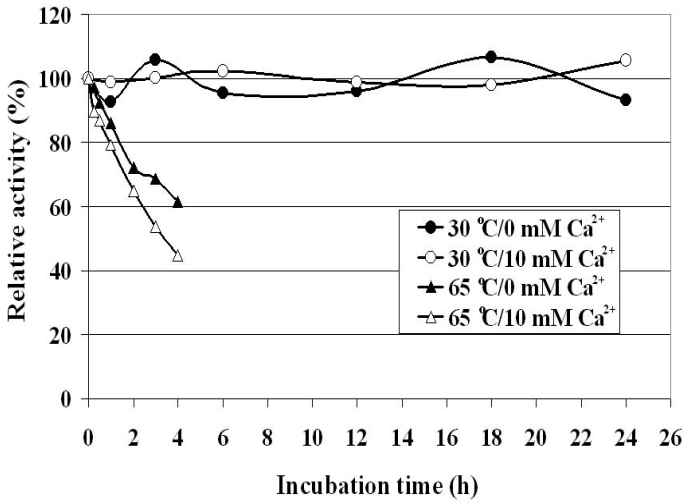

Figure 3.5 Thermal stability of *Virgibacillus* sp. SK37 proteinase in the absence of Ca^{2+} and 10 mM Ca^{2+} incubated at either 30 °C for 24 h or 65 °C for 4 h

This increased structural flexibility leading to an increase in activity. However, Ca^{2+} simultaneously decreased the rigid structure of enzyme, resulting in a decrease in conformational stability. In addition, Ca^{2+} might induce autolysis. Proteinase from archaebacterium *Desulfurococcus mucosus* was able to increase thermal stability when a protein substrate was used (Cowan, Smolenski, Daniel, and Morgan, 1987).

In the presence of substrate, proteinase tended to hydrolyze a substrate rather than underwent autolysis. It was postulated that protein substrate might protect the proteinase in the presence of Ca^{2+}. The effect of Ca^{2+} on *Virgibacillus* sp. SK37 proteinase appeared to be different from that on subtilisin which required Ca^{2+} for structural stabilization and protection against autolysis (Wong, 1995; Graycar et al., 2004). Ak.1 proteinase, subtilisin from *Bacillus* sp. Ak.1, sharply increased thermal stability with Ca^{2+} concentration up to 10 mM (Toogood, Smith, Baker, and Daniel, 2000). This result implied that *Virgibacillus* sp. SK37 proteinases were not subtilisin.

Proteinases from *Virgibacillus* sp. SK37 were more stable than others from moderately halophilic bacteria. About 80% of the original activity was retained at 65 °C, its optimal temperature, for 1 h (Figure 3.5). Activity of *Salinivibrio* sp. AF-2004 proteinase remained ~40% when incubated at the optimum temperature (65 °C) for 15 min (Karbalaei-Heidari et al., 2007). Proteinase from *Filobacillus* sp. RF2-5 and *Salinivibrio costicala* 18AG was unstable at its optimum temperature (60 °C) (Hiraga et al., 2005; Lama et al., 2005). For these reasons, our results demonstrated that proteinases from *Virgibacillus* sp. SK37 showed potential to be used in protein hydrolysis at high salt content.

3.4.5 Effect of NaCl on proteinase activity and activity staining

Activity of proteinases from *Virgibacillus* sp. SK37 gradually increased with NaCl concentration and showed maximum activity at 20% NaCl (Figure 3.6). Proteinase from *Pseudoalteromonas* sp. CP76 showed maximum activity at 0-1 M NaCl (0-5.8%) and its activity decreased at higher concentration (Sánchez-Porro et al., 2003). Activity of *Salinivibrio* sp. AF-2004 proteinase also decreased at >0.5 M

NaCl (2.9%) (Karbalaei-Heidari et al., 2007). These results indicated that activity of *Virgibacillus* sp. SK37 proteinase required more NaCl concentration than did other moderate halophile proteinases. Thermolysin from *Bacillus thermoproteolyticus* and proteinase from psychrophile PA-43 also activated by NaCl (Inouye et al, 1997; Irwin et al., 2001). Isoelectric point (pI) of thermolysin and PA-43 proteinase was 5.1 and 3.8, respectively. Therefore, their structures at neutral and alkaline pH contained charges on the surface, which could interact with hydrated NaCl. As a result, protein did not undergo aggregation under high NaCl content (Inouye et al, 1997; Irwin et al., 2001). It could be postulate that halophilic character of crude proteinase from *Virgibacillus* sp. SK37 might be due its low pI, allowing the enzyme to form electrostatic interaction between surface charges and hydrated salts.

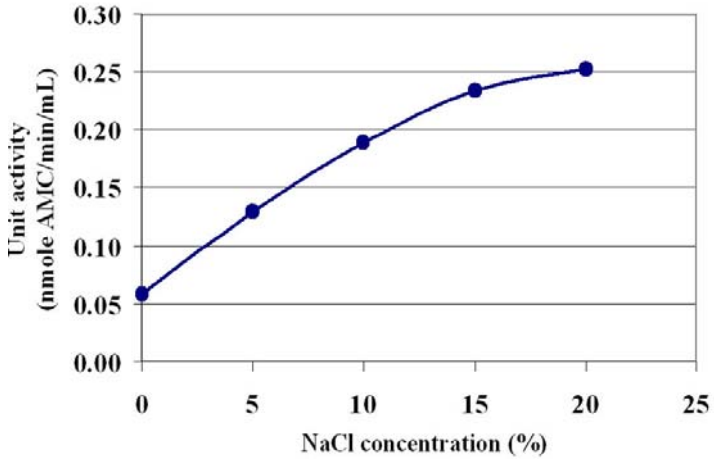

Figure 3.6 Effect of NaCl on the crude proteinase activity using Suc-Ala-Ala-Pro-Phe-AMC as a substrate

3.4.6 Molecular weight estimation

The dominant molecular weight (MW) of crude proteinase from *Virgibacillus* sp. SK37 was estimated to be 81, 67, 63, 50, 38 and 18 kDa, based on native-PAGE activity staining. All proteinases showed caseinolytic activity at high salt content (Figure 3.7). Crude proteinase from arhaeon *Natronococcus occultus* showed 7

distinct bands with MWs ranging from 50 to 120 kDa (Studdert, De Castro, Herrera Seitz, and Sánchez, 1997), while proteinases from *Brevibacterium linens* F exhibited MW varying from 37 to 325 kDa (Hayashi, Cliffe, and Law, 1990).

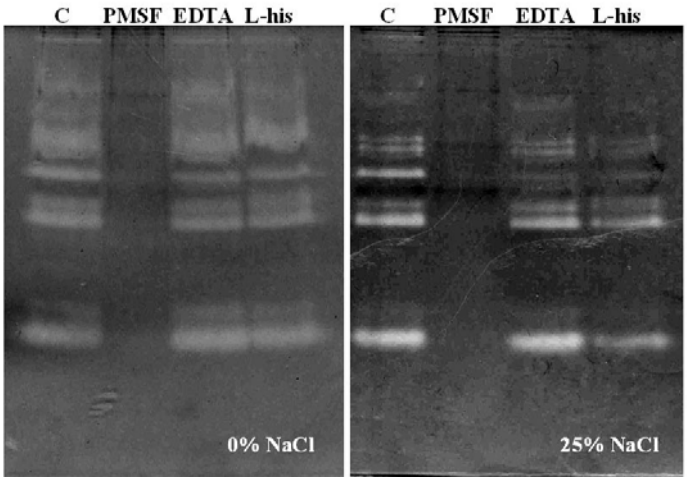

Figure 3.7 Activity staining (native-PAGE, 12.5% T) of crude proteinase from *Virgibacillus* sp. SK37 in the presence and absence of 25% NaCl. C = control (without inhibitor), PMSF = 1 mM, EDTA = 10 mM and L-his = 10 mM L-histidine

In the presence of metalloproteinase inhibitors, namely EDTA and L-histidine, all proteinases showed similar activity staining pattern to the that of control, but all proteinases were inactivated in the presence of PMSF (Figure 3.7). These results revealed that proteinases from *Virgibacillus* sp. SK37 were serine proteinases. In contrast, proteinases from *Paenibacillus* were zinc-metalloproteinases and inhibited by 1, 10-phenanthroline (Alvarez, von der weid, Seldin, and Santos, 2006). Crude proteinase from *Halobacillus thailandensis* sp. contained serine proteinase with MW of 100 and 17 kDa, and 42-kDa metalloproteinase (Chaiyanan et al., 1999). It should be noted that clear zone at high salt content increased with reaction time (Figure 3.8), confirming that crude proteinase was able to hydrolyze protein at high salt content for up to 2 h.

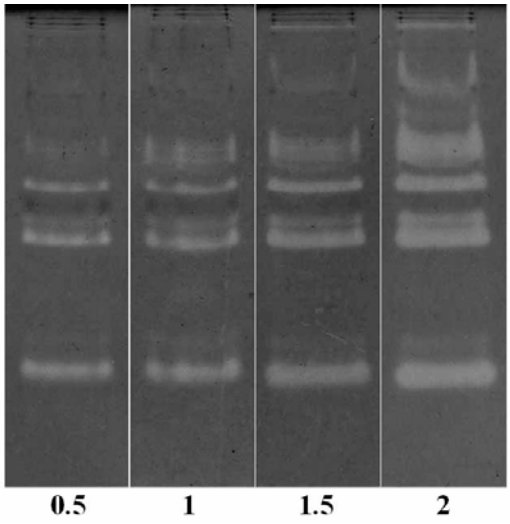

Figure 3.8 Activity staining (native-PAGE, 12.5% T) of crude proteinase from *Virgibacillus* sp. SK37 at 25% NaCl. Numbers indicate incubation time in h.

3.4 Conclusions

Crude proteinase exhibited the optimum temperature and pH at 65 °C and pH 8.0, respectively. Several proteinases were observed with dominant MW of 81, 67, 63, 50, 38 and 18 kDa. They were serine proteinases and showed caseinolytic activity at 25% NaCl. Activity of crude proteinase also required NaCl for maximum activity. Therefore, *Virgibacillus* sp. SK37 could be a potential strain to be developed for starter culture for fish sauce fermentation.

3.5 References

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CHAPTER IV

CHARACTERIZATION OF SALT TOLERANT CELL-BOUND PROTEINASE FROM *VIRGIBACILLUS* SP. SK37 ISOLATED FROM FISH SAUCE FERMENTATION

4.1 Abstract

Cell-bound proteinase from *Virgibacillus* sp. SK37 isolated from the 1st month of fish sauce fermentation was characterized. The enzyme showed the maximum activity at 65°C, pH 7.0 and 9.5, using azocasein as a substrate. Proteinase activity was activated by Ca^{2+} with the optimal concentration of 10 mM. Ethylenediaminetetraacetic acid (EDTA) and phenylmethanesulfonyl fluoride (PMSF) effectively inhibited the activity, indicating a metallo- and serine-proteinase characteristic. Among 6 synthetic substrates tested, the enzyme preferably hydrolyzed Suc-Ala-Ala-Pro-Phe-AMC, indicating a subtilisin-like proteinase. Although high salt concentration (20% NaCl) decreased 79% of the original activity, the enzyme showed high stability at 25% NaCl, 30°C. In addition, the cell-bound proteinase effectively hydrolyzed anchovy actomyosin at 5-20% NaCl. This was the first study to report biochemical characteristics of cell-bound proteinase from a moderately halophilic bacterium isolated from fish sauce.

Keyword: *Virgibacillus* sp., cell-bound proteinase, salt tolerant proteinase

4.2 Introduction

Most studies, thus far, have focused on extracellular proteinases secreted by extremely and moderately halophilic bacteria (Kamekura and Seno, 1990; Irwin, Alfredsson, Lanzetti, Gudmundsson, and Engel, 2001; Sánchez-Porro, Mellado, Bertoldo, Antranikian, and Ventosa, 2003; Karbalaei-Heidari, Ziaee, Schaller, and Amoozegar, 2007). Proteinases associated with cell envelope of extreme and moderate halophile have rarely been studied. Membrane-bound proteinase from *Halobacterium halobium* and *Natronomonas pharaonis* was reported (Fricke, Parchmann, and Aurich, 1993; Stan-Lotter et al., 1999). Cell-bound proteinases have been widely studied in lactic acid bacteria which are a starter culture of various fermented milk products. These proteinases play an important role in the initial stage of casein degradation to provide peptides for translocation into the cell and to contribute to flavor development of cheese (Bockelmann, 1995). C-terminal part of cell-bound proteinase involved in anchoring to cell envelope. C-terminus of cell-bound proteinases from *Lactoccoccus lactis* and *Streptococcus thermophilus* showed hydrophobic region and proline, glycine, threonine and serine rich region, which embedded to cell membrane and cell wall, respectively (Vos, Simons, Siezen, and de Vos, 1989; Piard et al., 1997; Rathsam and Jacques, 1998). C-terminal part of cell-bound proteinases from *Lactobacillus delbrueckii* subsp. *bulgaricus* contained lysine rich region, which could form electrostatic interaction with teichoic acids of cell wall (Gilbert et al., 1996; Germond, Delley, Gilbert, and Atlan, 2003). Cell-bound proteinases from *Lactococcus* sp., *Lactobacillus* sp. and *Streptococcus* sp. were a member of subtilisin family (Siezen and Leunissen, 1997; Fernandez-Espla, Garault, Monnet, and Rul, 2000). Molecular weight (MW) of cell-bound proteinase in soluble

form ranged from 37 to 180 kDa, which was larger than that of subtilisin. Typically, Ca^{2+} can activate and stabilize cell-bound proteinase at low concentration (1-5 mM) and showed inhibitory effect at higher concentration (Reid and Coolbear, 2004).

Recently, an isolate of moderately halophilic bacterium which was identified as *Virgibacillus* sp. SK37 according to 16S rRNA gene sequence exhibited high proteolytic activity at 25% NaCl. *Virgibacillus* sp. SK37 might be used as starter culture to reduce fish sauce fermentation time. Besides extracellular proteinase, this strain showed activity of cell-bound proteinase, which might play an important role in protein hydrolysis during fish sauce fermentation. Therefore, the objective of this study was to investigate biochemical characteristics of cell-bound proteinase from *Virgibacillus* sp. SK37.

4.3 Materials and methods

4.3.1 Chemicals

t-Butyloxycarbonyl(Boc)-Asp(oBzl)-Pro-Arg-4-methyl-7-coumarylamide (AMC), succinyl (Suc)-Ala-Ala-Pro-Phe-AMC and carbobenzoxy (Z)-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Z-Arg-Arg-AMC, leupeptin, trypsin inhibitor I (soybean), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), bestatin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidine)-butane (E-64), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), dithiothreitol (DTT), 2-mercaptoethanol (β-ME), bovine serum albumin, L-tyrosine and casein were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Ethylenediaminetetraacetic acid (EDTA), L-cysteine and L-histidine were purchased from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

4.3.2 Preparation of cell-bound proteinase

Virgibacillus sp. SK37 was cultivated by transferring a loopful of colony into neopeptone broth (NEO, 0.5% neopeptone, 1% $MgSO_4 \bullet 7H_2O$, 0.2% KNO_3, 0.0005% ferric citrate and 1% glycerol) containing 10% NaCl and incubating at 35 °C, 100 rpm, for 2 days. Subsequently, the culture (50 mL) was transferred to the same medium (450 mL) in a 1,000 mL erlenmeyer flask and incubated at 35 °C with a shaking speed of 100 rpm for 3 days. Bacterial growth was measured by Petroff-Hausser counter (Somasegaran and Hoben, 1994). Total cell count was approximately 10^9 cells/mL. Cell pellet collected by centrifugation at 10,000×g for 30 min, at 4 °C (RC 28S, Sorvall Co., Newtown, Conn., USA). Cell was resuspended in 50 mL of 50 mM Tris-maleate (pH 7.0) and used as cell-bound proteinase.

Residual extracellular proteinase of cell suspension was determined by the modified method of Barrett and Kirschke (1981) as described below. Cell suspension was centrifuged at 10,000×g for 30 min, at 4 °C (PK121K, ACCEL, Italy). Supernatant was collected and assayed in 200 mM Tris-HCl (pH 8.0) at 65 °C for 10 min using Suc-Ala-Ala-Pro-Phe-AMC as a substrate. Preliminarily results indicated that it was not necessary to remove extracellular proteinase(s) by washing cell pellet with cold buffer.

4.3.3 Casein hydrolysis

Cell-bound proteinase activity was determined using casein as a substrate. The reaction mixture (1 mL) contained 500 μL cell suspension, 2 mg/mL casein, 50 mM Tris-maleate (pH 7.0) and either 10 % NaCl, 10 mM Ca^{2+} or 10 % NaCl+10 mM Ca^{2+}. After incubation for 12 h at 50 ºC, the mixture was immediately centrifuged at 13,000×g for 5 min (Eppendorf AG 22331, Hamburg, Germany). Supernatant was collected and analyzed for casein degradation using 12.5% T sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1974).

Effect of Ca^{2+} on cell-bound proteinase activity was determined by 12.5% T SDS-PAGE (Laemmli, 1974). Cell-bound proteinase was incubated in 50 mM Tris-maleate (pH 7.0) and various Ca^{2+} concentrations (0 to 50 mM) at 50 ºC for 30 min.

4.3.4 Enzyme assay

Activity of cell-bound proteinase was assayed by the method of An, Seymour, Wu, and Morrissey (1994) using azocasein as a substrate (Sigma Chemical Co., St. Louis, Mo., USA). Cell suspension (500 μL) was added to the pre-incubated reaction mixture containing 2 mg/mL azocasein, 50 mM Tris-maleate (pH 7.0) and 10 mM Ca^{2+}, in a final volume of 1 mL, and incubated at 65 ºC for 30 min. The reaction was terminated by adding 500 μL of cold 50 % trichloroacetic acid (TCA) and kept at 4 ºC for 15 min to allow protein precipitation and subsequently centrifuged at 10,000×g for 10 min. One milliliter of supernatant was mixed with 100 μL of 10 N NaOH and determined the absorbance at 450 nm (GBC UV/VIS 916, GBC Scientific Equipment PTY, LTD., Australia). Blank was ran in the same manner except that cell suspension was added after the addition of TCA solution.

4.3.5 Temperature and pH optimum

Effect of temperature on cell-bound proteinase activity was carried out at 4 to 80 °C in 50 mM Tris-maleate (pH 7.0) by the method of An et al. (1994), using azocasein as a substrate. pH profile was measured at 65 °C at various pHs: pH 5, 5.5, 6 using 100 mM sodium acetate; pH 6.5, 7 using 50 mM Tris-maleate; pH 7.5, 8, 8.5, 9 using 200 mM Tris-HCl; and pH 9.5, 10, 11 using 200 mM glycine-NaOH.

