PURIFICATION AND CHARACTERIZATION OF PROTEASE ENZYME FROM *Bacillus* sp. strain S1-13

YODYING YINGCHUTRAKUL

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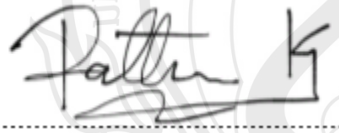
THESIS COMMITTEE



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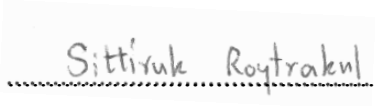

.....EXTERNAL EXAMINER
(Dr. Sittiruk Roytrakul)

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Yodying Yingchutrakul

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Author	Yodying Yingchutrakul
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ABSTRACT

Bacillus sp. strain S1-13 was identified as *Bacillus amyloliquefaciens* based upon 16s rRNA gene and submitted to Genbank database (Accession number: JX441363.1). The strain S1-13 produced alkaline serine protease enzyme (ASP1-13) when growths in nutrient broth containing 1% skim milk. The ASP1-13 enzyme was partially purified for 15 fold with a specific activity of 1324 U/mg and 2% yield by acetone precipitation and strong cation exchange chromatography, respectively. ASP1-13 enzyme played a broad range of activity at pH 7-12, optimum temperature at 50-60 °C and stable at pH 5-12 and 30-40 °C for 2 hr. In addition, this enzyme was stable in the presence of oxidant, surfactant and reducing agent such as CTAB, H_2O_2, and DTT. The organic solvent (acetone, acetonitrile, ethanol, isopropanol and methanol), and metal ion (K^+ Li^+, Mg^{2+}, Na^+, and Zn^{2+}) enhanced the protease activity. Furthermore, the ASP1-13 was completely inhibited with serine protease inhibitor and strongly inhibited with EDTA suggesting that ASP1-13 was a metal ion-dependent serine protease. Then, the molecular weight and isoelectric point of ASP1-13 enzyme was about 40 kDa and 8, respectively. The partial amino acid sequence of ASP1-13 obtained from LC/MS-MS technique showed high similarity with neutral protease precursor. Full coding sequence of ASP1-13 gene was amplified using PCR. The nucleotide sequence of PCR product was analyzed by bioinformatics tools including blast against

the GenBank database, and SignalP4.0 software. The result of ASP1-13 gene was showed 1,566 bp with deduced 521 amino acids. The full coding sequence of ASP1-13 and cleaved signal peptide ASP1-13 gene were cloned and expressed in *E. coli* system. The signal peptide was located in the first 28 amino acids of ASP1-13 protein. In addition of recombinant protein, the protease activity of expressed ASP1-13 and cleaved signal peptide ASP1-13 protein expression could not be determined by mean of spectrophotometric assay. However, only cleaved signal peptide ASP1-13 protein expression was detected by using LC/MS-MS technique. The apparent molecular mass of the expressed protein at 40 kDa was estimated by SDS-PAGE. The partial amino acid sequence of the expressed protein investigated by LC/MS-MS technique showed highly similar to neutral protease propeptide (GTG start codon) [*Bacillus subtilis*].

Keywords: alkaline serine protease enzyme/*Bacillus amyloliquefaciens*/purification/ LC/MS-MS/expression

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

Figure **Page**

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

Figure **Page**

ABBREVIATIONS AND SYMBOLS

ACN	=	Acetonitrile
$CaCl_2$	=	Calcium chloride
$CuSO_4.H_2O_2$	=	Copper (II) sulphate
°C	=	Degree Celsius
DNA	=	Deoxyribonucleic acid
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
FA	=	Formic acid
CTAB	=	Hexadecyltrimethylammonium bromide
hr	=	Hour
H_2O_2	=	Hydrogen peroxide
IPG	=	Immobilized pH gradient
IAA	=	Iodoacetamide
IEF	=	Isoelectric focusing
IPTG	=	Isopropyl β-D-1-thiogalactopyranoside

ABBREVIATIONS AND SYMBOLS (continued)

kb	=	Kilobase
kDa	=	Kilodalton
LC/MS-MS	=	Liquid chromatography mass spectrometry
LB	=	Lysogeny broth
MS/MS	=	Mass spectrometry
mA	=	Miliamps
µg	=	Microgram
µl	=	Microliter
µmole	=	Micromole
ml	=	Milliliter
mM	=	Millimole
min	=	Minute
M	=	Molar
OD	=	Optical density
PMSF	=	Phenylmethanesulfonylfluoride
PCR	=	Polymerase chain reaction

ABBREVIATIONS AND SYMBOLS (continued)

pH	=	Positive potential of the hydrogen ions
g	=	Relative centrifugal force
rpm	=	Revolutions per minute
rRNA	=	Ribosomal ribonucleic acid
BSA	=	Serum albumin
Na_2CO_3	=	Sodium carbonate
NaCl	=	Sobium chloride
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
NaOH	=	Sodium hydroxide
TBE	=	Tris/Borate/EDTA
TCA	=	Tri-chloroacetic acid
Tris-HCl	=	Tris (hydroxymethyl) aminomethane hydrochloride.
UV	=	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Protease enzymes are naturally produced by animals, fungal, plants, and microbes (Garcia-Carreno, 1992; Gupta, Beg, & Lorenz, 2002; Ray, 2012; Schallmey, Singh, & Ward, 2004). It catalyzes the digestion of long polypeptide chain into short peptide fragments, splitting the peptide bond that link amino acid residues (Gupta et al., 2002). Proteases are not only play an important role in the cellular metabolism, but also play crucial role in many field of industrial applications. Protease enzymes are one of the largest groups of industrial enzyme. There are many applications in detergents, feed processing, food processing, leather processing, and medical purposes (Ray, 2012). In the detergents application, these enzymes are used to remove stained protein on cloth. The protease enzymes are also used for hydrolyzing protein for preparation of high nutrition value in food and feed processing. The serine protease enzymes are essential for many organisms and the most important group for commercial enzyme. These enzymes are identified by the presence of serine group in active site. The alkaline serine proteases, largest group of serine protease, are active and stable in high pH value, optimal pH around 10 and their isoelectric point around 9 (Rao, Tanksale, Ghatge, & Deshpande, 1998). These properties of alkaline serine protease are suitable for use in the detergent application. Nowadays, most of commercial protease enzymes are produced by microbes because of low cost, fast growing and easy to manipulate genetic system. Numerous species of *Bacillus* are the majority microbes to produce protease enzymes (Gupta et al., 2002). *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus mojavensis*, and *Bacillus subtilis* are able to produce various alkaline protease enzymes with a primary application in

detergent (Rao et al., 1998; Schallmey, Singh, & Ward, 2004). The present study was showed the identification, purification, characterization, cloning and expression of an alkaline serine protease from *Bacillus amyloliquefaciens* strain S1-13 which is a potential for industrial application.

1.2 Objectives

1. To identify *Bacillus* sp. strain S1-13.
2. To purify protease enzyme from *Bacillus* sp. strain S1-13.
3. To characterize protease enzyme from *Bacillus* sp. strain S1-13.
4. To clone and express of protease enzyme gene in the *E. coli* system.