4.3.6 Effect of inhibitors and ions on cell-bound proteinase activity

Effect of inhibitors including leupeptin, trypsine inhibitor I (soybean), TLCK, TPCK, PMSF, EDTA, L-histidine, bestatine, pepstatin A, E-64, NEM, IAA, reducing agents (DTT, β-ME and L-cysteine) and metal ions (Cu^{2+}, Cd^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, Hg^{2+} and Zn^{2+}) on cell-bound proteinase activity was determined using azocasein as a substrate. Effect of mono- and di-valent cations (Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}) was determined in the absence of 10 mM Ca^{2+}.

4.3.7 Substrate specificity

Substrate specificity of cell-bound proteinase was determined by the modified method of Barrett and Kirschke (1981) using various synthetic substrates, including Boc-Asp(oBzl)-Pro-Arg-AMC, Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC. The reaction mixture (1 mL) containing 200 μL cell suspension, 1 μM synthetic substrate, 50 mM Tris-maleate (pH 7.0) and 10 mM Ca^{2+} was incubated at 65 °C for 10 min. The reaction was stopped by adding 1.5 mL of the stopping solution (30% butanol, 35% methanol and 35% deionized water). The mixture was centrifuged at 13,000×g for 5

min (Eppendorf AG 22331, Hamburg, Germany). Fluorescence intensity was measured at excitation wavelength of 380 nm and emission wavelength of 460 nm (RF-1501, Shimadzu Co., Kyoto, Japan).

4.3.8 Effect of NaCl on cell-bound proteinase activity

Effect of NaCl on cell-bound proteinase activity was determined using azocasein as a substrate. Cell suspension was incubated in 50 mM Tris-malelate (pH 7.0), 10 mM Ca^{2+} at various NaCl concentrations varying from 0 to 20%.

4.3.9 Enzyme stability

Stability of cell-bound proteinase was monitored by pre-incubating the cell suspension in 50 mM Tris-maleate (pH 7.0) containing either 10% or 25% NaCl. Samples were incubated at either 30 °C for 24 h or 65 °C for 4 h. Samples were rapidly cooled and centrifuged at 13,000 ×g for 5 min, 4 °C (PK121K, ACCEL, Italy). Cell pellet and supernatant were collected to determine enzyme stability and measure degraded peptide resulted from autolysis, respectively.

Activity of cell-bound proteinase was determined by the modified method of An et al. (1994), using casein as a substrate. Cell pellet was resuspened in 50 mM Tris-maleate (pH 7.0). Cell suspension (200 μL) was added to the pre-incubated reaction mixture containing 2 mg/mL casein, 50 mM Tris-maleate (pH 7.0) and 10 mM Ca^{2+}, in a final volume of 1 mL, and incubated at 65 ºC for 30 min. The reaction was immediately centrifuged at 13,000×g for 5 min, 4 °C and cooled in ice. Cold TCA was added to supernatant to contain final concentration of 5%. The mixture was kept at 4 ºC for 1 h to complete protein precipitation and subsequently centrifuged at

10,000×g for 10 min. TCA-soluble oligopeptide content was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using tyrosine as a standard. Blank was ran in the same manner except that cell suspension was added at 4 °C into reaction mixture and immediately centrifuged at 13,000×g for 5 min, 4 °C. Oligopeptide content resulted from autolysis was determined in supernatant by the method of Lowry et al. (1951), using tyrosine as a standard.

4.3.10 Hydrolytic activity towards anchovy actomyosin at various NaCl

Effect of cell-bound proteinase on fish protein hydrolysis was determined using actomyosin (AM) from anchovy (*Stolephorus indicus*) as a substrate. AM was prepared according to the method of Ogawa et al., (1999). Whole anchovy (100 g) was homoginized in cold 500 mL of 50 mM NaCl, 20 mM Tris-maleate, pH 7.0. The homogenate was centrifuged at 10,000×g for 5 min, 4 °C (RC 28S, Sorvall Co., Newtown, Conn., USA). The supernatant containing sarcoplasmic proteins was discarded. The precipitates were washed twice using the same buffer. Subsequently, the pellet was homogenized with cold 1 L of 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0. Suspension was centrifuged at 10,000×g for 5 min, 4 °C. Supernatant containing myofibrillar protein was filtered through three-layer cheese-cloth to remove the connective tissue. The filtrate was stirred in cold 3 L of deionized water to precipitate myofibrillar protein, and then centrifuged at 10,000×g for 15 min, 4 °C. The precipitate was washed in cold 500 mL of 50 mM NaCl, 20 mM Tris-maleate, pH 7.0. AM was collected by centrifugation at 10,000×g for 10 min, 4 °C. Water was removed from the AM pellet by centrifugation at 12,500×g for 15 min, 4 °C. AM was

solubilized in 20 mM Tris-maleate, pH 7.0 and various NaCl concentrations (0-20%) at 4 °C for 12 h. Samples were centrifuged at 8,000×g for 15 min, 4 °C (PK121K, ACCEL, Italy). Protein content of AM solution was determined by the dye binding method using bovine serum albumin as a standard (Bradford, 1976).

The reaction mixture (1 mL) contained cell suspension, 50 mM Tris-maleate (pH 7.0), 10 mM Ca^{2+} and 1.5 mg/mL AM solubilized at various NaCl concentrations varying from 0-20%. Final NaCl concentration in the reaction was adjusted to the respective NaCl concentration of the substrate. When incubation at 65 °C for 30 min was attained, the reaction was immediately centrifuged at 10,000×g for 10 min, 4 °C (PK121K, ACCEL, Italy). Supernatant was added TCA to contain final concentration of 5%. The samples were cooled at 4 ºC for 12 h to allow complete protein precipitation and subsequently centrifuged at 10,000×g for 10 min. TCA-soluble oligopeptide contents were determined by Lowry method (Lowry et al., 1951) using tyrosine as a standard. Two blanks were prepared to eliminate the effect of endogenous proteinase in the extracted AM and residual oligopeptide in cell suspension. The reaction mixture was prepared in the same manner but without cell suspension in order to eliminate the effect of endogenous proteinase. Another blank prepared without AM substrate was used to subtract out oligopeptide content derived from cell suspension. Unit activity was defined as released nmole of tyrosine per min.

4.4 Results and discussion

4.4.1 Casein degradation

Cell-bound proteinase from *Virgibacillus* sp. SK37 required Ca^{2+} for catalytic reaction (Figure 4.1). In the absence of 10 mM Ca^{2+}, cell-bound proteinase showed a

weak caseinolytic activity (lane C and 10% NaCl), but complete casein hydrolysis was observed when 10 mM Ca^{2+} was added. These results indicated that Ca^{2+} was an activator for *Virgibacillus* sp. SK37 cell-bound proteinase. Optimum concentration of Ca^{2+} for proteinase activation was shown in Figure 4.2. A slight degradation of casein was found at ≤5 mM Ca^{2+}, whereas complete casein hydrolysis occurred at ≥10 mM Ca^{2+}. Therefore, 10 mM Ca^{2+} was required for full activation. Activity of cell-bound proteinase from *Lactococcus lactis* SK11 increased with Ca^{2+} up to 10 mM (Exterkate and Alting, 1999), while that from *Streptococcus thermophilus* CNRZ 385 increased 2 to 10 times when Ca^{2+} was increased from 2 to 10 mM (Fernandez-Espla et al., 2000). An increase of Ca^{2+} concentration up to 10 mM did not affect activity of cell-bound proteinase from *Lactobacillus helveticus* L89 and *Lb. casei* subsp. *casei* IFPL 731 (Martín-Hernández, Alting, and Exterkate, 1994; Fernández de Palencia, Peláez, Romero, and Martín-Hernández, 1997).

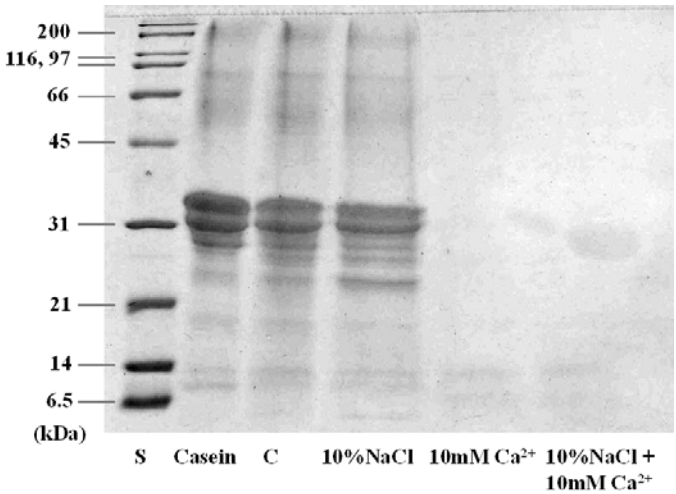

Figure 4.1 Casein substrate incubated with cell suspension of *Virgibacillus* sp. SK37 in the absence (C) or presence of 10% NaCl, 10 mM Ca^{2+} or 10% NaCl+10 mM Ca^{2+}, at 50 °C for 12 h. S = molecular weight standard, Casein = casein substrate incubated without cell suspension

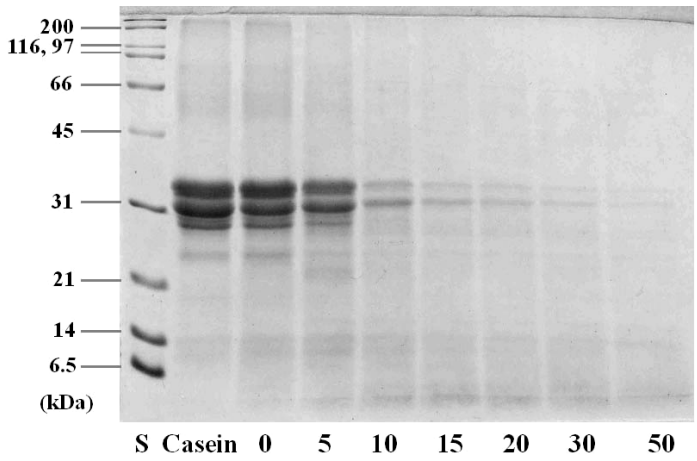

Figure 4.2 Casein substrate incubated with cell suspension of *Virgibacillus* sp. SK37 at various Ca^{2+} concentrations, at 50 °C for 30 min. S = molecular weight standard, Casein = casein substrate incubated without cell suspension, 0-50 = Ca^{2+} concentration at 0-50 mM

4.4.2 Temperature and pH optimum

Proteolytic activity of cell-bound proteinase from *Virgibacillus* sp. SK37 increased with temperature (Figure 4.3). Activity of cell-bound proteinase was maximum at 65 °C and rapidly decreased at higher temperature. Optimum temperature of cell-bound proteinase from *Virgibacillus* sp. SK37 was higher than that of proteinase from lactic acid bacteria. Cell-bound proteinase from *Streptococcus lactis* NCDO 763, *S. thermophilus* CNRZ 385, *S. cremoris* AC1, *Pediococcus* sp. LR, *Leuconostoc mesenteroides* ssp. *mesenteroides* CNRZ 1019, *Brevibacterium linens* CNRZ 944, *Propionibacterium acidipropionici* CNRZ 80, *Bifidobacterium infantis* 4038, *Lactobacillus casei* NCDO 151, *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 and *L. helveticus* L89 showed optimum temperature at 20-50 °C (Geis, Bockelmann, and Teuber, 1985; Monnet, Bars, and Gripon, 1987; Laloi, Atlan, Blanc, Gilbert, and Portalier, 1991; Næs, Charzanowska, and Blom, 1991; Ezzat, Soda, and El Shafei, 1993; Shahbal, Hemme, and Renault, 1993; Martín-Hernández et al., 1994).

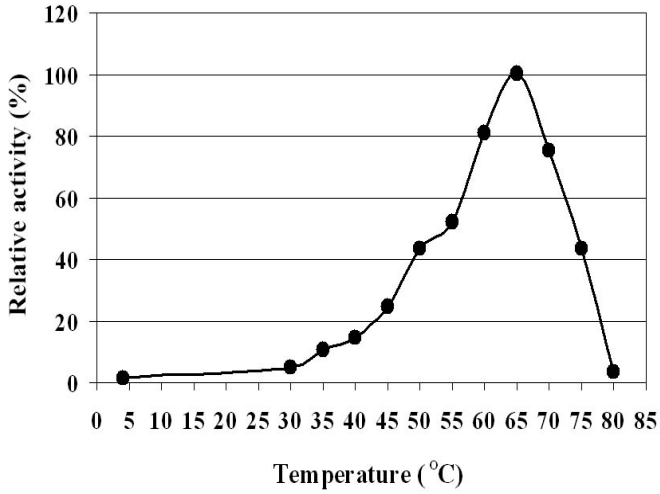

Figure 4.3 Temperature profile of cell-bound proteinase activity from *Virgibacillus* sp. SK37

Optimum pH of cell-bound proteinase from *Virgibacillus* sp. SK37 was found at pH 7 and 9.5 (Figure 4.4), implying that cell-bound proteinase might contain several proteinases. Typically, pH optimum of cell-bound proteinase from lactic acid bacteria was in acidic to neutral range. pH optimum of cell-bound proteinase from *Streptococcus lactis* NCDO 763, *S. thermophilus* CNRZ 385, *S. cremoris* AC1, *Pediococcus* sp. LR, *Leuconostoc mesenteroides* ssp. *mesenteroides* CNRZ 1019, *Brevibacterium linens* CNRZ 944, *Propionibacterium acidipropionici* CNRZ 80, *Bifidobacterium infantis* 4038, *Lactobacillus casei* NCDO 151, *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 and *L. helveticus* L89 was found at 5.5-7 (Geis et al., 1985; Monnet et al., 1987; Laloi et al., 1991; Næs et al., 1991; Ezzat et al., 1993; Shahbal et al., 1993; Martín-Hernández et al., 1994).

4.4.3 Effect of inhibitors and ions on proteinases activity

Activity of cell-bound proteinase from *Virgibacillus* sp. SK37 was strongly inhibited by EDTA, L-histidine and PMSF (Table 4.1), whereas chymotrypsin-like

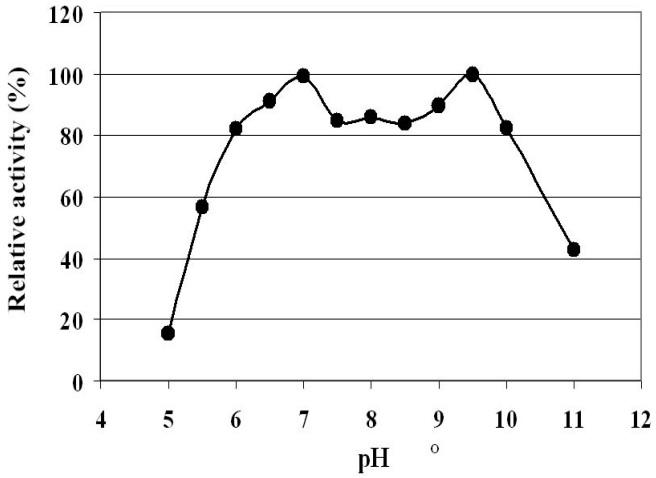

Figure 4.4 pH profile of cell-bound proteinase activity from *Virgibacillus* sp. SK37

proteinase inhibitor, TPCK, did not inhibit proteolytic activity. Trypsin-like proteinase inhibitors, namely leupeptin, soybean inhibitor, and TLCK, did not affect proteinase activity. Cysteine inhibitors (E-64, NEM and IAA) showed a slight inhibition. Based on these results, cell-bound proteinase from *Virgibacillus* sp. SK37 was metallo- and serine-proteinase. Moreover, reducing agents showed a moderate inhibition, suggesting that disulfide bond may be important in structural stabilization. Cell-bound proteinase from lactic acid bacteria was encoded by different genes (*prtP*, *prtS*, *prtH* and *prtB*), which were classified into subtilisin-like serine and subtilisin-like cysteine proteinases. *Lactococcus* sp., *Lactobacillus helveticus* and *Streptococcus* sp. expressed PrtP, PrtH and PrtS cell-bound proteinases, respectively, which were a subtilisin-like serine characteristic (Fernandez-Espla et al., 2000; Pederson, Mileski, Weimer, and Steele, 1999). Gilbert et al. (1996) reported PrtB cell-bound proteinase from *Lb. delbrueckii* subsp. *bulgaricus* belonged to cysteine subtilisin family. Based on the effect of inhibitors, cell-bound proteinase from lactic acid bacteria was classified into three groups, including serine proteinase

Table 4.1 Effect of various inhibitors and ions on enzyme activity of the cell-bound proteinase from *Virgibacillus* sp. SK37

Substances	Targeted enzyme	Final concentration	Relative activity (%)
Leupeptin	Trypsin-like and cysteine proteinases	100 μM	85
Trypsin inhibitor I (soybean)	Trypsin-like proteinase	0.02 mg/mL	102
TLCK	Trypsin-like proteinase	100 μM	99
TPCK	Chymotrypsin-like proteinase	100 μM	100
PMSF	Serine proteinase	1 mM	31
EDTA	Metallo proteinse	10 mM	2
L-Histidine	Metallo proteinase	10 mM	21
Bestatin	Aminopeptidase	10 μM	90
Pepstatin A	Acid proteinase	10 μM	77
E-64	Cysteine proteinase	10 μM	79
Iodoacetic acid	Cysteine proteinase	1 mM	63
N-Ethylmaleimide	Cysteine proteinase	1 mM	77
Dithiothereitol		10 mM	48
2-Mercaptoethanol		10 mM	49
L-Cysteine		10 mM	16
Metal ions			
Cu^{2+}		1 mM	43
Cd^{2+}		1 mM	42
Co^{2+}		1 mM	84
Fe^{3+}		1 mM	79
Mn^{2+}		1 mM	101
Hg^{2+}		1 mM	57
Zn^{2+}		1 mM	36
Mono- and di-valent cations [a]			
Li^{+}		10 mM	211
Na^{+}		10 mM	219
K^{+}		10 mM	202
Mg^{2+}		10 mM	339
Ca^{2+}		10 mM	324
Sr^{2+}		10 mM	318
Ba^{2+}		10 mM	296

[a] Reaction mixture without 10 mM Ca^{2+}

(Shahbal et al., 1993; Fernández de Palencia et al., 1997; Tsakalidou, Anastasiou, Vandenberghe, Beeumen, and Kalantzopoulos, 1999), cysteine proteinase (Laloi et al., 1991) and serine- and metallo-proteinase (Geis et al., 1985; Monnet et al., 1987;

Næs et al., 1991; Martín-Hernández et al., 1994). Activity of cell-bound proteinase from *Virgibacillus* sp. SK37 was strongly inhibited by metallo inhibitors, which were distinctly different from that of lactic acid bacteria. These resulted indicating that cell-bound proteinase from *Virgibacillus* sp. SK37 required ion for catalytic activity.

Mono- and di-valent cations increased the activity of cell-bound proteinase about two and three folds, respectively (Table 4.1), indicating that divalent cations effectively activated the activity of cell-bound proteinase from *Virgibacillus* sp. SK37. Most metal ions except for Mn^{2+} showed inhibitory effect (Table 4.1). Cu^{2+} was reported to inhibit cell-bound proteinase from *Streptococcus lactis* NCDO 763 (Monnet et al., 1987). Zn^{2+} and Cu^{2+} also inhibited the activity of cell-bound serine proteinase from various lactic acid bacteria (Næs et al., 1991; Tan, Pos, and Koning, 1991; Shahbal et al., 1993). This was because these ions can interact with histidine at the active site of serine proteinase (Malmström and Rosenderg, 1960; Fernández, Mohedano, Polanco, Medina, and Nuñez, 1996).

4.4.4 Substrate specificity

Cell-bound proteinase of *Virgibacillus* sp. SK37 preferentially cleaved Suc-Ala-Ala-Pro-Phe-AMC (Table 4.2), suggesting that P_1 of the proteinase preferred aromatic ring. Suc-Ala-Ala-Pro-Phe-chromogenic is a typical substrate for subtilisin (Graycar, Ballinger, and Wells, 2004). According to the effect of inhibitors and substrate specificity, the cell-bound proteinase from *Virgibacillus* sp. SK37 showed a subtilisin-like characteristic, which was similar to that from lactic acid bacteria. (Siezen and Leunissen, 1997). Extracellular proteinase from *Virgibacillus* sp. SK37 also displayed subtilisin-like characteristic, but showed different biochemical

properties from the cell-bound proteinase. It was likely that cell-bound and extracellular proteinases from *Virgibacillus* sp. SK37 was different enzyme.

Table 4.2 Substrate specificity of cell-bound proteinase from *Virgibacillus* sp. SK37

Synthetic substrates	Specificity	Relative activity (%)
Boc-Asp(oBzl)-Pro-Arg-AMC	Trypsin-like, α-thrombin-like	19
Boc-Gln-Ala-Arg-AMC	Trypsin-like	40
Boc-Val-Leu-Lys-AMC	Plasmin-like	9
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin-like, subtilisin-like	100
Z-Phe-Arg-AMC	Cathepsin L-like	19
Z-Arg-Arg-AMC	Cathepsin B-like	9

4.4.5 Effect of NaCl on cell-bound proteinase activity

Activity of cell-bound proteinase from *Virgibacillus* sp. SK37 gradually decreased with NaCl concentration (Figure 4.5A) as 21% activity was observed at 20% NaCl. It is expected that moderately halophilic bacteria can grow well at 3-14.5% NaCl (Kushner, 1992), thereby their enzymes might adapt well to NaCl concentration at this range. Activity of cell bound proteinase from *Streptococcus cremoris* AC1 was inactivated at >0.1 M NaCl (0.58%) (Geis et al., 1984), whereas that from *S. thermophilus* CNRZ 385 was 111 and 120% at 3.5 and 7% NaCl, respectively (Fernandez-Espla et al., 2000). Activity of cell-bound proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 increased with NaCl up to 1.6 M NaCl (9.4%) (Exterkate, 2000). Moreover, proteolytic activity of *Virgibacillus* sp. SK37 cell-bound proteinase towards actomyosin also decreased with NaCl concentration (Figure 4.5B). Cell-bound proteinase at 20% NaCl showed activity of 63% compared to that at 5% NaCl (Figure 4.5B). These results suggested that the cell-bound

proteinase from *Virgibacillus* sp. SK37 was able to hydrolyze fish protein at high salt content. Besides extracellular proteinase, cell-bound proteinase from a moderately halophilic bacterium, *Virgibacillus* sp. SK37, might play an important role in proteolysis during fish sauce.

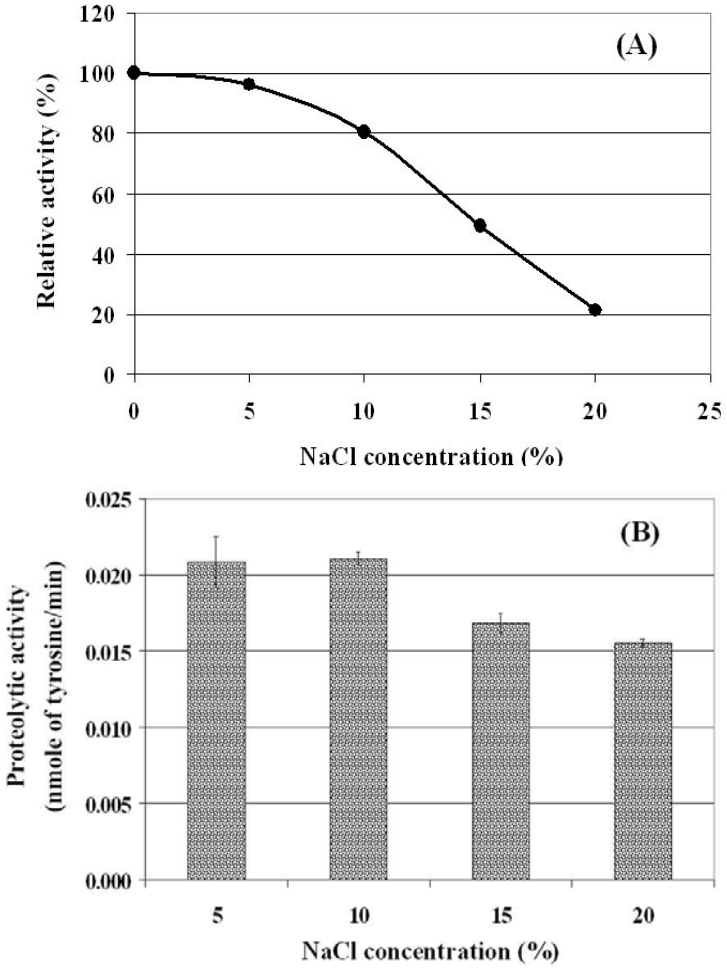

Figure 4.5 Proteinolytic activity of cell-bound proteinase from *Virgibacillus* sp. SK37 using casein (A) and anchovy actomyosin (B) as a substrate

4.4.6 Stability of cell-bound proteinase at high NaCl content.

Cell-bound proteinase from *Virgibacillus* sp. SK37 was less stable at its optimum temperature (65 °C) than at 30 °C, in the presence of either 10 or 25% NaCl (Figure 4.6). An increase of oligopeptide content in supernatant was observed

implying autolytic degradation throughout incubation time (Figure 4.7). This corresponded to a reduced stability of cell-bound proteinase (Figure 4.6). The enzyme may undergo autolysis at its optimum temperature. Since NaCl reduced its activity (Figure 4.5), cell-bound proteinase was less susceptible to autolysis. Typically, subtilisin-like cell-bound proteinase from lactic acid bacteria required Ca^{2+} for thermal stability and autolytic protection (Exterkate and Alting, 1999). Ca^{2+} may increase thermal stability of cell-bound proteinase from *Virgibacillus* sp. SK37 because it was subtilisin-like proteinase. In addition, cell-bound proteinase from *Virgibacillus* sp. SK37 was more thermo-stable than that of lactic acid bacteria. Cell-bound proteinases from lactic acid bacteria were instable at temperature >40 °C, while that from *Virgibacillus* sp. SK37 exhibited ~50% activity at 65 °C for 4 h (Figure 4.6). Cell-bound proteinase from *Lactococcus casei* NCDO 151, *Streptococcus thermophilus* CNRZ 385 and *Lactobacillus casei* subsp. *casei* IFPL 731 was inactivated when incubated at 50 °C for 20-30 min (Næs et al., 1991; Shahbal et al., 1993; Fernández de Palencia et al., 1997). Cell-bound proteinase from *Virgibacillus* sp. SK37 might be applied to hydrolyze protein at high temperature.

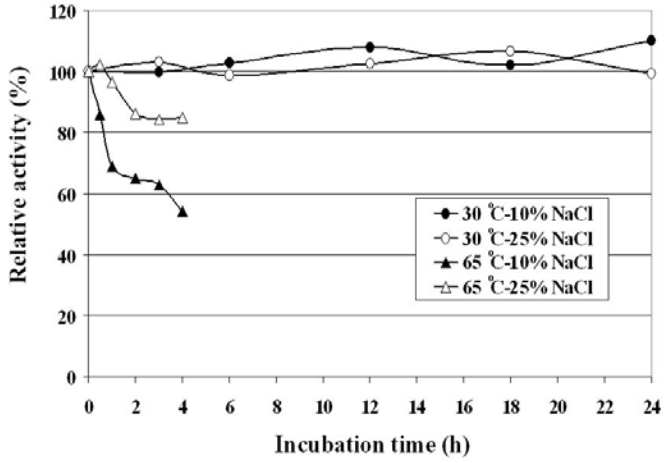

Figure 4.6 Effect of NaCl on the cell-bound proteinase stability at either 30 °C for 24 h or 65 °C for 4 h in the presence of 10% NaCl and 25% NaCl

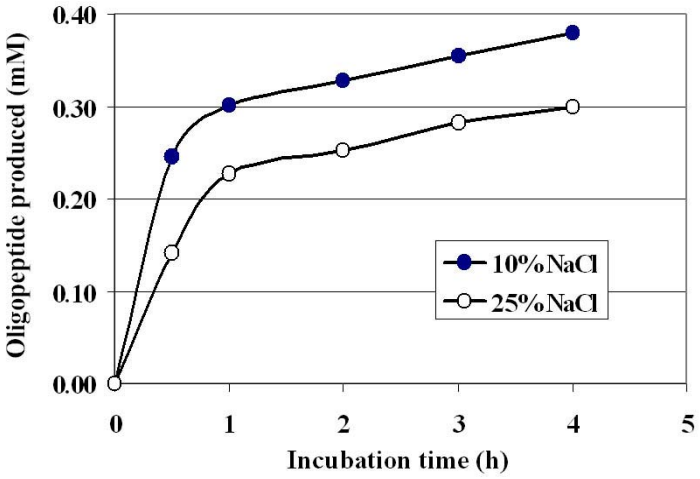

Figure 4.7 Oligopeptide produced during incubation of cell suspension at 65 °C for 4 h

4.5 Conclusions

Virgibacillus sp. SK37, a moderately halophilic bacterium, produced cell-bound proteinase with maximum activity at 65 °C and pH 7-9. The enzyme was metallo- and serine-proteinase with subtilisin characteristics. Divalent cations activated the enzyme, whereas metal ions inhibited its activity. Cell-bound proteinase from *Virgibacillus* sp. SK37 showed proteolytic activity at high salt content, suggesting an important role during fish sauce fermentation and potential application of the enzyme at high salt content.

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CHAPTER V

PRODUCTION AND CHARACTERIZATION OF NaCl-ACTIVATED PROTEINASE FROM *VIRGIBACILLUS* SP. SK33 ISOLATED FROM FISH SAUCE FERMENTATION

5.1 Abstract

Virgibacillus sp. SK33 newly isolated from 1 month-old Thai fish sauce was studied for its proteinase production. Neopeptone broth (NEO), halobacterium broth (HL) and HL without either yeast extract (HL-Y), peptone (HL-P) or casamino acid (HL-C) were found to be suitable for proteinase production, whereas fish broth (FB) and skim milk salts broth (SKS) appeared to suppress proteinase production. Moreover, yeast extract, peptone and casamino acid equally stimulated proteinase production. The optimal enzyme production for *Virgibacillus* sp. SK33 was at 5% NaCl, 40 °C. Maximum proteinase production was achieved at 36 h and maximum cell growth was obtained at 72 h in the modified HL supplemented with only yeast extract (Ym). Extracellular proteinase of *Virgibacillus* sp. SK33 exhibited optimum activity at 50 °C and pH 8, 10 and 11. Crude proteinase was strongly inhibited by phenylmethanesulfonyl fluoride (PMSF), indicating the presence of serine alkaline proteinase. The enzyme preferentially cleaved Suc-Ala-Ala-Pro-Phe-AMC, a substrate for subtilisin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) activity staining showed molecular weights of 56, 46, 42, 32, 25 and 19

kDa. All proteinases exhibited caseinolytic activity at 25% NaCl. Proteolytic activity towards synthetic substrate was increased with NaCl concentration up to 25%, indicating the characteristic of NaCl-activated proteinase. In addition, proteinase from *Virgibacillus* sp. SK33 showed higher proteolytic activity towards anchovy than commercial proteinases at 0-25% NaCl, indicating the potential application for protein hydrolysis at high salt content.

Keywords: *Virgibacillus* sp., fish sauce, serine proteinase, NaCl-activated proteinase

5.2 Introduction

Fish sauce is a hydrolysate used as a condiment in Southeast Asia and worldwide. Typically, production of fish sauce involves the addition of salt to uneviscerated fish at a ratio of approximately 1:3 and required about 1-1.5 years to complete fermentation. Long fermentation process is a major obstacle for the growth of fish sauce industry. Several methods have been proposed to reduce fermentation time, such as raising fermentation temperature (Gildberg, 1993), changing pH to alkaline or acidic (Gildberg 1993; Gildberg, Espejo-Hermes, and Magno-Orejana, 1984) and adding exogenous proteinases (Beddows and Ardeshir, 1979; Raksakulthai, Lee, and Haard, 1986). The use of commercial enzymes is limited by the cost and a reduced activity at high NaCl content. Protein hydrolysis during fish sauce fermentation is normally accomplished by the action of both fish endogenous and bacterial proteinases (Saisithi, 1994). Activity of endogenous proteinases decreased at high salt environment, resulting in slow rate of protein hydrolysis (Siringan, Raksakulthai, and Yongsawatdigul, 2006). Halophilic bacterial proteinase showing

activity and stability at high salt content could be a potential source to accelerate fish sauce fermentation process.

Thus far, halophilic and halotolerant proteinase-producing bacteria isolated from fish sauce fermentation included *Halobacterium salinarium* and *H. thailandensis* sp. (Thongthai, McGenity, Suninanalert, and Grant, 1992; Chaiyanan et al., 1999). These strains showed caseinolytic and gelatinolytic activity but the detailed characteristics of proteinases have not been thoroughly investigated. Proteinases from *Bacillus licheniformis*, *B. amyloliquefaciens* and *B. subtilis* have been characterized and these strains have been commercially used as a source of proteinase production (Adler-Nissen, 1993). However, their activity under high NaCl content is limited. Activity of *B. subtilis* JM-3 proteinase decreased with NaCl concentration (Kim and Kim, 2005) while proteinase activity from *Filobacillus* sp. RF2-5 increased 2.5 times in the presence of 15-25% NaCl, but it was not stable at the optimal temperature (Hiraga et al., 2005). To successfully accelerate protein hydrolysis at high salt content, proteinases exhibiting high stability and activity at 25-30% NaCl should be sought and characterized.

The composition of culture media greatly affects proteinase production of microorganisms. Soybean meal was used as the best nitrogen source for proteinase production of *Bacillus cereus* MCM B-326 (Nilegaonkar, Zambare, Kanekar, Dhakephalkar, and Sarnaik, 2007). Hiraga et al. (2005) reported that proteinase production of *Filobacillus* sp. RF2-5 depended on the presence of yeast extract. The content of nitrogen source in the medium is also a critical parameter. Proteinase production of *Bacillus firmus* was repressed by excessive amount of yeast extract (Moon and Parulekar, 1991). Optimum conditions of proteinase production varied

with species. Most studies have focused on proteinase production of alkaliphilic bacteria (Horikoshi, 1971; Takii, Kuriyama, and Suzuki, 1990; Johnvesly and Naik, 2001; Kanekar, Nilegaonkar, Sarnaik, and Kelkar, 2002; Singh, Vohra, and Sahoo, 2004). However, the optimal proteinase production of moderately halophilic bacteria and/or extremely halophilic bacteria has rarely been reported.

Recently, proteinase-producing bacteria from fish sauce fermentation have been isolated and identified as belonging to *Virgibacillus* sp. according to 16S rRNA gene sequence. The GenBank/NCBI accession number for the 16S rRNA gene sequence of *Virgibacillus* sp. SK33 was DQ910838. *Virgibacillus* sp. SK33 was Gram-positive/variable, rod shape of 0.5-0.7 x 2.1-4.1 μm, non-motile and terminal ellipsoidal spores. It can grow in wide pH (4-12) and in a narrow temperature range (30-45 °C). This strain showed high proteolytic activity toward anchovy at 25% NaCl and could be a promising strain for starter culture development for fish sauce fermentation. In addition, it could be a potential source for halophilic proteinase. However, factors affecting proteinase production of *Virgibacillus* sp. SK33 was still unknown. Thus, the objectives of this study were to investigate the optimum condition for proteinase production of *Virgibacillus* sp. SK33 and to elucidate biochemical characteristics of proteinases secreted from *Virgibacillus* sp. SK33.

5.3 Materials and methods

5.3.1 Chemicals

t-Butyloxycarbonyl(Boc)-Asp(oBzl)-Pro-Arg-4-methyl-7-coumarylamides (AMC), succinyl (Suc)-Ala-Ala-Pro-Phe-AMC and carbobenzoxy (Z)-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). Boc-Gln-Ala-Arg-

AMC, Boc-Val-Leu-Lys-AMC, Z-Arg-Arg-AMC, leupeptin, trypsin inhibitor I (soybean), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), bestatin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidine)-butane (E-64), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), dithiothreitol (DTT), 2-mercaptoethanol (β-ME), bovine serum albumin, L-tyrosine and casein were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Ethylenediaminetetraacetic acid (EDTA), L-cysteine, imidazole and L-histidine were purchased from Fluka (Buchs, Switzerland). Alcalase 2.4L and Protamex were supplied by Novo Nordisk A/S (Bagsvaerd, Denmark). All other chemicals used were of analytical grade. Plate count agar (PCA) was purchased from Merck (Merck KGaA, Darmstadt, Germany).