1.3 Scope of work

Bacillus sp. strain S1-13, isolated from Indonesia terasi shrimp paste, is an interested organism. The experiment starts with identification of *Bacillus* sp. strain S1-13 by 16s rRNA technique. Then, Effect of nutrient broth containing casein and skim milk on microbial growth and induction of the alkaline serine protease (ASP1-13) was studied. The cultured medium of strain S1-13 was used as crude enzyme. Azo-casein was used as a substrate for protease activity assay. Lowry's method was used to determine protein concentration. The enzyme purification was started with acetone precipitation and ion exchange chromatography. The protein profile and purity of ASP1-13 were determined by SDS-PAGE. Molecular mass and pI of the ASP1-13 enzyme were estimated by zymogram and isoelectric focusing technique, respectively. The partial purified ASP1-13 enzyme was characterized in term of optimal pH, optimal temperature, pH stability, temperature stability, effect of organic solvent, effect of surfactant, effect of reducing agent, effect of oxidant, effect of metal ion and effect of protease inhibitor. The ASP1-13 protein band on SDS-PAGE was analyzed by in-gel digestion, LC/MS-MS and MASCOT software. The primer was designed and used for amplification of ASP1-13gene. The purified PCR product was

cloned into TA cloning vector and sequenced. Then, the correct DNA fragment was subcloned into the pET-17b expression vectors. The recombinant DNA was transformed into *E. coli* BL21 (DE3). Finally, the expression of ASP1-13 gene in *E. coli* was studied.

CHAPTER 2

LITERATURE REVIEW

2.1 Genus *Bacillus*

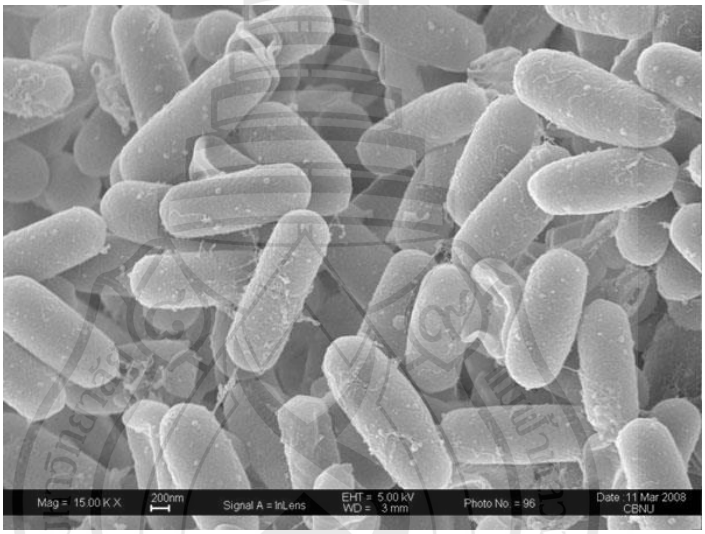


Figure 2.1 Scanning electron micrographs of *Bacillus* sp. (Madhaiyan, Poonguzhali, Kwon, & Sa, 2010)

Members of genus *Bacillus*, as shown in figure 2.1, are generally defined as gram positive, rod-shape, spore forming, aerobic or facultative anaerobic and the low G+C gram-positive bacteria (Schallmey et al., 2004). This genus was first classified by Ferdinand Julius Cohn in 1872. In addition of *Bacillus subtilis*, the strain was classified as the type species at 1932 based on morphology, nutrition, growth characteristics, various substrate utilization, and physiological assessments (Soule, 1932). Nowadays, more than 60 species of genus *Bacillus* are classified into 7 groups based on 16S rRNA phylogeny as shown in figure 2.2 (Wang & Sun, 2009)

Furthermore, the complete genome of *Bacillus subtilis* 168 was published. This knowledge of *Bacillus* sp. genomes are used in the further development. For bacterial classification, *Bacillus stearothermophilus* and *Bacillus brevis* have been classified as *Geobacillus stearothermophilus* and *Brevibacillus brevis*, respectively, by using other molecular techniques (Wang & Sun, 2009).

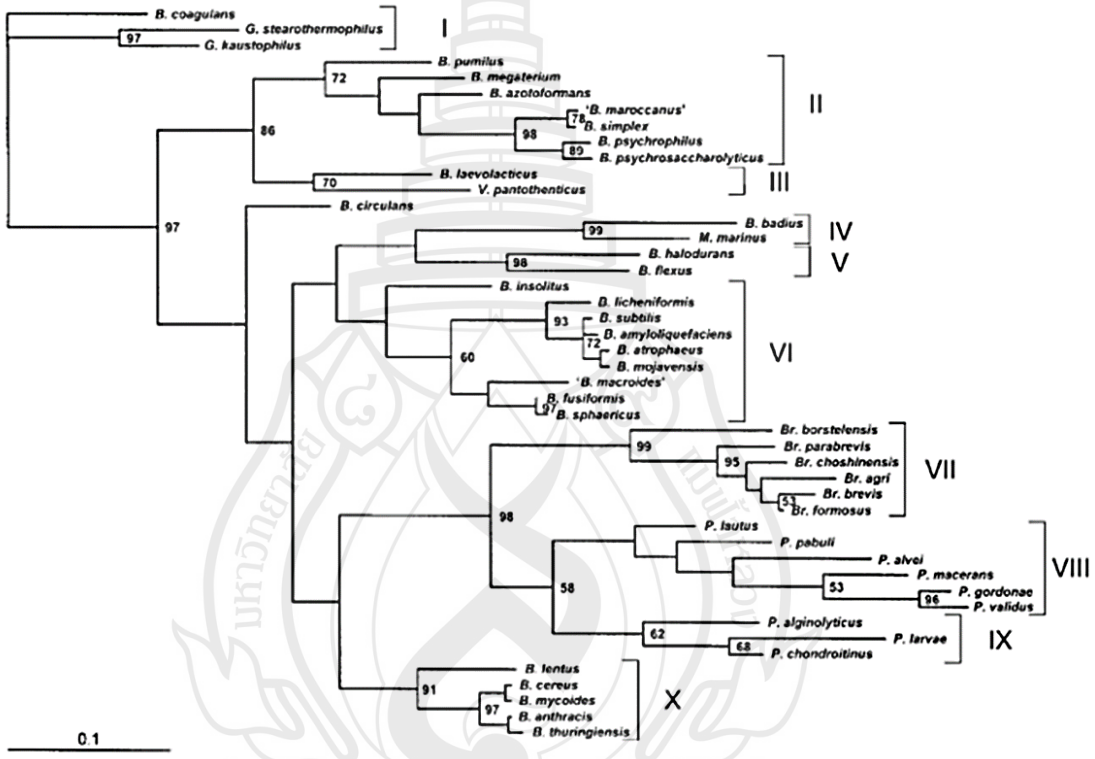


Figure 2.2 Phylogenic tree of genus *Bacillus* from the alignment of 16S rRNA gene (Wang & Sun, 2009)

Genus *Bacillus* is a major workhorse industrial microorganism with role of applied microbiology. Back to 1,000 years ago, *Bacillus subtilis* (natto) was used for production of solid-state fermentation of soybeans in japan (Schallmey et al., 2004). In this century, many industries have developed microbial strain and production by using molecular biology technique and strain development. Genus *Bacillus* is a famous used as industrial microorganism because of high growth rate, shot fermentation time, the

extracellular product and GRAS (generally regarded as safe) status with the food and drug Administration for species (Gupta, Beg, & Lorenz, 2002; Schallmey et al., 2004). In enzyme industries, genus *Bacillus* is used to produce many kind of enzyme as shown in table 2.1.