5.3.2 Effect of culture media on proteinase production

A loopful of *Virgibacillus* sp. SK33 was inoculated to halobacterium broth (HL, 1% yeast extract, 0.5% peptone, 1% casamino acid, 0.3% trisodium citrate, 0.2% KCl and 2.5% $MgSO_4 \bullet 7H_2O$) containing 10% NaCl, and incubated at 35 °C for 2 days. The cultured broth was centrifuged at 6,000×g, 4 °C for 10 min. Supernatant was discarded and equal volume of 100 mM phosphate buffer (pH 7.0) was added into the cell pellet and mixed. Resuspended cells were transferred to various media containing final concentration of 10% NaCl and incubated at 35 °C for 3 days. Media studied were fish broth (FB, FB was prepared by the modified method of Okuzumi and Awano (1983) as described below.), neopeptone broth (NEO, 0.5% neopeptone, 1% $MgSO_4 \bullet 7H_2O$, 0.2% KNO_3, 0.0005% ferric citrate and 1% glycerol), skim milk

salts broth (SKS, 1% skim milk, 0.5% neopeptone, 1% $MgSO_4 \bullet 7H_2O$, 0.2% KNO_3, 0.0005% ferric citrate and 1% glycerol), HL, HL without yeast extract (HL-Y), HL without peptone (HL-P) and HL without casamino acid (HL-C). For FB preparation, anchovy (*Stolephorus* spp.) was mixed with water (1:2) and boiled for 20 min. The mixture was filtered through three-layers of cheesecloth. The filtrate was centrifuged at 8,000×g for 30 min. The supernatant referred as fish tissue extract was added 1% glucose to prepare FB medium, then sterilized by autoclaving at 118 °C for 15 min.

The bacterial growth was monitored using spread plate technique on plate count agar (PCA) containing 10% NaCl, and incubated at 35 °C for 2 days. Production of extracellular proteinases was also monitored in each medium. Each cultured broth was collected by centrifugation at 8,000×g for 30 min. Proteinase activity was determined by the modified method of Barrett and Kirschke (1981) as described below.

5.3.3 Effect of nitrogen sources on proteinase production

Virgibacillus sp. SK33 was cultivated by transferring a loopful of colony to HL containing 5% NaCl and incubating at 40 °C for 2 days. Cells were collected by centrifugation at 6,000×g, 4 °C, for 10 min. The cell pellet was resuspended in 100 mM phosphate buffer (pH 7.0) with the equal volume of the discarded supernatant. One milliliter of cell suspension was transferred to various types of studied media (9 mL) containing final concentration of 5% NaCl and incubated at 40 °C for 3 and 6 days. Media studied were HL and the modified HL containing only a single nitrogen source of either yeast extract (Ym), peptone (Pm) or casamino acid (Cm). The

bacterial growth was monitored using spread plate technique on PCA agar containing 5% NaCl and incubated at 35 °C for 2 days. Production of extracellular proteinases was also monitored in each medium. Proteinase activity was determined by the modified method of Barrett and Kirschke (1981).

5.3.4 Optimal conditions for proteinase production

Virgibacillus sp. SK33 was cultivated by transferring a loopful of colony into HL containing 10% NaCl, and incubating at 35 °C for 2 days. Cell pellet was collected by centrifugation at 6,000×g, 4 °C for 10 min and was resuspended in 100 mM phosphate buffer (pH 7.0), then transferred to HL containing NaCl at final concentration of 0 to 25%, and incubated at various temperatures (30, 35 and 40 °C) for 1, 2, 3, 4 and 5 day, for the media contain 0, 5, 10, 15 and 20-25% NaCl, respectively. Bacterial growth was determined by total viable counts using spread plate technique on PCA agar containing 0-25% NaCl and incubated at 35 °C for 1-7 days, according to NaCl concentration in the HL medium. Proteinase activity was also determined by the modified method of Barrett and Kirschke (1981).

5.3.5 Time course of cell growth and proteinase production

Virgibacillus sp. SK33 was cultivated by transferring a loopful of colony into Ym containing 5% NaCl and incubating at 40 °C for 2 days. Subsequently, the culture (6 mL) was transferred to the same medium (54 mL) in a 250-mL erlenmeyer flask and incubated at 40 °C with a shaking speed of 100 rpm. Bacterial growth was measured spectrophotometrically at 600 nm and proteinase activity was determined at each time interval for 8 days.

5.3.6 Proteinase activity assay

Virgibacillus sp. SK33 was cultivated in Ym containing 5% NaCl, and incubated at 40 °C for 3 days. Crude extracellular proteinase was collected by centrifugation at 8,000×g for 30 min at 4 °C. The proteinase was dialyzed against 20 mM Tris-maleate (pH 7.0) at 4 °C overnight, using dialysis bag with molecular weight cut-off (MWCO) 10 kDa (Pierce Chemical Company, Rockford, IL USA). Proteinase activity was assayed by the modified method of Barrett and Kirschke (1981) using Suc-Ala-Ala-Pro-Phe-AMC as a substrate. The reaction mixture (1 mL) contained 50 μL crude proteinase, 1 μM synthetic substrate and 200 mM Tris-HCl (pH 8.0), and incubated at 50 °C for 5 min. The reaction was stopped by adding 1.5 mL of the stopping solution (30% butanol, 35% methanol and 35% deionized water). Fluorescence intensity was measured at excitation wavelength of 380 nm and emission wavelength of 460 nm (RF-1501, Shimadzu Co., Kyoto, Japan).

5.3.7 Temperature and pH optimum of proteinase

Effect of temperature on proteinase activity was carried out at 40 to 75 °C in 50 mM Tris-maleate (pH 7.0) by the modified method of Barrett and Kirschke (1981) as described above, using Suc-Ala-Ala-Pro-Phe-AMC as a substrate. pH profile was measured at 50 °C at various pHs: pH 5, 5.5, 6 using 100 mM sodium acetate; pH 6.5, 7 using 50 mM Tris-maleate; pH 7.5, 8, 8.5, 9 using 200 mM Tris-HCl; and pH 9.5, 10, 11 using 200 mM glycine-NaOH.

5.3.8 Substrate specificity

Activity of the crude proteinase was determined using various synthetic substrates at 1 μM, including Boc-Asp(oBzl)-Pro-Arg-AM), Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC. Specific activity was expressed as the release of AMC in nmole per min per mg protein of proteinase.

5.3.9 Effect of inhibitors and ions on proteinase activity

The effect of various inhibitors, including leupeptin, trypsin inhibitor I (soybean), TLCK, TPCK, PMSF, EDTA, L-histidine, bestatin, imidazole, pepstatin A, E-64, NEM, IAA and reducing agents (DTT, β-ME and L-cysteine) on proteinase activity was determined. The effect of metal ions, Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, Hg^{2+} and Zn^{2+}, was also investigated.

5.3.10 Molecular weight estimation

Molecular weight of proteinase was estimated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) activity staining according to Laemmli (1970) and García-Carreño, Dimes, and Haard (1993). Crude proteinase was loaded into gel at 0.2 U (nmole of AMC/min) per well. Acrylamide gel (12.5% T) was run at 100 V. Subsequently, it was immersed in 2% casein, 100 mM Tris-HCl, pH 8.0 at 4 °C for 30 min and was washed twice with 100 mM Tris-HCl (pH 8.0). Proteolytic reaction was carried out in 100 mM Tris-HCl, pH 8.0, containing either no NaCl or 25% NaCl and incubated at 50 °C for 30 min. Gel was stained in 0.1% Coomassie brilliant blue R-250, 40% methanol and 10% acetic acid for 1 h and

destained in 25% ethanol and 10% acetic acid. Clear zone indicated the presence of proteinase.

5.3.11 Effect of NaCl on proteinase activity

The reaction mixture contained 1 μM Suc-Ala-Ala-Pro-Phe-AMC and the appropriate buffer solution containing various NaCl concentrations (0-25%). Proteinase activity from *Virgibacillus* sp. SK33 was determined in 200 mM Tris-HCl (pH 8.0) at 50 °C for 5 min. Alcalase was assayed in 200 mM Tris-HCl (pH 8.0) at 60 °C for 5 min. Protamex was measured in 200 mM Tris-HCl (pH 9.5) at 50 °C for 5 min. In the presence of NaCl, deionized water was used instead of butanol to avoid phase separation. Subsequently samples were heated at 90 °C for 5 min to terminate the activity.

5.3.12 Hydrolysis of soluble anchovy proteins

Whole anchovy (*Stolephorus* spp.) was homoginized in cold 50 mM Tris-maleate, pH 7.0, containing various NaCl concentrations (0, 5, 10, 15, 20 and 25% NaCl) at the ratio of fish to buffer of 1:5. The homogenate was stirred at 4 °C for 30 min, then centrifuged at 13,000×g for 30 min. The supernatant was used as a substrate. Protein content was determined by a dye binding method (Bradford, 1976) using bovine serum albumin as a standard.

Since endogenous proteinase activity in anchovy (*Stolephorus* spp.) was relatively high, elimination of endogenous proteinase was required to evaluate the effect of exogenous proteinase addition. Endogenous proteinase of anchovy was inhibited by either trypsin inhibitor I, TLCK or leupeptin (Siringan et al., 2006).

Preliminary study indicated that activity of *Virgibacillus* sp. SK33 proteinase, Alcalase and Protamex was inhibited by PMSF, trypsin inhibitor I and leupeptin/trypsin inhibitor I, respectively. An inhibitor specific for anchovy proteinase without affecting activity of the studied enzyme was, therefore, added to the reaction mixture. The reaction of *Virgibacillus* sp. SK33 proteinase contained 0.02 mg/mL trypsin inhibitor I. Leupeptin at 100 μM was used in the reaction mixture of Alcalase. TLCK at 100 μM was added in the reaction mixture of Protamex.

The reaction mixture (1 mL) contained either crude proteinase from *Virgibacillus* sp. SK33, Alcalase or Protamex of 40 U (nmoles AMC/min) at the optimum temperature and pH of individual enzyme detailed in 2.3.6, an appropriate inhibitor. Anchovy protein (1.5 mg/mL) solubilized at various NaCl concentrations was used as a substrate. Final NaCl concentration in the reaction was adjusted to the respective NaCl concentration of the substrate. After incubating for 30 min, the reaction was terminated by adding trichloroacetic acid (TCA) to contain final concentration of 10%. The samples were cooled at 4 ºC for 1 h to allow complete protein precipitation and then centrifuged at 10,000×g for 10 min. TCA-soluble oligopeptide contents were determined by Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951) using tyrosine as a standard. Blank was prepared in the same manner except that the heated proteinase (90 °C for 5 min) was used.

5.3.13 Statistical analysis

Analysis of variance (ANOVA) was conducted using SAS program (SAS Institute Inc, Carry, NC., USA). Differences among mean values were established

using Duncan Multiple Range Test (DMRT) at $p<0.05$. The effect of culture media on proteinase production was analyzed using completely randomized design (CRD).

The optimal condition for proteinase production was analyzed using a split plot design. In the proteinase production study, incubation temperatures (30, 35 and 40 °C) were assigned as a main plot, and NaCl concentrations (0, 5, 10, 15, 20 and 25%) were assigned as a sub-plot. In the hydrolysis of soluble anchovy proteins, the effect of proteinases (*Virgibacillus* sp. SK33 proteinase, Alcalase and Protamex) and NaCl concentrations (0, 5, 10, 15, 20 and 25%) were analyzed using factorial experiments in CRD.

5.4 Results and discussion

5.4.1 Effect of culture media on proteinase production

Preliminary results revealed that extracellular proteinase production of *Virgibacillus* sp. SK33 was rather inconsistent in FB. Therefore, the effect of culture medium composition on proteinase production was investigated. Proteinase production of *Virgibacillus* sp. SK33 in NEO, HL, HL-Y, HL-P and HL-C was higher than that in FB and SKS ($p<0.05$) (Figure 5.1). These results indicated that proteinase production of *Virgibacillus* sp. SK33 was largely influenced by the composition of culture medium. Hydrolyzed peptides and amino acids from neopeptone, yeast extract, peptone and casamino acid were more favorable than large protein (fish and casein) for proteinase production by the bacterial isolate. Peptides and amino acids can be utilized by most bacteria. Many studies reported that peptides or amino acids were appropriate to be used as proteinase inducers. Proteinase production of marine

bacterium SA 1 was induced by free amino acid (L-phenylalanine and L-glutamic acid), peptone and casamino acid (Daatselaar and Harder, 1974). Peptone and bactopeptone effectively increased proteinase production for *Bacillus polymyxa* and *B. subtilis* NCIM no. 64, respectively (Fogarty and Griffin 1973; Kembhavi, Kulkarni, and Pant, 1993). Suitable nitrogen source for proteinase production of *Bacillus* sp. K30 was beef extract, yeast extract, tryptone and peptone (Naidu and Devi, 2005). Hence, the selection of suitable culture medium for microbial proteinase production was necessary. Our study revealed that hydrolyzed peptides were essential for inducing proteinase production of *Virgibacillus* sp. SK33.

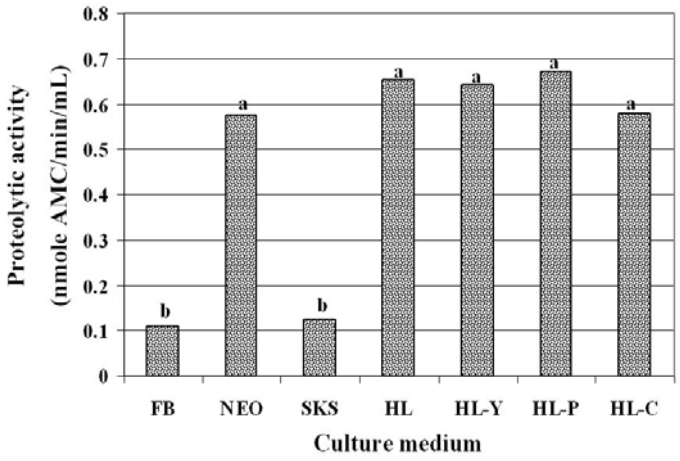

Figure 5.1 Effect of culture media containing 10% NaCl on proteinase production of *Virgibacillus* sp. SK33, incubated at 35 °C for 3 days. FB = fish broth; NEO = neopeptone broth; SKS = skim milk salt broth; HL = halobacterium broth; HL-Y = HL without yeast extract; HL-P = HL without peptone; HL-C = HL without casamino acid. Viable cell counts of *Virgibacillus* sp. SK33 at the end of cultivation in all media were approximately 7.55 Log CFU/mL. a and b indicate significant difference at $p<0.05$

Broths containing high molecular weigh proteins, like fish and skim milk, appeared to suppress proteinase production (Figure 5.1), but they had no effect on cell

growth as cell counts of all cultured media were approximately 7.55 Log CFU/mL. These results implied that the salt-soluble protein at the initial stage of fish sauce fermentation might reduce the proteinase secretion of *Virgibacillus* sp. SK33, leading to low proteinase activity. However, proteinase secretion of many bacteria was induced by high molecular weight protein. *Bacillus alcalophilus* was able to utilize gelatin and soy protein better than tryptone, yeast extract and peptone for its proteinase production (Kanekar et al., 2002). Soybean meal was the best nitrogen source to induce proteinase secretion of *Bacillus* sp. I-312 and *B. brevi*, while peptone caused significant reduction (Banerjee, Sani, Azmi, and Soni, 1999; Joo and Chang, 2005b). Media containing skim milk powder, soyabean flour, chick pea flour and pigeon pea flour induced the proteinase production of extremely halophilic bacterium, *Halogeometricum* sp. TSS101 (Vidyasagar, Prakash, and Sreeramulu, 2006). Bacteria secrete extracellular proteinase to digest large protein molecule to oligopeptides, dipeptides and amino acids, which are subsequently transferred across cell membrane for their metabolism. It can be seen that the effect of nitrogen source on extracellular proteinase secretion varies from strain to strain.

Proteinase production of *Virgibacillus* sp. SK33 in Ym, Pm and Cm was higher than in HL ($p<0.05$) (Figure 5.2). These results suggested that either yeast extract, peptone or casamino acid alone was sufficient to induce proteinase production. Thus, there is no need to incorporate 3 nitrogen sources all at once. *Pseudoalteromonas* sp. CP76 cultured in combination of casamino acid and NH_4Cl showed lower proteinase production than in either casamino acid or NH_4Cl alone (Sánchez-Porro, Mellado, Bertoldo, Antranikian, and Ventosa, 2003). Neopeptone, yeast

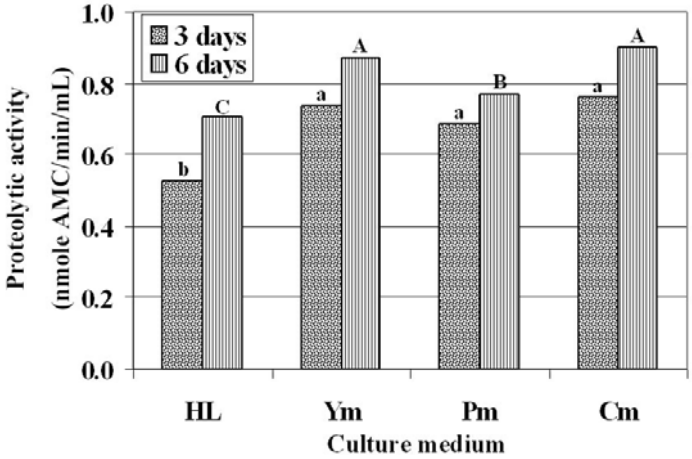

Figure 5.2 Effect of nitrogen source compounds on proteinase production of *Virgibacillus* sp. SK33. Media containing 5% NaCl and incubated at 40 °C for 3 and 6 days. HL = halobacterium broth; HL salt base containing either yeast extract (Ym), peptone (Pm), and casamino acid (Cm). Viable cell counts of *Virgibacillus* sp. SK33 at 3 and 6 days in all media were approximately 7.6 and 7.9 Log CFU/mL, respectively. a and b indicate significant difference (p<0.05) at day 3. A, B, and C indicate significant difference (p<0.05) at day 6.

extract, peptone and casamino acid have been reported to suppress proteinase production of various strains at high concentration. Higher yeast extract content (>0.03%) suppressed the proteinase production of *Bacillus firmus* (Moon and Parulekar, 1991), while proteinase production of *Bacillus* sp. SMIA-2 was gradually decreased with >0.2% peptone (do Nascimento and Martins, 2004). Proteinase production of alkaliphilic actinomycete decreased at either >1.5% peptone or >1% yeast extract (Mehta, Thumar, and Singh, 2006). High concentration (1%) of peptone, yeast extract and beef extract reduced proteinase secretion of *Bacillus* sp. JB-99 (Johnvesly and Naik, 2001). Higher amount of yeast extract (>0.5%) or peptone (>1.5%) decreased the proteinase production of *Conidiobolus coronatus* (Laxman et

al., 2005). Proteinase production is usually repressed by the presence of amino acid or ammonia, which is known as nitrogen catabolic repressor (Ward, 1992). Although neopeptone, yeast extract, peptone and casamino acid are easy to utilize in many bacteria, their content should be controlled so that the maximum proteinase production could be obtained.