Table 2.1 Industrial enzyme produced from genus *Bacillus* (Gupta et al., 2002)

Industry (%)	**Major *Bacillus* sp. enzymes**	***Bacillus* sp.**	**Comments**
Detergent (37)	Alkaline proteases	*B. clausii*	*Bacillus* proteases dominates the market, particularly the highly alkali stable enzyme from *B. clausii* Removal of starch stains
		B. amyloliquefaciens	
		B. halodurans	
	Alkaline amylase	*B. licheniformis*	
		B. halmapalus	
Starch (13)	α-Amylase	*B. licheniformis*	
	Pullulanase	*G. stearothermophilus*	
	Glucose isomerase	*B. acidipullulyticus*	
		B. deramificans	
Textile (6)	Amylase	*B. halodurans*	Desizing, dehairing hides, stain remover
	Pectate lyases, Alkaline amylase, Mannanase	*B.* sp.	
Dairy (14)	None	Calf and (or) fungal rennets	
Baking (9)	Amylase		
Beverage (6)	Amylase	*B. licheniformis*	
		G. stearothermophilus	
		B. amyloliquefaciens	
	β-glucanase	*B. subtilis*	

The main application for industrial enzyme is detergents. Most of detergent proteases used in the market are alkaline serine protease. Numerous species of *Bacillus* have been explored for alkaline protease production, but most potential alkaline protease producers are strains of *Bacillus licheniformis*, *Bacillus mojavensis*, *Bacillus*

subtilis and especially in *Bacillus amyloliquefaciens* (Gupta et al., 2002). The proteases from these strains, having pH optimal in the range 9–12 and good thermo stability, have found commercial applications in detergents.

2.1.1 *Bacillus amyloliquefaciens*

Bacillus amyloliquefaciens is a species of microbial in the genus *Bacillus.* Its close affinity with *Bacillus subtilis* has long been recognized. The organism has been included in *Bacillus subtilis* as a variant that produces extensive quantities of extracellular enzyme. The separated species form *Bacillus subtilis* to *Bacillus amyloliquefaciens* was identified based on biochemical test and DNA sequence (Priest, Goodfellow, Shute, & Berkeley, 1987; Welker & Leon-Campbell, 1967). *Bacillus amyloliquefaciens* is closely related to *Bacillus subtilis* and the other four species which compose the *Bacillus subtilis* group VI, *Bacillus atrophacus*, *Bacillus licheniformis, Bacillus mojavensis* and *Bacillus pumilus*, shown in figure 2.2 (Wang & Sun, 2009). *Bacillus amyloliquefaciens* is a potential source for α-amylase and especially protease enzyme. It also produces antibiotic and many secondary metabolites for use in industrial application such as *Bam*H1 restriction enzyme (Gould, May, & Elliott, 1975). Several studies are available on the protease enzyme. The protease enzyme produced from *Bacillus amyloliquefaciens* was developed for widely use. In Korea, *Bacillus amyloliquefaciens* was used as a starter for fermentation of soybean sauce (Cho, Oh, Pridmore, Jutillerat, & Lee, 2003). It was also used to hydrolyze a protein in milk for making high nutrient value product (Son & Kim, 2002). For the medical purposes, *Bacillus amyloliquefaciens* produced a protease enzyme for used as fibrinolytic enzyme (Agrebi, Hmidet, Hajji, Ktari, Haddar, Fakhfakh-Zouari, & Nasri, 2010). In the other hand, the protease enzyme produced from *Bacillus amyloliquefaciens* was used to hydrolyze feather for making feed product (Cortezi, Contiero, De-Lima, Lovaglio, & Monti, 2008).

2.2 Protease enzyme

Protease enzymes are among the oldest, known and essential constituents of all organisms, including prokaryotes, fungal, plants and animals. It catalyzes the digestion of long polypeptide chain into short peptide fragments, splitting the peptide bond that link amino acid residues (Garcia-Carreno, 1992; Rao et al., 1998; Ray, 2012). Protease enzymes differ widely in their properties such as catalytic mechanism, substrate specificity, active site, pH and temperature in terms of stability and activity. In microbe, intracellular proteases are important for various cellular and metabolic processes such as differentiation, sporulation and maturation of enzymes. The extracellular protease enzymes are important for hydrolysis of protein in environments and enable the cell to absorb or utilize hydrolytic product (Rao et al., 1998). However, protease enzymes are not only play an important role in the cellular metabolism, but also play crucial role in many field of industrial applications. Protease enzymes become widely used in detergent industrial since 1914. Nowadays, microbial protease enzymes are one of the largest groups of industrial enzyme. They are a perfect source of protease enzyme because of low cost, fast growing, and easy to manipulate genetic system. There are many applications of protease enzymes in detergents, feed processing, food processing, leather processing, and medical purposes (Ray, 2012). Microbial protease enzymes have been studied for long times. The study focused on selection and fermentation of protease enzymes, whereas mainly dealt with the sources of microbial and their protease enzymes functional role in nature. This detail was descripted to the type of protease and their industrial application. Many microbes are known to produce of protease enzyme although very few are recognized as industrial enzyme. Only those microbe produced substantial extracellular protease enzyme were launched successfully in the marker as shown in table 2.1 (Gupta et al., 2002).

2.2.1 Classification of protease enzyme

Protease enzymes can be classified into various groups dependent on their active condition (acidic, neutral and alkaline condition), and active site (aspartic-, cysteine-, metallo- and serine- group) (Deng, Wu, Zhang, Zhang, & Wen, 2010; Dutt, Gupta,

Saran, Misra, & Saxena, 2009; Garcia-Carreno, 1992; Rao et al., 1998). The classification of protease enzyme based on their active site was grouped according to their mechanism of peptide hydrolysis. The mechanism was dependent on the amino acid residues involved at the active center of protease enzyme (Rao et al., 1998). Protease inhibitor was also used to identify the protease type as shown in table 2.2. The protease inhibitor blocked a catalytic amino acid in the active center or chelate the required cation (Garcia-Carreno, 1992).

Table 2.2 Inhibitor for classification of protease enzyme (Garcia-Carreno, 1992)

Protease types	**Protease inhibitor**
Aspartic	Pepstation A
Cycteine	Iodoacetamide (IA)
	N-Ethyl-maleimide (NEM)
Metallo	Ethylenediaminetetraacetic acid (EDTA)
Serine	Phenylmethysulfonyl fluoride (PMSF)
	Soybean typsin inhibitor (SBTI)
	Tosyl-phenylalanine chloromethyl ketone (TPCK)

The most important group of enzyme exploited industrial applications is alkaline serine protease enzyme (Ray, 2012). They are defined which the active condition in a neutral to alkaline range. It has a serine residue located at active site (serine protease). These protease enzymes are also differentiated with aspartate and histidine residue, alone with serine residue, forms the catalytic triad (Garcia-Carreno, 1992). Serine protease enzymes are important in terms of their activity and stability at alkaline pH that have applications in a number of industries as shown in table 2.3 (Ray, 2012). The property of this enzyme could be used in detergent because compatibility with a detergent components such as surfactant, good activity at relevant washing pH and temperature. In addition of food and feed process, these protease enzymes have

been used in the preparation of protein hydrolysates of high nutritional value product from various natural protein substrates (Gupta et al., 2002; Ray, 2012).