5.4.2 Optimal conditions for proteinase production

Optimal conditions for proteinase production of *Virgibacillus* sp. SK33 in HL was at 40 °C and 5% NaCl (Figure 5.3A). Proteinase production dramatically decreased in the absence of NaCl and >5% NaCl ($p<0.05$). Temperature significantly affected proteinase production of *Virgibacillus* sp. SK33 ($p<0.05$). The highest proteinase activity was obtained at low salt content (5%) and at 40 °C. These results suggested that *Virgibacillus* sp. SK33 minimally produced proteinase during fish sauce fermentation, which contained approximately 25-28% NaCl. Consequently, protein hydrolysis contributed from *Virgibacillus* sp. SK33 could be negligible during the natural fermentation. Optimal condition found in this study was in agreement with that of moderately halophile *Salinivibrio* sp. AF-2004 (Amoozegar, Fatemi, Reza, Karbalaei-Heidari, and Razavi, in press). Haloalkaliphilic bacterium *Bacillus* sp. Ve1 also exhibited the optimum proteinase production at 10% NaCl (Patel, Dodia, and Singh, 2005). *Pseudoalteromonas* sp. CP76 showed the highest proteinase production at 7.5% NaCl (Sánchez-Porro et al., 2003).

Similar to proteinase production, viable cell counts slowly decreased with salt content and temperature greatly affected cell growth ($p<0.05$) (Figure 5.3B).

Optimum cell growth was at 35 and 40 °C at 5% NaCl, indicating that *Virgibacillus* sp. SK33 was moderately halophilic bacterium. Limited growth at 25% NaCl also

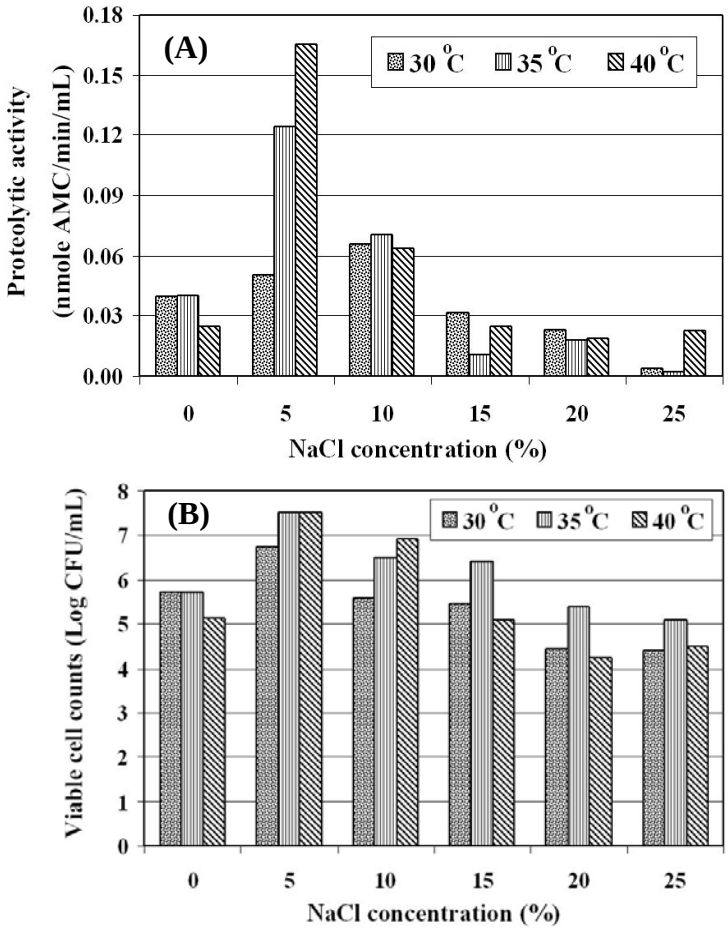

Figure 5.3 Effect of NaCl concentration and temperature on proteinase production (A) and viable cell counts (B) of *Virgibacillus* sp. SK33 cultivated in halobacterium broth (HL) for 1-5 days. Initial viable cell count was ~4.8 Log CFU/mL.

explained why protein hydrolysis caused by microbial proteinases during fish sauce fermentation was rather minimal. Therefore, an increase of cell concentration of *Virgibacillus* sp. SK33 in the form of starter culture could be a potential means to effectively increase microbial proteinase activity and protein hydrolysis of fish sauce fermentation. This would eventually shorten fermentation time.

5.4.3 Time course of cell growth and proteinase production

Cell growth of *Virgibacillus* sp. SK33 gradually increased and reached maximum cell density early stationary phase at 72 h (Figure 5.4). Cell density decreased slowly after 120 h, corresponding to the death phase. Proteinase production also increased rapidly and reached the maximum activity at 36 h, and remained constant thereafter (Figure 5.4). Maximum proteinase activity of *Virgibacillus* sp. SK33 was observed at the middle of log phase (36 h), whereas *Bacillus subtilis* NCIM No. 64 showed the highest production at the late log phase (Kembhavi et al., 1993). *Bacillus polymyxa* and *Halogeometricum* sp. TSS101 showed proteinase production at the end of log phase (Fogarty and Griffin 1973; Vidyasagar et al., 2006). In contrast, *Bacillus* sp. Ve1 showed the optimum proteinase production at early stationary phase (Patel et al., 2005). *Bacillus sphaericus* exhibited maximum proteinase production at death phase (Singh et al., 2004). Our results indicated that *Virgibacillus* sp. SK33 produced proteinase at the earlier stage than other bacteria reported. It should be mentioned that proteinase activity remained constant despite of cell death, suggesting that proteinase from *Virgibacillus* sp. SK33 could be stable at 5% NaCl, 40 °C for up to 192 h.

5.4.4 Temperature and pH optimum of proteinase

Proteinase activity increased with temperature and reached maximum activity at 50 °C. The activity decreased at temperature >50 °C (Figure 5.5A). Optimum temperature of proteinase from *Bacillus subtilis* CN2 isolated from Vietnamese fish

sauce (nuoc-mam), *Halobacillus* sp. SR5-3 isolated from Thai fish sauce (nampla), and *B. subtilis* and *B. licheniformis* isolated from budu (fish sauce produced from

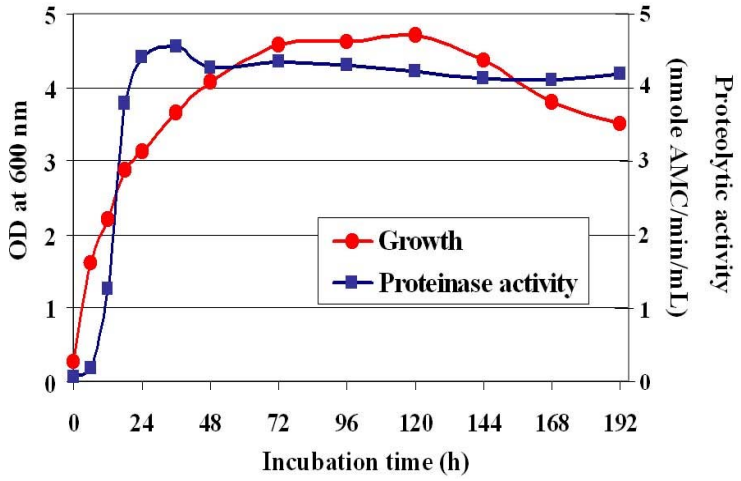

Figure 5.4 Growth curve and proteinase production of *Virgibacillus* sp. SK33 in Ym containing 5% NaCl, 40 °C and shaking speed at 100 rpm

Southern part of Thailand) was at 50 °C (Choorit and Prasertsan, 1992; Uchida et al., 2004; Namwong et al., 2006). Proteinase of *Filobacillus* sp. RF-25 and *B. subtilis* JM-3 isolated from fish sauce showed maximum activity at 60 °C (Hiraga et al., 2005; Kim and Kim, 2005). At 40 °C, a typical temperature of traditional fish sauce fermentation, 72% of maximum activity was retained. This indicated that extra energy supply might not be necessary if the enzyme is added to accelerate the fermentation process.

Optimum pH was found at 8, 10 and 11 (Figure 5B). Bacterial proteinases isolated from fish sauce fermentation have been reported to be active over a wide pH range. Optimum pH of proteinases from *Bacillus subtilis* CN2, *Halobacillus* sp. SR5-3, *B. subtilis*, *B. licheniformis* and *Filobacillus* sp. RF-25 was about pH 7-11 (Choorit and Prasertsan, 1992; Uchida et al., 2004; Hiraga et al., 2005; Namwong et al., 2006),

while proteinase from *B. subtilis* JM-3 showed optimum pH at 5.5 (Kim and Kim, 2005). Several optimum pH values observed in this study implied that crude extract might contain several proteinases.

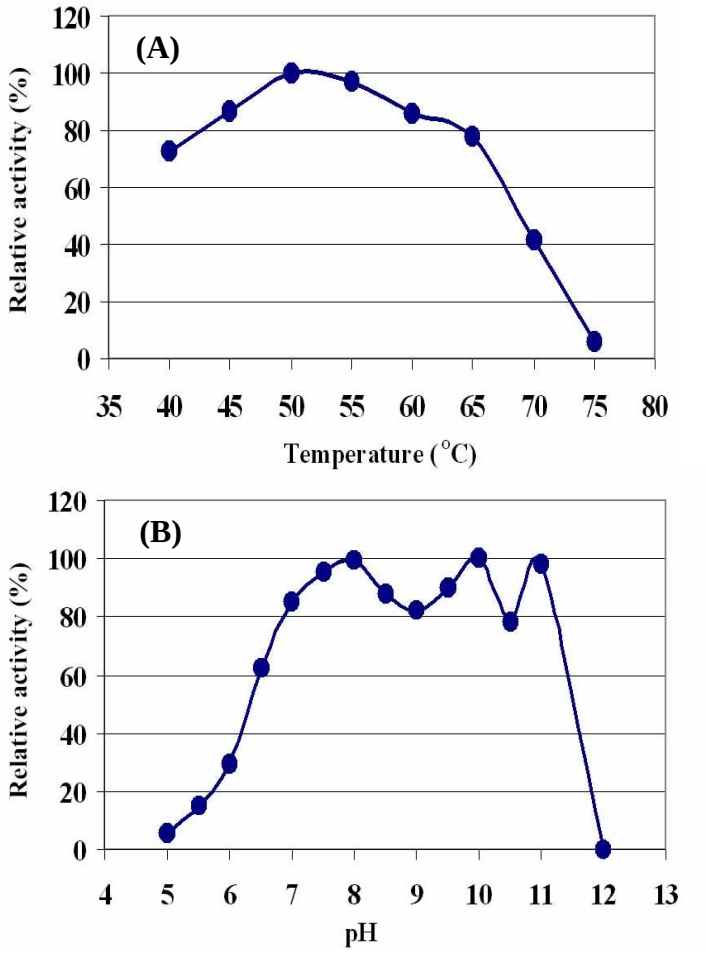

Figure 5.5 Temperature (A) and pH (B) optimum of proteinases from *Virgibacillus* sp. SK33

5.4.5 Effect of inhibitors, ions and synthetic substrates on proteinase activity

Proteinase activity of *Virgibacillus* sp. SK33 was strongly inhibited by PMSF, a serine proteinase inhibitor (Table 5.1). Trypsin-like proteinase inhibitors, namely leupeptin, soybean inhibitor and TLCK, did not inhibit proteinase activity.

Chymotrypsin-like proteinase inhibitor also had no effect on activity. Metalloproteinase and cysteine inhibitors showed moderate inhibition. These results indicated that crude proteinase from *Virgibacillus* sp. SK33 was serine proteinase. The partial inhibition of cysteine proteinase inhibitors was likely due to a similar configuration of active site between serine and cysteine proteinases (Fernández, Mohedano, Polanco, Medina, and Nuñez, 1996).

Proteinase from *Virgibacillus* sp. SK33 was not inhibited by all metal ions studied (Table 5.1). Monovalent cations (Li^+, Na^+ and K^+) did not affect proteinase activity, whereas divalent cations, namely Mg^{2+}, Ca^{2+} and Sr^{2+}, showed moderate activation (Table 5.1). Hydrated salts would form specific electrostatic interaction on enzyme surface and at the active site, thereby their effect on proteinase activity may be either activation or inactivation (Inouye, Kuzuya, and Tonomura, 1998). Crude proteinase from *Bacillus brevis* was slightly inhibited by Na^+ and K^+, but was activated by Ca^{2+} (Banerjee et al., 1999), while proteinase from *Pseudoalteromonas* sp. CP76 was slightly inhibited by Mg^{2+}, Ca^{2+} and Ba^{2+} (Sánchez-Porro et al., 2003).

Proteinases of *Virgibacillus* sp. SK33 only hydrolyzed Suc-Ala-Ala-Pro-Phe-AMC (Table 5.2), suggesting that P_1 and P_2 position of the enzyme preferred aromatic amino acid and proline residue, respectively. Suc-Ala-Ala-Pro-Phe-chromogenic is a typical substrate for subtilisin (Graycar, Ballinger, and Wells, 2004). According to the effect of inhibitors and substrate specificity, the crude proteinase from *Virgibacillus* sp. SK33 showed a subtilisin-like characteristic.

Table 5.1 Effect of various inhibitors and ions on activity of crude proteinase from *Virgibacillus* sp. SK33

Substances	Targeted proteinase	Final concentration	Relative activity (%)
Leupeptin	Trypsin-like and some cysteine proteinases	100 μM	91
Trypsin inhibitor I (soybean)	Trypsin-like proteinase	0.02 mg/mL	91
TLCK	Trypsin-like proteinase	100 μM	96
TPCK	Chymotrypsin-like proteinase	100 μM	93
PMSF	Serine proteinase	1 mM	45
EDTA	Metallo proteinase	10 mM	95
L-Histidine	Metallo proteinase	10 mM	90
Imidazole	Metallo proteinase	10 mM	89
Bestatin	Aminopeptidase	10 μM	92
Pepstatin A	Acid proteinase	10 μM	92
E-64	Cysteine proteinase	10 μM	85
N-Ethylmaleimide	Cysteine proteinase	1 mM	83
Iodoacetic acid	Cysteine proteinase	1 mM	77
Dithiothreitol		10 mM	90
2-Mercaptoethanol		10 mM	96
L-Cysteine		10 mM	95
Mono- and di-valent cations			
Li^{+}		10 mM	103
Na^{+}		10 mM	94
K^{+}		10 mM	97
Mg^{2+}		10 mM	125
Ca^{2+}		10 mM	143
Sr^{2+}		10 mM	121
Ba^{2+}		10 mM	66
Metal ions			
Cu^{2+}		1 mM	103
Cd^{2+}		1 mM	131
Co^{2+}		1 mM	93
Fe^{3+}		1 mM	104
Mn^{2+}		1 mM	87
Hg^{2+}		1 mM	91
Zn^{2+}		1 mM	133

Table 5.2 Substrate specificity of crude proteinase from *Virgibacillus* sp. SK33

Synthetic substrates	Specificity	Specific activity (nmole AMC/min/mg)
Boc-Asp(oBzl)-Pro-Arg-AMC	Trypsin-like, α-thrombin-like	0
Boc-Gln-Ala-Arg-AMC	Trypsin-like	0
Boc-Val-Leu-Lys-AMC	Plasmin-like	0
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin-like, subtilisin-like	164
Z-Phe-Arg-AMC	Cathepsin L-like	0
Z-Arg-Arg-AMC	Cathepsin B-like	0

5.4.6 Molecular weight estimation

Molecular weight (MW) of crude proteinase from *Virgibacillus* sp. SK33 was estimated to be 56, 46, 42, 32, 25 and 19 kDa, based on activity staining (Figure 5.6). Crude proteinase from *Brevibacterium linens* ATCC 9172 showed four main MWs of 280, 220, 130 and 43 kDa (Buchinger, Tomaschová, Zemanovic, and Hampel, 2001). Proteinase patterns observed in 25% NaCl were the same as those in the absence of NaCl, demonstrating that all proteinases showed hydrolytic activity at high salt content (25% NaCl). *Virgibacillus* sp. SK33 produced several proteinases with different molecular sizes, which might have different biochemical characteristics as three peaks of pH profile were observed. Proteinases with MW of 32 and 19 kDa could be the abundant component due to their distinctive intensity.

5.4.7 Effect of NaCl on proteinase activity

Activity of crude proteinase from *Virgibacillus* sp. SK33 gradually increased with NaCl concentration and showed maximal activity at 15-25% NaCl (Figure 5.7A). Lower activity was observed in the studied commercial enzymes, Alcalase and

Protamex. Extreme halophile proteinase usually requires ≥1 M NaCl (5.8%) for optimal activity and stability (Lanyi, 1974). Our study found that optimum activity of proteinase from *Virgibacillus* sp. SK33 also required high NaCl concentration. Activity of *Bacillus licheniformis* SMI 4.C.1 proteinase was increased 3 times with 1-1.5 M NaCl (5.8-8.8%) (Manachini and Fortina, 1998). Proteinase from *Halobacillus* sp. SR5-3 also showed maximal activity at 25% NaCl (Namwong et al., 2005), while psychrophilic bacterium PA-43 proteinase increased its activity to 3.6 times at 4.5 M NaCl (26.3%) (Irwin, Alfredsson, Lanzetti, Gudmundsson, and Engel, 2001).