Table 2.3 Commercial microbial alkaline protease, applications and their industrial supplies (Ray, 2012)

Supplier	Product trade name	Microbial source	Application
Novo Nordisk	Alcalase	*Bacillus licheniformis*	Detergent, silk degumming
	Savinase	*Bacillus* sp.	Detergent, textile
	Esperase	*B. lentus*	Detergent, food, silk degumming
Genencor International, USA	Purafact	*B. lentus*	Detergent
	Primatan	*Bacterial source*	Leather
Gist-Brocades, Netherlands	Subtilisin	*B. alcalophilus*	Detergent
	Maxacal	*Bacillus* sp.	Detergent
	Maxatase	*Bacillus* sp.	Detergent
Solvay Enzymes, Germany	Opticlean	*B. alcalophilus*	Detergent
	Optimase	*B. licheniformis*	Detergent
	Protease	*B. licheniformis*	Food, waste
Amano Pharmaceticals,	Proleather	*Bacillus* sp.	Food
Enzyme Development,	Alkaline protease	*B. licheniformis*	Industrial
USA Enzeco	Enzeco alkaline protease-L FG	*B. licheniformis*	Food

2.2.2 Cloning and expression of protease enzyme

Many microbial strain improvement by either conventional mutagenesis technique (UV or chemical exposure) or recombinant DNA technique. The modified strain is also used for improving protease enzyme production and studying protease enzyme in terms of structural, functional, and mechanism. Recombinant DNA technology is used as instrument for the construction of genetically modified microbial strain. Many protease genes have been cloned and expressed in new hosts. The *E. coli*

and *Bacillus subtilis* are major organisms of choice for cloning and expression as shown in table 2.4 (Gupta et al., 2002; Rao et al., 1998; Ray, 2012; Schallmey et al., 2004).

Table 2.4 Cloning and expression of microbial protease genes (Gupta et al., 2002)

Parent strain	Host strains for cloning and expression	Gene	X-Fold increase in protease activity
B. amyloliquefaciens	*B. subtilis* I-168	Subtilisin	200
B. subtilis I-168	*E. coli* and *B. subtilis*	Subtilisin E	5
B. amyloliquefaciens ATCC 23844	*E. coli* GX1210, supE thi; *B. subtilis* BR151, IS4	Alkaline and neutral protease	n.s.
B. licheniformis NCIB 6816	*E. coli* HB101, JM101; *B. subtilis* 168	Subtilisin Carlsberg	n.s.
B. subtilis IFO3013	*E. coli*	Intracellular protease	n.s.
Bacillus strain YaB	*B. subtilis* DB104	Subtilisin	17
Bacillus sp. 221	*E. coli* MV1184, XL1 blue; *B. subtilis* DB104	Alkaline serine protease	n.s.
B. alcalophilus PB92	*B. subtilis* 1-A40	Alkaline serine protease	1.5
B. stearothermophilus	*E. coli* MC1061, JM109; *Bacillus subtilis* DB104	Subtilisin J	46
Bacillus sp. AH-101	*E. coli* MV1184, XL 1 blue*; B. subtilis* DB-104	Thermostable alkaline protease	n.s.
Bacillus sp. G-825-6	*E. coli* HB101	Subtilisin	n.s.

n.s. Not specified

2.3 LC/MS-MS

Liquid chromatography mass spectrometer (LC/MS-MS) is a particularly powerful separation technique in the life science and related fields of chemistry such as proteins, peptides, carbohydrates, DNA, and drugs or metabolites. It has been described as the smallest scale in the world because of a weighs of molecule or a microanalytical technique. The basic mass spectrometry instrument are consisted of sample injection, ionisation, mass analyzer sorting and detector as shown in figure 2.3 (Kang, 2012).

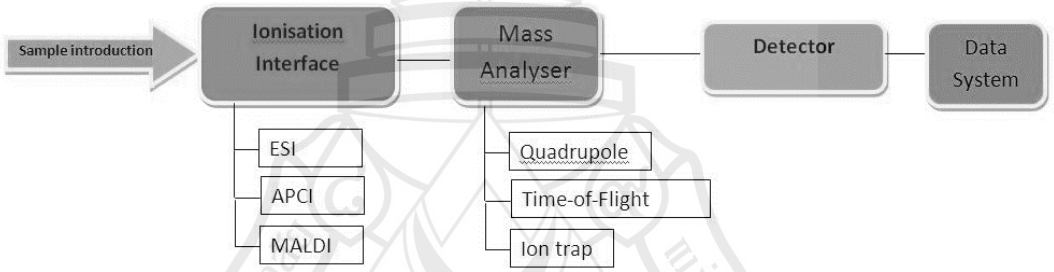


Figure 2.3 Overview of liquid chromatography mass spectrometer (LC/MS-MS)

For the protein analyses, there are two approaches that can be used; a gel base approach for example, SDS-PAGE or two-dimensional-PAGE and a gel free approach. Before analysis with LC/MS-MS, the protein sample need to be cleaved into smaller compound (peptides) with specific proteases enzyme, most commonly trypsin. After analysis, the information obtained from mass spectrometry experiments (MS-MS data) are used for the study of proteins and peptides. It can be identified or characterized based on their peptide map and primary structure. MS-MS data is used to confirm the assigned protein identification. Additionally, the information obtained allows localizing of post-translational or chemical modifications at the amino acid residues level (Soares, Pires, Almeida, Santos, Gomes, KoÃi, Franco, & Coelho, 2012).

CHAPTER 3

RESEARCH METHADOLOGY

3.1 Bacterial strain and growth condition

Bacillus sp. strain S1-13 isolated from Indonesia terasi shrimp paste was used in this study. The stock culture was kept in 20% glycerol at -80 °C. The strain S1-13 was refreshed on nutrient agar. For inoculums preparation, the colony appeared on nutrient agar was picked up and inoculated into 5 ml nutrient both (NB) consisting of 0.5% peptone, 0.1% meat extract, 0.2% yeast extract and 0.5% NaCl pH 7.0, and incubated at 37 °C with shaking for 16 hr. The culture broth was used as crude enzyme in the later experiment.

3.2 Identification of *Bacillus* sp. strain S1-13

3.2.1 16s rRNA gene primer design

All references 16s rRNA sequence of genus *Bacillus* was retrieved from public GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned with MEGA 5.0 program. Primers were designed by using those conserved sequence. Then, sequences of primers were compared by using BlastN program (nucleotide blast) with GenBank database. Finally, primers were synthesized from Biodesign, Thailand as shown in table 3.1.

3.2.2 Genomic DNA extraction

Bacillus sp. strain S1-13 was inoculated in nutrient broth (NB) and incubated at 37 °C for overnight with shaking at 180 rpm. After that, 1.5 ml of each overnight

was centrifuged at 5,000 g for 5 min. The pellets was resuspended in 1 ml of lysis buffer containing of 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.5% (w/v) SDS and 400 µg/ml proteinase K. After incubated at 56 °C for 30 min, 500 µl of phenol : chloroform (1:1) was added to mixture solution. The mixture was centrifuged at 12,000 g for 5 min. The upper aqueous phase was transferred to a new centrifuge tube. The 500 µl of chloroform was added, inverted for 5 min and centrifuged at 12,000 g for 5 min. The upper aqueous phase was transferred to a new centrifuge tube. Then, the genomic DNA in solution was precipitated by adding 1 volume of isopropanol, incubating -20 °C for 1 hr and centrifuging at 12,000 g for 5 min. Finally, DNA pellet was washed twice with 70% (v/v) ethanol and resuspended with 50 µl of sterile water (Malik, Kain, Pettigrew, & Ogram, 1994). The DNA concentration was measured by nano-drop (NanoDrop® ND1000 Spectrophotometer, Wilmington, USA).

Table 3.1 Oligonucleotide primer used for 16s rRNA amplification and sequencing

Primer name	Direction	Sequence (5'>3')	T_M (°C)
117F	Forward	GGCGGACGGGTGAGTAA	65
743F	Forward	GGAGGAACACCAGTGGCG	66
743R	Reverse	CGCCACTGGTGTTCCTCC	66
1472R	Reverse	GTGTGACGGGCGGTGTG	67

3.2.3 16s rRNA gene amplification

The PCR reaction mixture was consisted of 100 ng of DNA template, 2X PCR master mixes (Fermentas, USA), 0.2 µM of 117F primer, 0.2 µM of 1472R primer and sterile nano water. The PCR reaction conditions were composed of one initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, extension step at 72 °C for 10 min and stored at 4 °C. Then, the amplified product was analyzed with gel electrophoresis technique.

3.2.4 Gel electrophoresis

PCR products were analyzed with 1% agarose gel in 0.5X TBE buffer. The samples were mixed with 6X DNA loading dye and loaded in to agarose gel. Gene Ruler 1 kb DNA Ladder (Thermo scientific, USA) was used as DNA standard. Then, electrophoresis was performed at 100 volt for 20 min using 0.5X TBE as running buffer. After that, the gel was stained with 10 µg/ml of ethidium bromide solution for 10 min and destained with distilled water. Finally, DNA band was visualized by Genedoc system (Syngene Gene Genius Gel BioImaging Unit, Frederick, USA).

3.2.5 Purification of PCR product from agarose gel

PCR product in agarose gel was extracted using NucleoSpin® Extract II kit (Macherey-Nagel, Germany). The PCR product was excised from the ethidium bromide stained agarose gel and transferred into 1.5 ml sterile microcentrifuge tube. Sample was dissolved with buffer NT (200 µl per 1 mg of agarose gel) at 50 °C for 5 min until the gel was completely dissolved. Then, dissolved sample was transferred to NucleoSpin® Extract II column and centrifuged at 11,000 g. for 1 min. After that, 700 µl of buffer NT3 was added into NucleoSpin® Extract II column and centrifuged at 11,000 g for 1 min. The column was further centrifuged at 11,000 g for 2 min to remove buffer NT3. Finally, column was placed into a clean 1.5 ml microcentrifuge tube. PCR product was eluded by using 30 µl of NE buffer. The purified PCR product concentration was measured by nano-drop (NanoDrop® ND-1000 Spectrophotometer, Wilmington, USA).

3.2.6 16s rRNA gene sequencing and analyzing

The nucleotide sequence of 16s rRNA gene was determined by First BASE Laboratories (Sdn Bhd, Malaysia) using primers shown in table 3.1. The 16s rRNA gene sequence data was analyzed by BlastN program with GenBank database available at NCBI public database. Phylogenic tree was constructed by MEGA 5.0 program using the neighbor-joining method, bootstrap analysis for 1,000 replications (Wang & Sun, 2009; Xu & Côté, 2003). Finally, the analyzed 16s rRNA gene sequence was submitted to GenBank database.

3.3 Optimization of media composition

Three different media (NB, NB+1% casein, and NB+1% skim milk) was used in this study (Cheng, Wang, & Wanga, 2012; Vidyasagar, Prakas, Jayalakshmi, & Sreeramulu, 2007). The 2.5 ml of overnight cultured Strain S1-13 in NB was inoculated into 250 ml media and incubated at 37 °C, 180 rpm. Then, samples were taken and measured the absorbance at 600 nm (OD_{600}) between 6, 12, 24, 30, and 16 hr. Finally, crude enzymes were separated by centrifugation at 5,000 g for 10 min and stored at -20 °C.

3.4 Protease activity assay

Protease activity was determined using azocasein as substrate. The sample (20 µl) was mixed with 380 µl of 2% azocasein solution in 0.1 M phosphate buffer pH 6.5. The mixture was incubated at 37 °C for 30 min. The reaction was terminated with 1 ml of 0.44 M tri-chloroacetic acid (TCA), and centrifuged at 12,000 g for 10 min. Then, 0.5 ml of supernatant was mixed with 0.5 ml of 1 M NaOH and incubated at 37 °C for 30 min. Finally, absorbance at 440 nm (OD_{440}) of the solution was measured (Cho et al., 2003; Hutadilok-Towatana, Painupong, & Suntinanalert, 1999; Son & Kim, 2002). One unit of protease enzyme was defined as the amount of enzyme required to cause an increase of 0.001 A_{440} unit per minute under assay condition (Son & Kim, 2002).

3.5 Protein determination

The total protein concentration was determined by Lowry's method using bovine serum albumin (BSA) as standard (Lowry, Rosebrough, Farr, & Randall, 1951). The absorbance at 750 nm (OD_{750}) was measured. The protein concentration was calculated from protein standard curve of BSA data, The 5 µl of samples were

mixed with 200 µl of solution A that consist of 0.2% $CuSO_4.H_2O_2$, 20% Na_2CO_3, 0.8 N NaOH, 5% SDS and 0.4% tatalic acid. The mixtures were incubated at room temperature for 30 min before adding 50 µl of 20% Folin & Ciocalteu's phenol reagent. The mixture was mixed and incubated at room temperature for 30 min. Finally, absorbance at 750 nm (OD_{750}) was measured using Spectra Max M5 spectrophotometer (Molecular device, USA).

3.6 Purification procedure

3.6.1 Acetone precipitation

Crude enzyme was precipitated by addition of 2 volumes of cold acetone solution and kept at -20 °C for 2 hr. Then, the mixture solution was centrifuged at 12,000 g, 4 °C for 10 min. The precipitate was dissolved and dialyzed against in 10 mM phosphate buffer pH 6.0 at 4°C overnight.