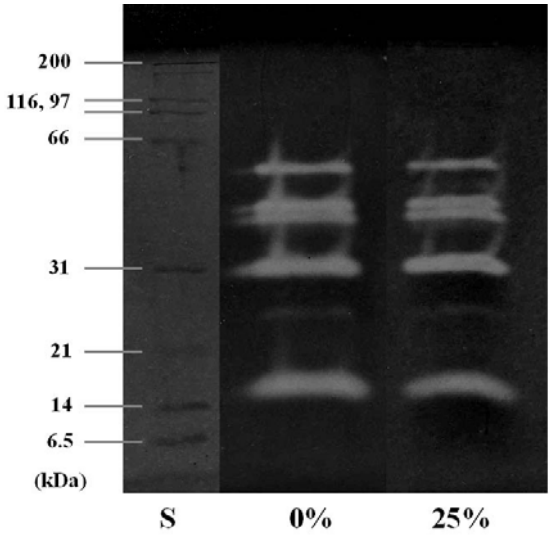

Figure 5.6 Activity staining (SDS-PAGE, 12.5% T) of *Virgibacillus* sp. SK33 proteinases in the presence and absence of 25% NaCl. S = molecular weight standard

Virgibacillus sp. SK33 proteinase also hydrolyzed anchovy protein to a greater extent than did commercial proteinases studied ($p<0.05$) (Figure 5.7B). Activity of crude proteinase and Alcalase gradually increased with NaCl and reached the maximum at about 20-25% NaCl, corresponding to the results of synthetic substrate

(Figure 5.7A). NaCl may induce structural changes, which increased the flexibility of substrate-binding site, leading to increment of substrate binding. Protamex showed

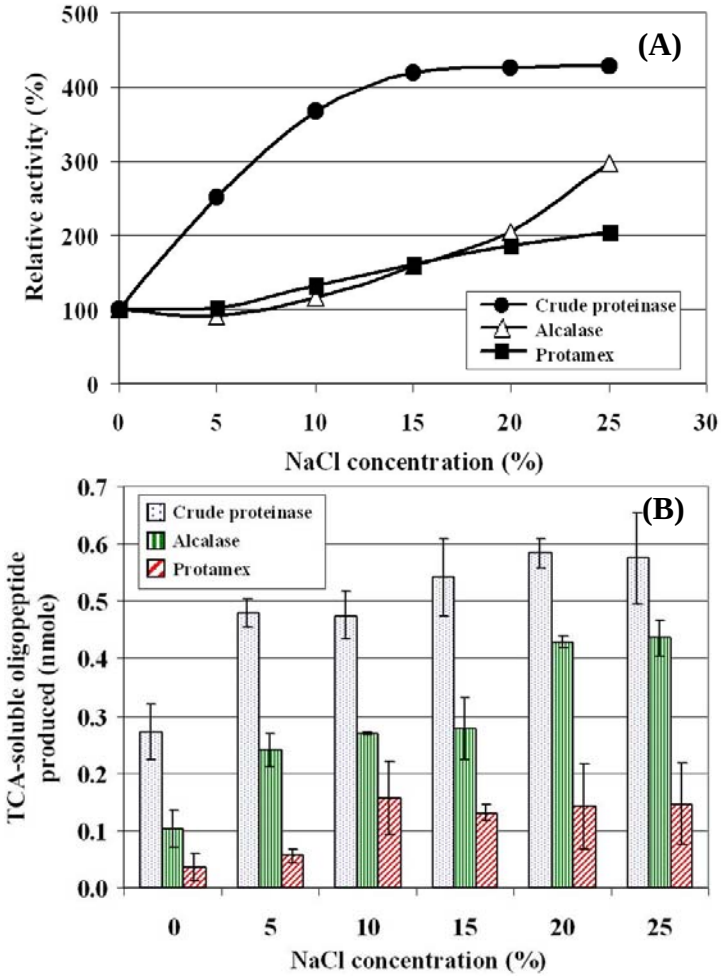

Figure 5.7 Effect of NaCl on proteolytic activity of crude proteinase from *Virgibacillus* sp. SK33 compared with commercial endoproteinases using Suc-Ala-Ala-Pro-Phe-AMC (A) and anchovy (B) as a substrate.

the lowest proteolytic activity when compared to others (p<0.05). It should be noted that protein composition at each salt content would be different because solubility and extractability of muscle proteins varied with NaCl concentration. Proteinases from *Bacillus* sp. Ve1 gradually decreased activity with increasing NaCl up to 0.17 M (1%) (Gupta et al., 2005), while proteinase from moderately halophilic *Bacillus* sp. no.21-1 showed its maximum activity at 0.5 M NaCl (2.9%) and no activity was observed at 3

M NaCl (17.5%) (Kamekura and Onishi, 1974). Proteinase from *Bacillus clausii* I-52 and *Salinivibrio costicola* 18AG exhibited optimal activity at 1% and 2% NaCl, respectively, and its activity gradually decreased at higher NaCl concentration (Joo and Chang, 2005a; Lama, Romano, Calandrelli, Nicolaus, and Gambacorta, 2005). Although *Virgibacillus* sp. SK33 could not grow well and optimally secreted proteinases under high salt content, the secreted proteinases exhibited high activity towards synthetic substrate and anchovy at 20-25% NaCl, a typical salt concentration of fish sauce fermentation. The enzymes also showed activity at relatively higher NaCl compared to other commercial microbial proteinases. Based on these characteristics, *Virgibacillus* sp. SK33 proteinases could be employed to increase the degree of protein hydrolysis at high NaCl content like fish sauce or soy sauce fermentation.

5.5 Conclusions

Virgibacillus sp. SK33 has been newly isolated from fish sauce fermentation. Yeast extract, peptone or casamino acid could be used as a single nitrogen source to induce proteinase production of *Virgibacillus* sp. SK33. *Virgibacillus* sp. SK33 did not grow well and produced proteinase under high salt content. But, proteinase activity increased with NaCl concentration. Proteinases from *Virgibacillus* sp. SK33 showed subtilisin-like characteristic with broad optimal pH range (7-11). Several proteinases with different MWs were observed under high NaCl concentration (25%). Proteinases from *Virgibacillus* sp. SK33 showed higher activity than two commercial enzymes, Alcalase and Protamex, under high salt content, demonstrating its potential application for the process containing high NaCl.

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CHAPTER VI

PURIFICATION AND CHARACTERIZATION OF A NaCl-ACTIVATED PROTEINASE FROM *VIRGIBACILLUS* SP. SK33 ISOLATED FROM THAI FISH SAUCE

6.1 Abstract

Extracellular proteinase from *Virgibacillus* sp. SK33, a moderately halophilic bacterium isolated from 1 month-old fish sauce, was purified to electrophoretic homogeneity using hydrophobic interaction chromatography and hydroxyapatite with purification fold of 2.5 and 7% yield. The enzyme was a dimer with molecular weight (MW) of ~43 kDa with a subunit of ~19 kDa. The dimer was stabilized by hydrophobic interactions and showed caseinolytic activity in the dissociated form. The isoelectric point (pI) value was 4.28. Optimum conditions for catalytic activity were at 55 °C and pH 7.5. Phenylmethanesulfonyl fluoride (PMSF) strongly inhibited the activity. The enzyme preferentially hydrolyzed Suc-Ala-Ala-Pro-Phe-AMC, indicating a subtilisin-like characteristic. The enzyme was stable at 30-60 °C and 0-25% NaCl. Michaelis constant (K_m) and maximum velocity (V_{max}) of the purified proteinase were 27 μM and 0.35 μM/s, respectively. Proteinase activity towards both synthetic and anchovy substrates was activated by NaCl up to 25%.

Key word: *Virgibacillus* sp., NaC-activated proteinase, purification, fish sauce

6.2 Introduction

Microbial proteinases from *Bacillus* and various strains of fungi have been extensively studied and commercially exploited. Typically, these enzymes were isolated from mesophilic microorganisms and exhibited activity at neutral to alkaline pH range, mild temperature and low ionic strength. Activity of *Bacillus* proteinases exhibited the optimal pH at 5.5-9 and temperature at 50-60 °C (Petersen, 1981). Low thermal stability of these enzymes is major limitation for application of the proteinase (Anwar and Saleemuddin, 1998). Proteinase from *Bacillus amyloliquefaciens* could not function at >50 °C, while *B. licheniformis* proteinase readily inactivated near pH 4 (Adler-Nissen, 1993). In addition, activity of *Bacillus* proteinases decreased at high NaCl concentration. Proteinase activity from *Bacillus* sp. decreased with NaCl concentration (Gupta et al., 2005). Thus, proteinases from mesophilic bacteria have a limitation in applying in harsh conditions (Demirjian, Morís-Varas, and Cassidy, 2001).

Extremophilic microorganisms are considered to be an important source of enzymes that are able to function under harsh conditions (Kumar and Takagi, 1999). Extermophiles are organisms thriving in extreme conditions, which include thermophiles, acidophiles, alkalophiles, psychrophiles and halophiles. Halophiles can produce proteinases that are able to perform catalytic reaction at relatively high NaCl concentration (5.8-30%). Archaebacteria maintain an osmotic balance in their cytoplasm against salt environments by accumulating high salt concentration (Margesin and Schinner, 2001). These results might be the adaptation towards

extreme condition of the enzymes. Halophilic bacteria produced varieties of proteinases. Stepanov et al. (1992) purified subtilisin-like proteinase from *Halobacterium mediterranei*. A chymotrpysin-like proteinase was purified from arhaeon *Natrialba asiatica* 172 P1 and showed optimum activity at 30% NaCl (Kamekura and Seno, 1990), whereas optimum activity of the purified chymotrypsin-like proteinase from arhaeon *Natrialba magadii* was at 1-1.5 M NaCl (5.8-8.8%) (Giménez, Studdert Sánchez, and De Castro, 2000). Crude enzyme usually contains several proteinases. Seven proteinases with MWs ranging from 50 to 120 kDa were found in crude proteinase from arhaeon *Natronococcus occultus* (Studdert, De Castro, Herrera Seitz, and Sánchez, 1997). Purification and characterization is necessary to elucidate characteristics of the interested proteinase.

Moderate halophiles are microorgainisms growing well at 3-15% NaCl (Ventosa, Nieto, and Oren, 1998) and its proteinase activity also increased with NaCl concentration. Recently, proteinase-producing bacteria from fish sauce fermentation have been isolated and identified to be *Virgibacillus* sp. according to 16S rRNA gene sequence. Activity of crude proteinases from *Virgibacillus* sp. SK33, a moderately halophilic bacterium, increased with NaCl concentration up to 25% and showed higher proteolytic activity towards anchovy than Alcalase and Protamex. However, the proteinase from *Virgibacillus* sp. SK33 has never been purified and characterized. In addition, very few proteinases from bacteria isolated from fish sauce fermentation have been purified (Uchida et al., 2004; Kim and Kim, 2005; Hiraga et al., 2005; Namwong et al., 2006). Therefore, our objective was to purify the proteinase from *Virgibacillus* sp. SK33 and to elucidate biochemical characteristics of the purified proteinase.

6.3 Materials and methods

6.3.1 Chemicals

t-Butyloxycarbonyl(Boc)–Asp(oBzl)-Pro-Arg-4-methyl-7-coumarylamide (AMC), succinyl(Suc)-Ala-Ala-Pro-Phe-AMC and carbobenzoxy(Z)-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Z-Arg-Arg-AMC, leupeptin, trypsin inhibitor I (soybean), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), bestatin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidine)-butane (E-64), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), dithiothreitol (DTT), 2-mercaptoethanol (β-ME), bovine serum albumin, L-tyrosine and casein were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Ethylenediaminetetraacetic acid (EDTA), L-cysteine, imidazole and L-histidine were purchased from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

6.3.2 Purification

Virgibacillus sp. SK33 was cultivated in halobacterium salt broth supplemented with yeast extract (1% yeast extract, 0.3% trisodium citrate, 0.2% potassium chloride, 2.5% magnesium sulfate and 5% NaCl) at 40 °C and a shanking speed of 100 rpm, for 3 days. Crude proteinase was collected by centrifugation at 8,000×g for 30 min, at 4 °C.

All purification steps were carried out at ~4 °C using the Purifier 10 (ÄKTA, GE Healthcare, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Ammonium

sulfate was slowly added to the crude proteinase to attain a final concentration of 1 M, centrifuged at 10,000×g for 30 min and filtered through a 0.45 μm-membrane filter. The filtrate was loaded onto a phenyl-Sepharose (GE Healthcare, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column (2.6 × 6.5 cm) equilibrated with 1 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 8.0 and washed with same buffer for 3 bed volumes. Elution was performed with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 8.0. Fractions of 5 mL were collected at a flow rate of 1 mL/min. Active fractions were pooled. Diafiltration against 50 mM Tris-HCl (pH 8.0) was performed using a membrane with molecular weight cut-off (MWCO) of 10 kDa (Vivaspin, Sartorius AG, Goettingen, Germany).

Sample obtained after diafiltration was applied to a hydroxyapatite column (5 mL) (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 50 mM Tris-HCl (pH 8.0). The column was washed with equilibrating buffer for 3 bed volumns and was eluted for 2 bed volumes with a linear gradient of 0 to 0.3 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 8.0. Fractions of 2.5 mL were collected at a flow rate of 0.5 mL/min. Active fractions were pooled. Protein content was monitored at 280 nm and proteinase activity was determined as described below.

6.3.3 Activity staining

Activity staining was determined according to García-Carreño, Dimes, and Haard (1993). Purified proteinase was mixed with equal volume of loading buffer containing β-ME (125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% sodiumdodecyl sulfate (SDS) and 10% β-ME) and in the absence of β-ME. The gel containing 12.5% acrylamide was run at 100 V (Laemmli, 1970). Subsequently, it was immersed in 2%

casein, 100 mM Tris-HCl, pH 8.0 at 4 °C for 30 min and washed twice with 100 mM Tris-HCl (pH 8.0). Proteolytic reaction was carried out in 100 mM Tris-HCl (pH 7.5) at 55 °C for 30 min. Gel was stained in 0.1% Coomassie brilliant blue R-250, 40% methanol and 10% acetic acid for 1 h and destained in 25% ethanol and 10% acetic acid. Clear zone indicated the presence of proteinase activity.

6.3.4 Estimation of molecular weight by gel filtration

The molecular weight of the native proteinase was determined using a Superose 6 (GE Healthcare, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column (1.6 × 85 cm) equilibrated and eluted with 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0. Fractions of 0.85 mL were collected at a flow rate of 0.85 mL/min. Void volume (V_o) was determined using blue dextran (Sigma Chemical Co., St. Louis, Mo., USA). The ratio of elution volume (V_e) to V_o was calculated. The column was calibrated using a protein standard consisting of β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrom C (13 kDa) (Sigma Chemical Co., St. Louis, Mo., USA).

6.3.5 Isoelectric point determination

The isoelectric point (pI) of the purified proteinase was determined by PhastGel isoelectric focusing (IEF) with a PhastGel IEF 3-9 (GE Healthcare, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The operating condition was as follows: (1) prefocusing step: 2000 V, 2 mA, 3.5 W, 15 °C, 75 Vh; (2) sample application: 100 V, 2 mA, 3.5 W, 15 °C, 15 Vh; (3) focusing step: 2000 V, 5 mA, 3.5

W, 15 °C, 75 Vh. The broad range pI standard (3-10) was used (GE Healthcare, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Silver staining was performed.

6.3.6 Assay of proteinase activity

Proteinase activity was assayed by modified method of Barrett and Kirschke (1981) using Suc-Ala-Ala-Pro-Phe-AMC as a substrate. The reaction mixture (1 mL) contained 50 μL of the purified enzyme, 1 μM synthetic substrate, 200 mM Tris-HCl (pH 7.5) and was incubated at 55 °C for 5 min. The reaction was stopped by adding 1.5 mL of the stopping solution (30% butanol, 35% methanol and 35% deionized water). Fluorescence intensity was measured at excitation and emission wavelength of 380 and 460 nm, respectively (RF-1501, Shimadzu Co., Kyoto, Japan). Unit activity was defined as nmole of AMC released/min.

6.3.7 Effect of temperature and pH

The optimal temperature of proteinase activity was measured at 4, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C in 200 mM Tris-HCl (pH 8.0), which was the optimal pH of crude proteinase. pH profile was measured at 55 °C using various pHs: pH 5.0, 5.5, 6.0 (100 mM sodium acetate); pH 6.5, 7.0 (100 mM Tris-maleate); pH 7.5, 8.0, 8.5, 9.0 (200 mM Tris-HCl); pH 9.5, 10.0 (100 mM borate buffer); pH 11.0 (200 mM carbonate buffer); and pH 12.0 (200 mM phosphate buffer).

6.3.8 Effect of NaCl

Effect of NaCl on proteinase activity was determined by incubating the purified proteinase in 200 mM Tris-HCl (pH 7.5) containing various NaCl concentrations (0-25%) at 55 °C for 5 min. Deionized water was used instead of butanol to avoid phase separation in the presence of NaCl. All reactions were heated at 90 °C for 5 min to terminate the reaction. The residual activity was calculated as the percentage of sample without NaCl as 100%.

6.3.9 Thermal and salt stability

Temperature stability of the enzyme was investigated by pre-incubating the purified enzyme in 200 mM Tris-HCl (pH 7.5) at 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C for 30 min. When incubation time was reached, samples were immediately cooled in iced water. The residual activity was determined by the method described above.

Salt stability was monitored by pre-incubating the purified proteinase in 5, 10, 15, 20 and 25% NaCl, in 200 mM Tris-HCl, pH 7.5 at 55 °C for 60 min. The sample were rapidly cooled and assayed at the optimum condition, 200 mM Tris-HCl (pH 7.5), 20% NaCl, at 55 °C.

6.3.10 Effect of inhibitors and ions

The effect of various inhibitors on proteinase activity was determined using various substances, namely leupeptin, trypsine inhibitor I (soybean), TLCK, TPCK, PMSF, EDTA, L-histidine, imidazole, bestatin, pepstatin A, E-64, IAA, NEM, DTT and β-ME. The effect of metal ions, Cu^{2+}, Cd^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, Hg^{2+}, Zn^{2+}, Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}, was also investigated. Reaction without

substances was taken as 100% activity. In addition, effect of divalent ions, Ca^{2+}, Sr^{2+} and Mg^{2+}, on proteinase activity was performed at various concentrations ranging from 0-100 mM.

6.3.11 Substrate specificity

Activity of the purified proteinase was determined using various synthetic substrates at 1 μM, including Boc-Asp(oBzl)-Pro-Arg-AMC, Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC. Activity was determined under the standard assay condition. Specific activity was expressed as the released amount of AMC in nmole per min per mg protein.

6.3.12 Michaelis constant

Michaelis constant (K_m) and maximum velocity (V_{max}) value of the purified proteinase were carried out in 200 mM Tris-HCl (pH 7.5) at 55 °C. Suc-Ala-Ala-Pro-Phe-AMC at various concentrations from 10 to 200 μM was used as a substrate. Kinetic parameters were determined using linear regression of Hanes-Wolff plot (Copeland, 2000).

6.3.13 Hydrolysis of anchovy proteins

Whole anchovy (*Stolephorus indicus*) was homogenized in cooled 50 mM Tris-maleate, pH 7.0, containing various NaCl concentrations (0, 5, 10, 15, 20 and 25% NaCl) at the ratio of fish to buffer of 1:5. The homogenate was stirred at 4 °C for 30 min. The homogenate was centrifuged at 13,000×g for 30 min. The

supernatants were used as a substrate of the purified enzyme. Protein content was determined by a dye binding method (Bradford, 1961) using bovine serum albumin as a standard.

The reaction mixture (1 mL) contained the purified proteinase 30 U (nmole AMC/min), 200 mM Tris-HCl (pH 7.5), 100 μM leupeptin and 1.5 mg/mL fish protein solubilized at various NaCl concentrations. Concentration of NaCl in the reaction was adjusted with respect to NaCl concentration of the substrate. The mixture was incubated at 55 °C for 30 min. The reaction was stopped by adding trichloroacetic acid (TCA) to final concentration of 10%. The samples were cooled at 4 ºC for 1 h to allow complete protein precipitation and then centrifuged at 10,000×g for 10 min. TCA-soluble oligopeptide contents were determined by Lowry method (Lowry, Rosenbrough, Farr, and Randall, 1951) using tyrosine as a standard. Blanks were prepared in the same manner except that the heated proteinase (90 °C for 5 min) was used.