3.6.2 Ion exchange chromatography

SP-Sepharose (strong cation exchanger) and Q-Sepharose (strong anion exchanger) was packed in 10 ml syringe (diameter 15.8 mm) at a flow rate 2 ml/min. The column was equilibrated with 10mM phosphate buffer pH 6.0. Then, the concentrated enzyme was applied to SP-Sepharose column at a flow rate of 1 ml/min. The column was washed to remove unbound proteins with 2 bed volumes of 10 mM phosphate buffer pH 6.0. After that, stepwise gradient of 0.1 to 1.0 M NaCl in 10 mM phosphate buffer pH 6.0 was used to elute bound proteins. The fraction was collected at a flow rate of 1 ml/min. After that, all fractions were monitored protease activity with standard protease activity assay and total protein content with OD_{280}. The fractions containing protease activity was dialyzed against the same buffer at 4°C overnight. The dialysate of active fraction from SP-Sepharose was applied to Q-Sepharose column. The column was washed and eluted with the same buffer. Finally, all fractions were monitored with standard protease activity assay and OD_{280}, respectively.

3.6.3 Hydrophobic exchange chromatography

Dialysate of active fractions from SP-Sepharose was applied to Sep-Pak® Vac 35cc C8 cartridges (weak hydrophobicity), and Sep-Pak® Vac 35cc C18 cartridges (strong hydrophobicity) (waters, Ireland). The column was pre-equilibrated with 5% acetonitrile, 0.1% formic acid, 10 mM phosphate buffer pH 6.0 at a flow rate of 1 ml/min. Then, sample was mixed 0.1% formic acid and subjected into a column. After that, the sample was stepwise eluted with 30%, 50%, 70% and 100% acetonitrile, 0.1% formic acid, 10 mM phosphate buffer pH 6.0. Finally, the protease activity and protein content was monitored with standard protease activity assay and OD_{280}, respectively.

3.6.4 Gel filtration chromatography

Dialysate of active fractions from SP-Sepharose was applied to a Superose 12 10/300 GL column (10 × 300 mm) and ÄKTA purifier 10 systems (GE Healthcare, USA). The Superose 12 10/300 GL (GE Healthcare, USA) column was previously pre-equilibrated with 10 mM phosphate buffer pH 6.0 at a flow rate of 0.5 ml/min. Protease enzyme was eluted with the same buffer. Then, the fractions was monitored for protein concentration with OD_{280} and assayed for protease activity. Finally, the active fraction was stored at -20°C.

3.7 SDS-PAGE and Zymogram analysis

3.7.1 Sample preparation

For standard SDS-PAGE technique, 25-100 µg of samples and Low Molecular Weight SDS Marker Kit (GE Healthcare, USA) were mixed with 5X loading buffer that consist of 0.02% bromophenol blue, 0.2M DTT, 20% glycerol, 4% SDS and 0.125 M Tris-HCl pH 6.8. Then, the mixture solution was heated at 95 °C for 10 min before loaded into a gel. For zymogram technique, 25 µg of sample was mixed with 5X zymogram loading buffer that consist of 0.02% bromophenol blue, 20% glycerol, 4% SDS and 0.125 M Tris-HCl pH 6.8. After mixed, the mixture samples were loaded

into a gel without heating step (Garcia-Carreno, Dimes, & Haard, 1993; Wilkesman & Kurz, 2009). For isoelectric focusing, 250 µg of sample was mixed with 2% IPG buffer (GE Healthcare, USA) before loaded into a gel.

3.7.2 Preparation of SDS-PAGE

SDS-PAGE was prepared on mini PAGE chamber, AE-6530 (ATTO, Japan). The 12.5% acrylamide gel was prepared according to method of Laemmli (Laemmli, 1970). The solution mixture for separating and stacking gel were prepared according to table 3.2.

Table 3.2 Preparation of separating and stacking gel for SDS-PAGE

Reagents	Separating gel (12.5%)	Stacking gel (5%)
40% acrylamide	3.13 ml	0.2 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	-
0.5 M Tris-HCl pH 6.8	-	0.371 ml
10% SDS	125 µl	15 µl
Sterile nano water	4.23 ml	0.908 ml
10% ammonium persulfate	50 µl	12.5 µl
TEMED	10 µl	0.85 µl

3.7.3 Running Condition

SDS-PAGE was carried out at 20 mA per gel using electrophoresis power supply EPS-601 (GE Healthcare, USA) until blue line of protein loading dye reached the end of gel.

3.7.4 Coomassie staining

After standard electrophoresis, the gel was fixed with 10% acetic acid and 40% methanol for 20 min. Protein bands were visualized by staining with coomassie

Brilliant Blue R-250 solution that consist of 0.1% coomassie G-250, 10% acetic acid, and 50% methanol. After stained for overnight, the gel was washed with destain solution that consist of 10% acetic acid and 40% methanol until blue background was clear (Steinberg, 2009). Finally, the gel was washed with sterile nano water and stored in 0.1% acetic acid at 4 °C.

3.7.5 Zymogram

The mixed zymogram sample was applied to standard SDS-PAGE. After standard electrophoresis, the gel was immersed in casein solution (2% casein in 50 mMTris-HCl pH 7.5) at 4 °C for overnight. After incubating in casein solution at 37°C for 90 min, the gel was washed with distilled water and stained with coomassie brilliant blue R-250 solution (0.1% coomassie brilliant blue R-250, 40% ethanol, and 10% acetic acid) for 2 hr. Finally, the gel was washed with destain solution (40% ethanol, 10% acetic acid). The clear zone on blue background, indicating protease activity, was observed at this step (Garcia-Carreno et al., 1993; Wilkesman & Kurz, 2009).

3.7.6 Isoelectric focusing

The mixed with 2% IPG buffer sample was applied to isoelectric focusing gel strip (IEF), with a pH gradient of 3-10 (GE Healthcare, USA). The running condition was accorded to the protocol recommended by the manufacturer (GE Healthcare, USA). Then, IEF strip was covered with 12.5% SDS-Poly acrylamide gel. Finally, zymogram technique was used to determine the isoelectric point.

3.8 Characterization of the native protease enzyme

3.8.1 Optimization and stability of temperature

The optimum temperature of protease enzyme against azocasein was determined by incubating the enzyme reaction mixture at different temperatures (30°C to 90°C) (Fernanda Susana, Anita, Vanja, Artur, & Gubitz, 2007). Thermal stability

was carried out by pre-incubating enzyme solution at various temperatures (30°C to 80°C) for 2 hr before standard protease activity assay (Deng et al., 2010; Fernanda et al., 2007).

3.8.2 Optimization and stability of pH

The optimum pH of protease enzyme against azocasein was measured at different pH value (4.0 to 12.0). For pH stability, the enzyme solution was pre-treatment at various pH buffers at 4 °C for 12 hr. The residual enzyme activity was determined under protease activity assay condition (Charles, Devanathan, Anbu, Ponnuswamy, Kalaichelvan, & Hur, 2008; Deng et al., 2010; Fernanda et al., 2007).

3.8.3 Determination of Protease Type

The partial purified protease enzyme was incubated at 37 ºC for 30 min with 10 mM of protease inhibitor reagents including: ethylenediaminetetraacetic acid (EDTA), iodoacetamide (IA), pepstatin, and phenylmethanesulfonylfluoride (PMSF). The remaining activity was measured under protease activity assay conditions (Deng et al., 2010; Garcia-Carreno, 1992; Phrommao, Yongsawatdigul, Rodtong, & Yamabhai, 2011).