6.4 Result and discussion

6.4.1 Purification and molecular weight estimation of proteinase

Proteinase from *Virgibacillus* sp. SK33 was purified to homogeneity using phenyl-Sepharose and hydroxyapatite (HA). In hydrophobic interaction chromatography (HIC), one protein peak with activity was eluted around 20 mM $(NH_4)_2SO_4$ (Figure 6.1), indicating that proteinase strongly bound to the phenyl ligand. This step achieved 1.8-fold with a yield of 11% (Table 6.1). The purified proteinase was obtained after HA chromatography. Typically, protein elution from HA can be achieved by phosphate buffer. This is based on the competition between

Table 6.1 Purification table of the purified proteinase from *Virgibacillus* sp. SK33

Step	Total Unit activity (Unit)	Protein concentration (mg/mL)	Specific activity (Unit/mg protein)	Purifi-cation fold	Yield (%)
Crude in 1 M $(NH_4)_2SO_4$	696	0.04	154	1	100
Phenyl-Sepharose	62	0.36	276	1.8	11
Hydroxyapatite	37	0.02	374	2.5	7

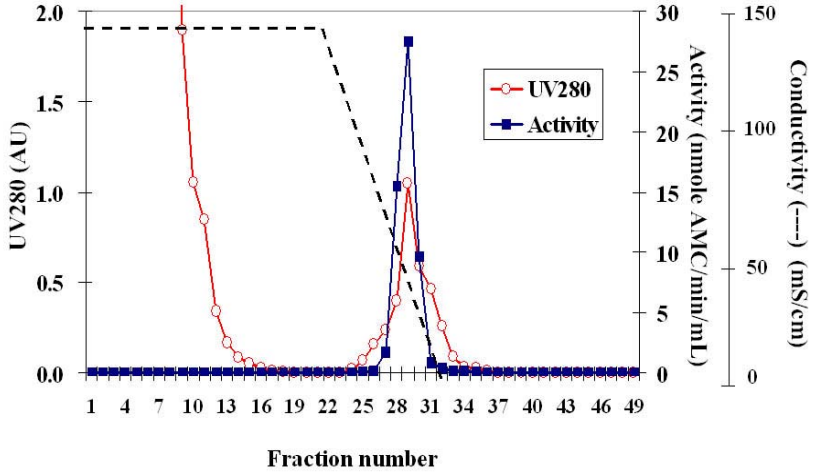

Figure 6.1 Chromatogram of the purified proteinase on a phenyl-Sepharose column

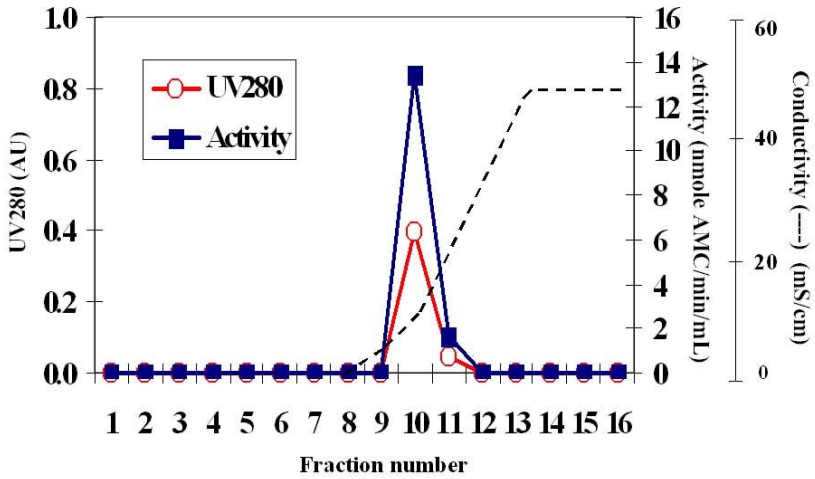

Figure 6.2 Chromatogram of the purified proteinase on a hydroxyapatite column

phosphate ion and protein for interaction with calcium group of HA. However, our results demonstrated that $(NH_4)_2SO_4$ was a more effective eluent. Purity of proteinase increased about 2.5 folds with a yield of 7% (Table 6.1).

Virgibacillus sp. SK33 proteinase did not bind to benzamidine Sepharose, suggesting that the proteinase is not a lysine-specific enzyme, like trypsin. Moreover, the proteinase was not successfully purified using diethylaminoethyl (DEAE) Sephacel and Source 15 quaternary ammonium (Q). However, proteinase from archaebacterium *Pyrococcus abyssi* was successfully purified using DEAE Trisacryl M, sulfopropyl (SP) Trisacryl M and high Q (Dib, Chobert, Dalgalarrondo, Barbier, and Haertlé, 1998). *Halobacterium* sp. TuA4 proteinase was also accomplished using carboxymethyl (CM) Sehphadex, Mono Q and phenyl-Sepharose (Schmitt, Rdest, and Goebel, 1990). Purification of *Virgibacillus* sp. SK33 proteinase was similar to halophilic proteinase from *Desulfurococcus mucosus* using CM Sepharose and HA (Cowan, Smolenski, Daniel, and Morgan, 1987).

Molecular weight (MW) of the purified proteinase determined from gel filtration was estimated to be ~43 kDa (Figure 6.3) and ~19 kDa under denature condition, implying that proteinase was a dimer. Proteinase from *Brevibacterium linens* and *B. linens* ATCC9174 was found to be a dimer with MW of 95 kDa (Juhasz and Škárka, 1990) and 126 kDa (Rattray, Bockelmann, and Fox, 1995), respectively. Serine endoproteinases from archaea and eubacteria are rarely reported to be dimer. Proteinase from arhaeabacterium, *Pyrococcus abyssi* 549, was oligomer with predominant MWs of 150, 105 and 60 kDa (Dip et al., 1998), while *P. furiosus* proteinase was hexamer with MW of 124 kDa and trimer with MW of 59 kDa (Halio, Bauer, Mukund, Adams, and Kelly, 1997). Extracellular proteinases secreted by

extremely and moderately halophilic bacteria, *Natrialba magadii*, *Na. asiatica* 172 P1, *Salinivibrio costicola* 18AG, *Halobacterium* sp. TuA4, *H. mediterranei* and *H. halobium*, were a single polypeptide chain with MW of 45, 44-46, 38, 60, 41 and 66 kDa, respectively (Kamekura and Seno, 1990; Schmitt et al., 1990; Stepanov et al., 1992; Ryu, Kim, and Dordick, 1994; Giménez et al., 2000; Lama, Romano, Calandrelli, Nicolaus, and Gambacorta, 2005). Subtilisin, a *Bacillus* proteinase, is a monomer with MW of ~27 kDa (Graycar, Ballinger, and Wells, 2004). Thus, the purified proteinase from *Virgibacillus* sp. SK33 appeared to show different MW from other microbial serine proteinases previously reported.

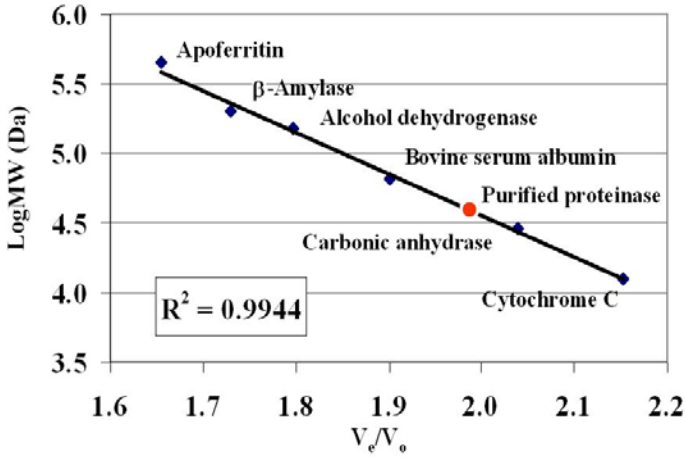

Figure 6.3 Estimation of molecular weight of the purified proteinase on Superose 6

Protein pattern of the purified enzyme in the presence of β-ME was not different from that without β-ME, indicating that monomer was not linked by disulfide bonds (Figure 6.4A). It was postulated that hydrophobic interaction was the main force stabilizing dimer. It should be noted that a subunit of the proteinase exhibited caseinolytic activity even under denaturing condition (Figure 6.4B). This implied that each subunit contained active site. Moreover, 2% SDS might not completely denature monomeric form of proteinase. Native proteinase structure of *Thermococcus stetteri* was maintained in the presence of 2% SDS (Klingeberg,

Galunsky, Sjoholm, Kasche, and Antranikian, 1995). The activity of *Bacillus* sp. AH-101 proteinase was also not affected by 0.2% SDS (Takami, Akiba, and Horikoshi, 1990), whereas archaeon *Natrialba magadii* and *Bacillus amyloliquefaciens* S94 proteinases were strongly inhibited by 2% and 0.1% SDS, respectively (Giménez et al., 2000; Son and Kim, 2002).

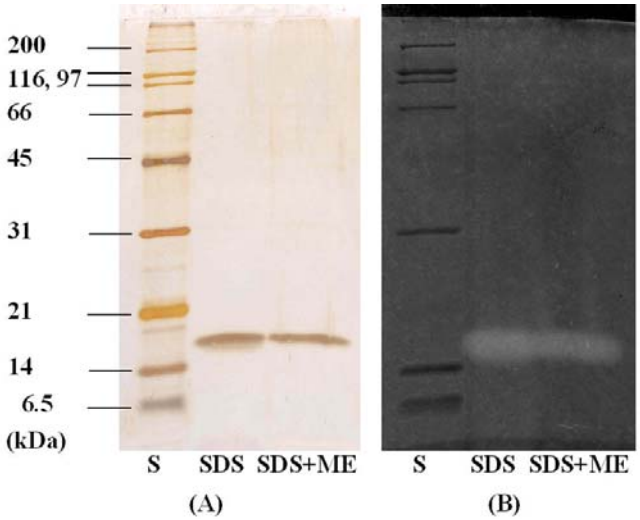

Figure 6.4 SDS-PAGE (12.5% T) of the purified proteinase visualized by silver staining (A) and activity staining (B). S = standard molecular weight; SDS = treatment buffer containing SDS and SDS+ME = treatment buffer containing SDS and 2-mercaptoethanol

6.4.2 Isoelectric point

The isoelectric point (pI) of the purified proteinase was ~4.28 (Figure 6.5). This was similar to pI of proteinase from *Brevibacterium linens* ATCC 9174 and *Bacillus subtilis* which was 4.1 (Rattray et al., 1995; Yamagata, Yoshida, Noda, and Ito, 1995). Proteinase from extremely halophilic bacteria *Natrialba asiatica* 172 P1, *Haloferax mediterranei* R4 and *Thermococcus kodakaraensis* showed pI value of 3.7, 4.1 and 4.2, respectively (Sacco, Tortora, and Vanoni, 2004). Psychrophilic bacterium PA-43 and *Flavobacterium balustinum* P104 produced serine proteinases with low pI value at ~3.8 and ~3.5, respectively (Morita et al., 1998; Irwin,

Alfredsson, Lanzetti, Gudmundsson, and Engel, 2001). pI of *Bacillus thermoruber* proteinase was 5.3 and that of *Bacillus* sp. SS103 was 5.5 (Manachini, Fortina, and Parini, 1988; Cha, Park, and Yoon, 2005). However, archaebacteria and eubacteria typically produced serine proteinases with pI varying from neutral to alkaline range. Proteinase from archaebacteria *Desulfurococcus mucosus*, *Halobaterium mediterranei*, and *Pyrobaculum aerofilum* showed pI of 8.7, 7.5 and 6.6, respectively (Cowan et al., 1987; Stepanov et al., 1992; Sacco et al., 2004). Proteinase from *Bacillus alcalophilus* subsp. *halodurans* KP1239, *B. pumilus*, *Bacillus* sp. Y and *Bacillus* sp. NKS-21 showed pI of 8.2-10.1 (Tsuchida et al., 1986; Takii, Kuriyama, and Suzuki, 1990; Shimogaki et al., 1991; Huang, Peng, Li, Wang, and Zhang, 2003). Therefore, pI of *Virgibacillus* sp. SK33 proteinase was rather different from that of other serine proteinases. Based on pI value in the acidic range, the purified proteinase from *Virgibacillus* sp. SK33 might possess numerous acidic amino acids in its structure.

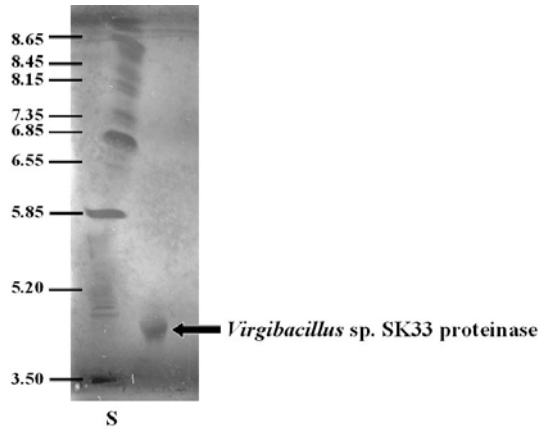

Figure 6.5 Isoelectric focusing of the purified proteinase. S = isoelectric focusing standard

6.4.3 Temperature/pH optimum and thermal stability

Optimum temperature of the purified proteinase was at 55 °C (Figure 6.6). The activity was gradually increased with temperature, suggesting that the enzyme is a heat-activated proteinase. Archaebacteria *Halobacterium mediterranei*, *Halogeometricum* sp. TSS101 and *Natrialba magadii* produced proteinase with the maximum activity at 55-60 °C (Stepanov et al., 1992; Giménez et al., 2000; Vidyasagar, Prakash, and Sreeramulu, 2006). Proteinase of *Halobacterium halobium* S9 showed maximal activity at 40 °C (Capiralla, Hiroi, Hirokawa, and Maeda, 2002), whereas *Natrialba asiatica* 172 P1 produced proteinases with optimal activity at 75-80 °C in the presence of 25% NaCl (Kamekura and Seno, 1990). It should be noted that the relative activity of the purified proteinase at 35-40 °C was about 75-80%. High activity at moderate temperature is beneficial for protein hydrolysis application as far as the energy saving is concerned.

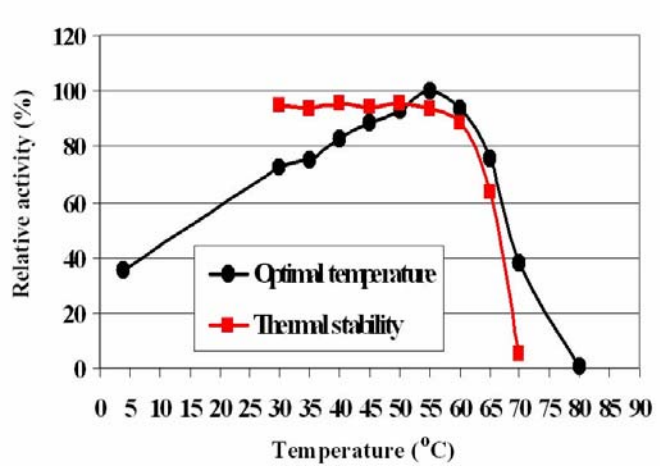

Figure 6.6 Temperature profile and thermal stability of the purified proteinase

The purified proteinase exhibited high stability at 30-60 °C (Figure 6.6), but its thermal stability decreased at >60 °C. The proteinase also showed high thermal stability at its optimal temperature, 55 °C. Typically, proteinase from archaebacteria

and eubacteria was unstable at the optimal temperature. Proteinase from archaebacteria *Natrialba magadii* and *Na. asiatica* 172 P1, and moderately halophilic bacteria *Pseudoalteromonas* sp. CP76 and *Filobacillus* sp. RF2-5 was not stable at its optimal temperature (Kamekura and Seno, 1990; Giménez et al., 2000; Sánchez-Porro, Mellado, Bertoldo, Antranikian, and Ventosa, 2003; Hiraga et al., 2005). Activity of *B. subtilis* JM-3, *Salinivibrio costicola* 18AG and *Salinivibrio* sp. AF-2004 proteinases decreased to ~20-30% of the original activity after incubated at the optimal temperature (60-65 °C) for 30 min (Kim and Kim, 2005; Lama et al., 2005; Karbalaei-Heidari, Ziaee, Schaller, and Amoozegar, 2007). Psychrophilic bacterium PA-43 proteinase underwent extensive autolysis at the optimal temperature, 60 °C (Irwin et al., 2001). It can be seen that the purified proteinase from *Virgibacillus* sp. SK33 showed relatively higher thermal stability, which is another prominent characteristic for enzyme application.

Optimum pH of the purified proteinase was 7.5 (Figure 6.7). Activity decreased markedly at either acid or basic pH range, suggesting that the enzyme is a neutral proteinase. Proteinase from *Desulfurococus mucosus* showed maximum activity at pH 7.2 (Cowan et al., 1987). Proteinase from *Halogeometricum* sp. TSS101, *Halobacterium halobium*, *H. mediterranei*, *Natrialba asiatica* 172 P1 and *Na. magadii* exhibited high activity at alkaline pH, about 8-10.7 (Izotova et al., 1983; Kamekura and Seno, 1990; Giménez et al., 2000; Capiralla et al., 2002; Vidyasagar et al., 2006), whereas PfpI-C1 proteinase from *Pyrococcus furiosus* showed optimum pH at 6.3 (Halio et al., 1997).

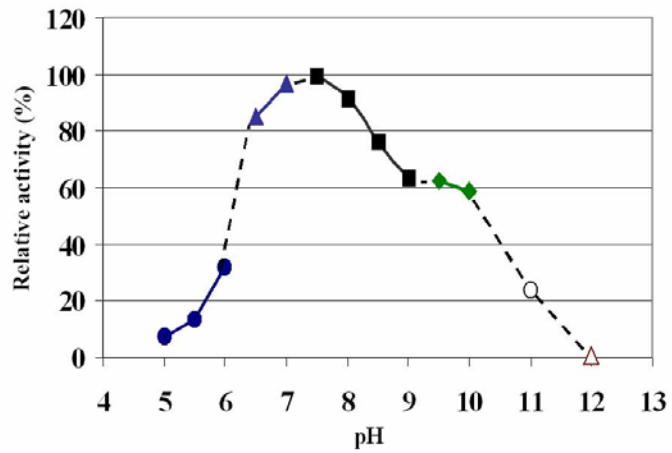

Figure 6.7 pH profile of the purified proteinase. = sodium acetate; = Tris-maleate; = Tris-HCl; = borate buffer; = glycine-NaOH and = phosphate buffer.