3.8.4 Effect of surfactant, oxidant and reducing agent

The partial purified protease enzyme was incubated with 5% CTAB, 5% SDS, 5% triton x100, 5% tween 80, 1% H_2O_2, and 10 mM DTT at 4 °C for 1 hr. Then, the protease activity was measured under protease activity assay conditions (Deng et al., 2010; Hutadilok-Towatana et al., 1999; Phrommao et al., 2011; Vishwanatha, Appu-Rao, & Singh, 2010).

3.8.5 Effect of organic solvent

The effect of organic solvent on the protease enzyme was measured at different concentration (0-80%) of the following organic solvents: acetone, acetonitrile, ethanol, isopropanol, and methanol under protease activity assay

condition (Castro, 1999; Saborowski, Sahling, Navarette-del-Toro, Walter, & Garc´-Carreño, 2004; Verma & Ghosh, 2010).

3.8.6 Effect of metal ion

The protease activity was estimated immediately after the addition of 10 mM Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, and Zn^{2+} into the reaction mixture. The protease activity was determined under protease assay condition (Deng et al., 2010; Phrommao et al., 2011).

3.8.7 Protein identification

The sample preparation for LC/MS-MS analysis was prepared using in-gel digestion method (Shevchenko, Tomas, Havlis, Olsen, & Mann, 2006). The protein band from SDS-PAGE was cut into thin slices. The gel pieces was washed with distilled water and dehydrated with 100% acetonitrile (ACN) at room temperature (RT) for 5 min. Then, gel pieces was reduced with 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate at RT for 1 hr and alkylated with 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate at RT for 1 hr in dark. After that, gel was washed twice with 100% ACN at RT for 5 min. The proteins in gel pieces was digested with 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) at 4°C for 20 min. After that, 20 µl of 30% ACN was added to keep the gels immerse at 37 °C for 3 hr or overnight. After digestion, peptides were extracted from gel pieces by 30 µl of 50% ACN in 0.1% formic acid (FA) with shaking at RT for 10 min. The extracted solution was dried at 40 °C for 3 hr or overnight. Then, dried samples were dissolved with 0.1% formic acid, and the digested protein samples were analyzed with Liquid Chromatography Mass Spectrometry Analysis (Waters SYNAPT™ HDMS™ system) (Waters Corp., Milford, MA). Finally, MS-MS data results were used for peptide identification using the MASCOT software (Matrix Science, London, UK) and the NCBI (National Center for Biotechnology Information) database.

3.9 Expression of recombinant protease enzyme

3.9.1 Protease gene primer design

The primers (full length of ASP1-13 gene) were designed based on protein sequence of neutral protease precursor (accession number: gi|83972424) as shown in figure 3.1. Then, the primers were checked for its specificity with BlastN program. The nucleotide sequences of primers were shown in figure 3.2.

```
LOCUS       ABC49679               521 aa            linear   BCT 30-APR-2006
DEFINITION  neutral protease precursor [Bacillus subtilis].
ACCESSION   ABC49679
VERSION     ABC49679.1  GI:83972424
DBSOURCE    accession DQ291130.1
SOURCE      Bacillus subtilis
  ORGANISM  Bacillus subtilis
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae;
Bacillus.
REFERENCE   1  (residues 1 to 521)
  AUTHORS   Bai,W., Zhang,R., Niu,T.G. and Guo,S.D.
  TITLE     A novel neutral protease from Bacillus Subtilis B111 with
            antifungal activity against Verticillium
  JOURNAL   Unpublished
ORIGIN
        1 mglgkklsvt vaasfmslti slpgvqaaen pqlkenltnf vpkhslvqse lpsvsdkaik
       61 qylkqngkvf kgnpserlkl idhttddlgy khfryvpvvn gvpvkdsqvi thvdksnnvy
      121 aingelnnda saktanskkl sanqaldhaf kaigkspeav sngnvanknk aelkaaatkd
      181 gkyrlaydvt iryiepepan wevtvdaetg kvlkkqnkve haaatgtgtt lkgktvslni
      241 ssengkyvmr dlskptgtqi itydlqnrqy nlpgtlvsst tnqfttssqr aavdahynlg
      301 kvydyfyqtf krnsydnrgg kivssvhygs rynnaawigd qmiygdgdgs ffsplsgsmd
      361 vtahemthgv tqetanlnye nqpgalnesf sdvfgyftdt edwdigegit vsqpalrsls
      421 nptkygqpdh yknyqnlpnt dagdyggvht nsgipnkaay ntitkigvkk aeqiyyralt
      481 vyltpsssfk dakaaliqsa rdlygsqdaa sveaawnavg l
```

Figure 3.1 The protein sequence of neutral protease precursor [*Bacillus subtilis*] (Accession number: gi|83972424)

ASP1-13 Forward primer: 5' GAA TTC TAA GTA GGA GGC ATT AAT ACA TAT GGG TTT AGG TAA GAA ATT GTC TG 3'

***Eco*RI RBS *Nde*I**

bp: 53, Tm: 66.1 °C, CG: 34%

ASP1-13_USN Forward primer: 5' GAA TTC TAA GTA GGA GGC ATT AAT ACA TAT GGC TGA GAA TCC TCA GCT TAA AGA A 3'

***Eco*RI RBS *Nde*I**

bp: 54, Tm: 64.6 °C, CG: 36%

ASP1-13Reverse primer: 5' CTC GAG TCT AGA AAG CTT ACA AGC CGA CCG CAT TC 3'

***Xho*I *Xba*I *Hind*III**

bp: 35, Tm: 66.8°C, CG: 51%

Figure 3.2 Oligonucleotide primer used for ASP1-13 amplification

3.9.2 PCR amplification

The PCR reaction mixture was consisted of 100 ng of DNA template, 2X PCR master mixes (Fermentas, USA), 0.2 µM of forward and reverse primer, ASP1-13 forward (full length of ASP1-13 gene) and ASP1-13_USN forward (cleaved signal peptide ASP1-13 gene) as shown in figure 3.2. The PCR reaction conditions were consisted of one initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, extension step at 72 °C for 10 min and stored at 4 °C. Then, the amplified products will be analyzed with 1% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator. Finally, PCR product will be purified by NucleoSpin® Extract II kit and sequenced.

3.9.3 Preparation of competent cell

The competent cells of *E. coli* strain DH5α and *E. coli* strain BL21 (DE) pLysS were prepared by using $CaCl_2$ solution. Single colony of *E. coli* was transferred into 3 ml of LB broth and incubated at 37 °C for 16 hr under shaking condition. After that, 1 ml of culture was inoculated to 100 ml of LB broth and incubated at 37°C with shaking until mid-log phase (OD_{600}~0.4-0.6). Then, the cell was collected by

centrifugation at 5,000 g at 4 °C for 10 min. The cell pellet was resuspended with 5 ml of ice cold 50 mM $CaCl_2$ and incubated on ice for 20 min. After centrifuged at 5,000 g at 4 °C for 10 min, the cell pellet was resuspended with 5 ml of ice cold 50mM $CaCl_2$ and 18% glycerol solution. Finally, 50 µl competent cells was transferred to new 1.5 ml sterile tube and stored at -80 °C (Li, Sui, Zhang, Sun, Zhao, Zhai, & Wang, 2010; Singh, Yadav, Ma, & Amoah, 2010).