6.4.4 Substrate specificity and kinetic constant

The purified proteinase preferentially cleaved peptide bond with phenylalanine at P_1 position and proline at P_2, whereas other synthetic substrates were not hydrolyzed (Table 6.2). Subtilisin can hydrolyze large and non-β-branched hydrophobic residues at P_1 and small neutral side chains at P_2 (Graycar et al., 2004). Ak.1 proteinase, a thermostable subtilisin from *Bacillus* sp. Ak.1, was very specific to Suc-Ala-Ala-Pro-Phe-*p* nitroanilide (*p*NA) but unable to hydrolyze bond with arginine and tyrosine at P_1 (Toogood, Smith, Baker, and Daniel, 2000). Therefore, the purified enzyme showed subtilisin–like characteristic. Proteinase from *Natrialba asiatica* 172 P1 and *Na. magadii* showed a chymotrypsin-like characteristic that preferentially hydrolyzed phenylalanine and tyrosine at P_1 (Kamekura and Seno, 1990; Giménez et al., 2000). *Halobacterium mediterranei* proteinase showed subtilisin characteristic that favored a substrate with leucine at P_1 (Stepanov et al., 1992).

Michaelis constant (K_m) and maximum velocity (V_{max}) value of purified proteinase analyzed by the Hanes-Wolff plot was 27 μM and 0.35 μM/s, respectively. K_m of *Filobacillus* sp. RF2-5 and *Bacillus clausii* I-52 proteinase was 226 μM and 84 μM, respectively, whereas psychrophilic bacterium PA-43 was 3200 μM using *N*-Suc-Ala-Ala-Pro-Phe-*p*NA as a substrate (Irwin et al., 2001; Hiraga et al., 2005; Joo and Chang, 2005).

Table 6.2 Substrate specificity of the purified proteinase

Synthetic substrates	Specificity	Specific activity (nmole AMC/min /mg)
Boc-Asp(oBzl)-Pro-Arg-AMC	Trypsin-like, α-thrombin-like	0
Boc-Gln-Ala-Arg-AMC	Trypsin-like	0
Boc-Val-Leu-Lys-AMC	Plasmin-like	0
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin-like, subtilisin-like	2,361
Z-Phe-Arg-AMC	Cathepsin L-like	0
Z-Arg-Arg-AMC	Cathepsin B-like	0

6.4.5 Inhibitors and other substances

Table 6.3 shows the effect of various substances on activity of the purified proteinase. The activity was strongly inhibited by PMSF. Leupeptin, trypsin inhibitor I (soybean) and TLCK which are trypsin-like inhibitors did not inhibit the activity. Chymotrypsin-like inhibitor, TPCK, also had no effect the activity. A group of metallo proteinase inhibitors, including pepstatin A, EDTA, L-histidine and imidazole, as well as cysteine proteinase inhibitors slightly inhibited the enzyme activity. The thiol reagents had no effect on the enzyme activity. According to the substrate specificity and effect of inhibitors, the purified enzyme is classified as subtilisin-like proteinase. Activity of *Desulfurococcus mucosus* proteinase was also

Table 6.3 Effect of various substances on the activity of the purified proteinase

Substances	Targeted proteinase	Final concentration	Relative activity (%)
Leupeptin	Trypsin-like and some cysteine proteinases	100 μM	95
Trypsin inhibitor I (soybean)	Trypsin-like proteinase	0.02 mg/mL	91
TLCK	Trypsin-like proteinase	100 μM	92
TPCK	Chymotrypsin-like proteinase	100 μM	92
PMSF	Serine proteinase	1 mM	2
EDTA	Metallo proteinase	10 mM	94
L-Histidine	Metallo proteinase	10 mM	85
Imidazole	Metallo proteinase	10 mM	88
Bestatin	Aminopeptidase	10 μM	88
Pepstatin A	Acid proteinase	10 μM	81
E-64	Cysteine proteinase	10 μM	93
N-Ethylmaleimide	Cysteine proteinase	1 mM	88
Iodoacetic acid	Cysteine proteinase	1 mM	101
Dithiothreitol		10 mM	95
2-Mercaptoethanol		10 mM	100
L-Cysteine		10 mM	97
Metal ions			
Cu^{2+}		1 mM	106
Cd^{2+}		1 mM	113
Co^{2+}		1 mM	108
Fe^{3+}		1 mM	119
Mn^{2+}		1 mM	108
Hg^{2+}		1 mM	96
Zn^{2+}		1 mM	127
Mono and divalent cation			
Li^{+}		10 mM	102
Na^{+}		10 mM	105
K^{+}		10 mM	99
Mg^{2+}		10 mM	125
Ca^{2+}		10 mM	122
Sr^{2+}		10 mM	119
Ba^{2+}		10 mM	65

inhibited by seine inhibitors, namely PMSF and di-isopropyl phosphorofluoridate (DPF), but TLCK, TPCK and EDTA did not affect on the activity (Cowan et al.,

1987). Proteinase from archaebacterium, *Sulfolobus solfataricus*, was identified as metalloproteinase (Villa et al., 1993).

It is well known that Cu^{2+} and Zn^{2+} can interact with histidine residue (Malmström and Rosenderg, 1960), thereby all serine proteinases containing histidine at catalytic site could be inhibited. However, proteinase from *Virgibacillus* sp. SK33 was not inhibited by these metal ions (Table 6.3). Activity of many serine microbial proteinases including proteinase from *Bacillus* sp. KSM-K16 (proteinase H), *B. mojavensis*, *B. stearothermophilus* F1, *Bacillus* sp. Ve1 and psychrophilic bacterium PA-43 was not affected by Cu^{2+} and Zn^{2+} (Rahman et al., 1994; Kobayashi, Hakamada, Hitomi, Koike, and Ito, 1996; Irwin et al., 2001; Beg and Gupta, 2003; Gupta et al., 2005). These suggested that Cu^{2+} and Zn^{2+} did not necessary inhibit bacterial serine proteinase.

Monovalent cations (Li^{+}, Na^{+} and K^{+}) did not affect the purified proteinase activity, whereas divalent cations (Ca^{2+}, Sr^{2+} and Mg^{2+}) at 10 mM slightly activated the enzyme (Table 6.3). Moreover, activity increased with an increased concentration of Ca^{2+}, Sr^{2+} and Mg^{2+} to almost ~2 times at 100 mM (Figure 6.8). These divalent cations might induce structural changes leading to more flexibility of substrate-binding site. However, the effect of divalent ions varied with strains. Proteinases from *Bacillus pumilus*, *B. subtilis* PE-11, *Bacillus* sp. PS719 and *Brevibacterium linens* ATCC 9174 were also activated by Mg^{2+} and Ca^{2+} (Rattray et al., 1995; Hutadilok-Towatana, Painupong, and Suntinanalert, 1999; Kumar, 2002; Adinarayana, Ellaiah, and Pradad, 2003; Huang et al., 2003), whereas *Natrialba asiatica* 172 P1 proteinase activity was not affected by Ca^{2+}, Sr^{2+} and Mg^{2+} (Kamekura and Seno, 1990). Activity of *Pyrococcus abyssi* proteinase decreased

with Mg^{2+} and Ca^{2+} (Dib et al., 1998). It has been reported that Ca^{2+}, Mn^{2+} and Mg^{2+} protected the *Bacillus* proteinase against thermal inactivation (Paliwal, Singh, and Garg, 1994). It would be postulated that divalent cation did not only increase the activity of the purified proteinase from *Virgibacillus* sp. SK33 but also stabilize the enzyme structure against thermal inactivation.

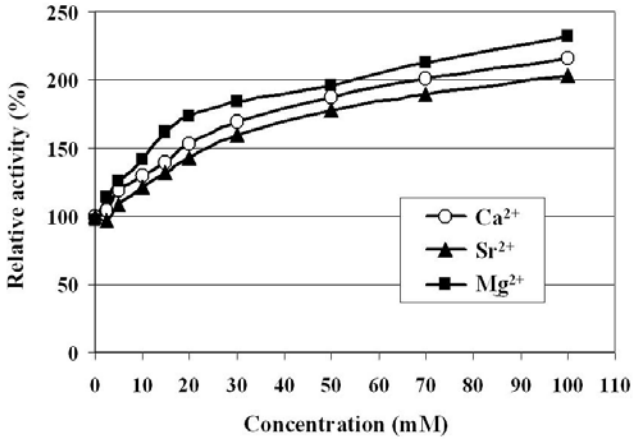

Figure 6.8 Effect of Ca^{2+}, Sr^{2+} and Mg^{2+} at various concentrations on the purified proteinase activity

6.4.6 Effect of NaCl and salt stability

Activity of purified proteinase from *Virgibacillus* sp. SK33 increased with NaCl and stable under high salt concentration (Figure 6.9). Activity at 10-25% NaCl was ~2.5 times greater than that without NaCl. In addition, the proteinase showed salt stability up to 25% NaCl at optimum condition (55 °C, pH 7.5) (Figure 6.9). Proteinases from extremely halophilic bacteria normally show activity in 4 M NaCl (23.4% NaCl), and irreversibly inactivated under low salt concentration. Archaebacterium proteinase from *Halobacterium halobium* was completely and irreversibly inactivated at <2 M NaCl (11.7% NaCl) (Izotova et al., 1983), whereas *H. halobium* ATCC 43214 proteinase was irreversibly inactivated at <4 M NaCl (Kim

and Dordick, 1997). Proteinases from *Natrialba asiatica* 172 P1, *Halobacterim halobium* S9 and *H. halobium* l538 reached maximum activity at 23.4-30% NaCl (Kamekura and Seno, 1990; Stepanov et al., 1992; Capiralla et al., 2002). On the other hand, activity of eubacterial proteinase was often unstable under high salt content. Activity of *Bacillus subtilis* JM-3 proteinase decreased to ~10% of the original at 30% NaCl (Kim and Kim, 2005). *Bacillus* sp. proteinase decreased to ~80% of the original at 0.17 M NaCl (1% NaCl) (Gupta et al., 2005). Proteinase activity of marine bacterium, γ-Proteobacterium DGII, retained ~50% of original activity under 30% NaCl (Sana, Ghosh, Saha, and Mukherjee, 2006). NaCl is an antichaotropic salt, which increases the intramolecular hydrophobic interaction of the protein molecule and increases surface tension of water promoting protein-folding (Arakawa and Timasheff, 1982). Therefore, NaCl could reduce structure flexibility. This could limit the use of nonhalphilic proteinase in high salt content. Interestingly, proteinase from moderate halophiles, *Virgibacillus* sp. SK33, showed similar characteristics to those of halophilic proteinase. Low pI value of *Virgibacillus* sp. SK33 proteinase implied that surface of enzyme contained many acidic amino acids, which is an unique characteristic of halophilic proteinase. *Halobacterium halobium* proteinase contained higher aspartic and glutamic acid residues and/or their amides in the molecule than did subtilisin BPN' from *Bacillus amyloliquefaciens* (Izotova et al., 1983; Stepanov et al., 1992). Excess acidic amino acids of halophile protein were found on surface of structure. These allow the enzyme to interact with hydrated salt ions and water (Rao and Argos, 1981), thereby halophile proteinase is protected against the salting-out effect from antichaotropic salt. Thus, high salt and temperature

stability of *Virgibacillus* sp. SK33 proteinase would be suitable for application at high salt content.

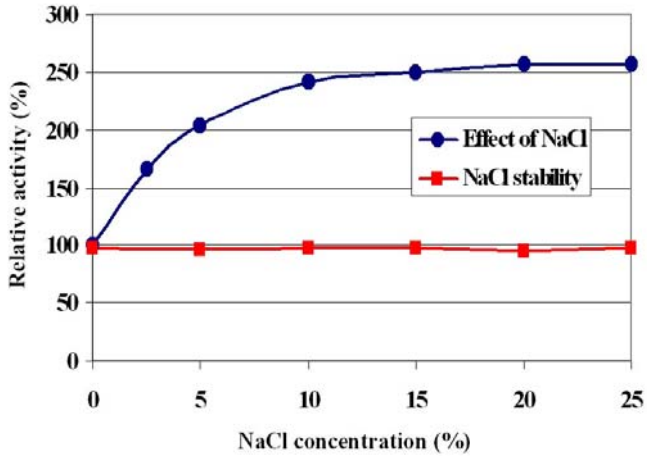

Figure 6.9 Effect of NaCl on purified proteinase activity of *Virgibacillus* sp. SK33 using Suc-Ala-Ala-Pro-Phe-AMC as a substrate and salt stability incubated at 55 °C for 60 min

Proteolytic degradation of anchovy protein by the purified proteinase increased with NaCl, and reached the maximum at 20-25% NaCl (p<0.05) (Figure 6.10). These results corresponded with those obtained from a synthetic substrate. Assay of proteinase activity with protein substrate at high NaCl usually encounters with substrate precipitation. Our results compared activity at various salt content at the same protein concentration basis. These results, therefore, reflect a true activity of proteinase under high NaCl. It should be noted that muscle proteins are extractable with various NaCl concentrations, thereby protein composition at each salt content would be different. Caseinolytic activity of nonhalophile and halophile proteinases has been reported. Activity of *Halobacterium halobium* S9 proteinase was found to gradually decrease with NaCl and retained ~50% activity at 4 M NaCl (Capiralla et al., 2002). Activity of archaeon *Natrialba magadii* proteinase exhibited maximal at 1-1.5 M NaCl (5.8-8.8% NaCl) and gradually decreased at higher NaCl concentration

(Giménez et al., 2000). Proteinase from *Pseudoalteromonas* sp. CP76 remained the activity of ~40% at 4 M NaCl (Sánchez-Porro et al., 2003), while *Salinivibrio* sp. AF-2004 proteinase showed maximal activity at 0-0.5 M NaCl (0-2.9% NaCl) and only ~25% residual activity retained at 4 M NaCl (Karbalaei-Heidari et al., 2007). *Filobacillus* sp. RF2-5 proteinase showed ~10% residual activity at 30% NaCl (Hiraga et al., 2005). These results indicated that the purified proteinase from *Virgibacillus* sp. SK33 had a potential to hydrolyze protein under high salt content better than other proteinases previously reported.

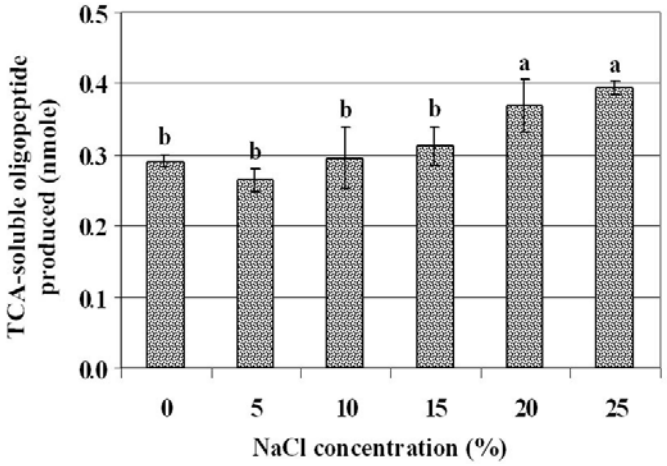

Figure 6.10 Effect of NaCl on proteolytic activity of the purified *Virgibacillus* sp. SK33 proteinase (30 nmole AMC/mL) using anchovy proteins as substrate

6.5 Conclusions

The purified proteinase from *Virgibacillus* sp. SK33 was a serine neutral proteinase existing in a dimer with MW of ~43 kDa and a subunit with MW of ~19 kDa. The proteinase was activated and stable under high NaCl content (25%). Therefore, the enzyme can be used to hydrolyze protein under high salt content.

6.6 References

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CHAPTER VII

SUMMARY

Virgibacillus sp. SK37 isolated from the fish sauce fermented for 1 month produced both cell-bound and extracellular proteinases, which exhibited subtilisin-like characteristics. Cell-bound enzyme was classified as metallo- and serine-proteinase, while serine proteinase was observed in the extracellular fraction. Proteinases from both fractions required Ca^{2+} for maximum activity. NaCl decreased the activity of cell-bound proteinase, but prevented thermal inactivation of the enzyme during incubation at the optimum temperature (65 °C). Extracellular proteinase showed maximum activity at 20% NaCl. Several extracellular proteinases with molecular weight of 81, 67, 63, 50, 38 and 18 kDa were observed and showed caseinolytic activity at 25% NaCl.

Another strain of *Virgibacillus* sp. SK33 showed maximum proteinase production in the modified halobacterium broth containing a single nitrogen source of either yeast extract, peptone or casamino acid. Although *Virgibacillus* sp. SK33 did not grow well at high salt concentration, proteinase activity increased with NaCl concentration up to 25%. Molecular weight of proteinases was estimated to be 56, 46, 42, 32, 25 and 19 kDa as evaluated by activity staining at 25% NaCl. Proteinases from *Virgibacillus* sp. SK33 showed higher activity than Alcalase and Protamex under high salt content (5-25% NaCl), demonstrating its potential application for the process containing high NaCl. In addition, purified proteinase showed optimal catalytic activity at 55 °C and pH 7.5. This proteinase was a dimer with MW of ~43

kDa with a subunit of ~19 kDa. At 25% NaCl, the proteinase was activated about 2.5 times and showed high stability. Therefore, *Virgibacillus* sp. SK37 and SK33 produced NaCl-activated proteinase that might be applied as a stature culture to increase proteolysis during fish sauce fermentation.

CURRICULUM VITAE

Sornchai Sinsuwan was born in November 19, 1979 in Phichit, Thailand. In 1998, he got his high school diploma (M. 6) from Phichitpitayakom School. In 2002, he received Bachelor's degree in Food Technology from Suranaree University of Technology.

In 2002-2003, he worked as a research assistant at the School of Food Technology, Suranaree University of Technology. He has co-published the article entitled "Aggregation and conformational changes of tilapia actomyosin as affected by calcium ion during setting" in Food Hydrocolloids (2007, Vol. 21, P. 359-367). Then, he received scholarship from the National Center for Genetic Engineering and Biotechnology (BIOTEC), to study for a Master degree at Suranaree University of Technology. During his graduate study, he presented several poster presentations including:

- Calcium induces conformational changes in tilapia actomyosin. Sornchai Sinsuwan and Jirawat Yongsawatdigul. The 7th Agro-Industrial Conference, 22-24 June 2005, BITEC, Bangkok.
- Salt tolerant cell-bound proteinase from *Halobacillus* sp. SK37. Sornchai Sinsuwan, Sureelak Rodtong, and Jirawat Yongsawatdigul. BioThailand 2005, 2-3 November 2005, The Queen Sirikit National Convention Center, Bangkok.
- Characterization of extracellular proteinases from *Halobacillus* species isolated from Thai fish sauce. Sornchai Sinsuwan, Siriwan Nawong, Sureelak

Rodtong, and Jirawat Yongsawatdigul. BioThailand 2005, 2-3 November 2005, The Queen Sirikit National Convention Center, Bangkok.

- Production and characterization of proteinases from *Brevibacillus* sp. isolated from fish sauce fermentation. Sornchai Sinsuwan, Sureelak Rodtong, Nongnuch Raksakulthai, and Jirawat Yongsawatdigul. The 8th Agro-Industrial Conference, 15-16 June 2006, BITEC, Bangkok.