3.9.4 Ligation cloning vector

Purified PCR product was ligated to pTZ57R/T plasmid with InsTAclone PCR cloning Kit (Thermo Scientific, USA). The reaction mixture contained 1.5 µl of pTZ57R/T plamid vector, 200 ng of purified PCR product, 3 µl of 5X T4 DNA ligase buffer, and 0.5 µl of T4 DNA ligase enzyme. Then, the volume was adjusted to 15 µl with sterile nano water. Finally, the mixture solution was incubated at 4 °C overnight.

3.9.5 Transformation

The competent cells of *E.coli* strain DH5α were thawed on ice for 2 hr. Then, ligated PCR product (2 µl) was added to thawed competent cells (50 µl). After incubated for 30 min on ice, the mixture was heat shocked at 42 °C for 90 sec and immediately transferred to ice box for 5 min. Then, 450 µl of LB was added into the mixture before incubated at 37 °C for 1 hr under shaking condition (Singh et al., 2010). Finally, the culture was spreaded on LB agar contained 50 ng/ml ampicillin and incubated at 37 °C for 16-18 hr.

3.9.6 Isolation of recombinant plasmid

The recombinant clone was confirmed by colony PCR technique with M13 universal primer (Woodman, 2008). The appeared colony on LB agar contained 50 ng/ml ampicillin was picked and placed into a new culture media plate, and resuspended in the PCR reaction mixture. The PCR mixture was contained of 2X PCR master mixes (Fermentas, USA), 0.2 µM of M13 forward primer (5'- GTA AAA CGA CGG CCA GT -3'), M13 reverse primer (5'- GCG GAT AAC AAT TTC ACA CAG G -3'), and sterile nano water. Then, PCR reaction conditions consist of one

initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, extension step at 72 °C for 10 min and stored at 4 °C. Finally, the amplified products was analyzed with 1% agarose gel and visualized under UV transilluminator.

3.9.7 Purification of recombinant plasmid DNA

The plasmid DNA was isolated from overnight cultured of recombinant clone by using NucleoSpin® plasmid kit (Macherey-Nagel, Germany). The colonies of recombinant clone were picked and placed into 6 ml of LB broth containing 50 ng/ml ampicillin and incubated at 37 °C overnight. Then, the cell was collected by centrifugation at 10,000 g for 1 min. The cell pellet was resuspended with 250 µl of buffer A1 by vortex or pipetting and followed by adding 250 µl of buffer A2. After incubated at room temperature for 5 min, the mixture was gently mixed with 300 µl of buffer A3 by inverting and centrifuged at 10,000 g for 10 min. The supernatant was transferred to NucleoSpin® plasmid column and followed by centrifugation at 10,000 g for 1 min. The dry column was washed with preheated 500 µl of buffer AW and washed again with 600 µl of buffer A4. After dryed column by centrifugation at 10,000 g for 2 min, the plasmid DNA was eluted by using 30 µl of AE buffer. Finally, the purified plasmid DNA concentration was measured by nano-drop.

3.9.8 DNA sequencing, analyzing and primer design for cleaved signal peptide ASP1-13 gene

The plasmid DNA from recombinant clone was sequenced by First BASE Laboratories (Sdn Bhd, Malaysia). The results were analyzed by BlastN program with GenBank database available at NCBI public database. Then, the nucleotide sequence was translated into the protein sequence by using Open Reading Frame Finder program (ORF finder) available at NCBI public (http://www.ncbi.nlm. nih.gov). The signal peptide was predicted using Signal P4.1 program available at CBS public (http://www.cbs.dtu.dk/services/SignalP/). After that, the primer for cleaved signal peptide ASP1-13 gene was designed as ASP1-13_USN primer, as shown in figure 3.1, using those data. The primer was synthesized from Biodesign, Thailand. Finally,

the cleaved signal peptide ASP1-13 gene was amplified, ligated, transformed and sequenced with the same protocol.

3.9.9 Restriction enzyme digestion

The pET18a expression vector, pET28a expression vector, recombinant ASP1-13 plasmid and ASP1-13_USN plasmid were digested using restriction enzyme according to the manufacture recommend. The reaction mixture contained 1 µg of purified vector or plasmid, 2 µl of 10X buffer O, 1 µl of 10 U/µl *Nde*I, 2 µl of 10 U/µl *Xho*I, and 10 µl of sterile water. After incubated at 37 °C overnight, the mixture was analyzed with 1% agarose gel, stained with ethidium bromide and visualized under UV transilluminator. Finally, the digested DNA band was purified using NucleoSpin® Extract II kit.

3.9.10 Construction of expression vector

Digested ASP1-13 gene and cleaved signal peptide ASP1-13 gene were ligated to pET17B expression vector, pET28a expression vector with T4 DNA ligase enzyme. The reaction mixture contained 100 ng of digested expression vector, 300 ng of digested gene, 3 µl of 5X T4 DNA ligase buffer and 0.5 µl of T4 DNA ligase enzyme. The reaction volume was adjusted to 15 µl with sterile nano water and incubated at 4 °C overnight. Finally, the mixture was transformed to *E.coli* strain BL21 (DE3) pLysS.

3.9.11 Isolation of transformed expression vector by using casein agar plate

The transformed *E.coli* strain BL21 (DE3) was spreaded on LB agar containing 50 ng/ml ampicillin, 1% casein, and 0.5 mM IPTG. After incubated at 37 °C for 18 hr, the clear zone forming colony was picked and placed into a new culture media plate.

3.9.12 Isolation of transformed expression vector by using colony PCR technique

The transformed *E.coli* strain BL21 (DE3) was spreaded on on LB agar containing 50 ng/ml ampicillin and incubated at 37 °C for 18 hr. Then, the colony PCR technique was used to identify *E.coli* strain BL21 (DE3) harboring recombinant plasmid.

3.9.13 Optimum condition for protease enzyme expression

The *E.coli* strain BL21 (DE3) harbouring recombinant plasmid was grown in 5 ml of LB broth containing 50 ng/ml ampicillin and incubated at 37 °C overnight. Then, 1% cultured bacteria was inoculated to 100 ml of LB broth containing 50 ng/ml ampicillin and incubated at 37 °C under shaking condition until mid-log phase (OD_{600}~0.4-0.6). After that, bacteria cell was induced by adding IPTG to final concentrations of 1, 3 and 5 mM. The OD_{600} of the bacterial samples were collected at 0, 3, 6, and 24 hr after induction. Supernatant (crude enzyme) and cell pellet was harvested by centrifugation at 5,000 g for 10 min and stored at -20 °C.

3.9.14 Extraction of recombinant ASP1-13 enzyme

The induced of *E.coli* cell was resuspended in 10 mM phosphate buffer pH 6.0 and lysed by ultrasonic (Sonic vibracell VCX130PB, Connecticut, USA) on ice for 30 sec. Finally, the cell debris was removed by centrifugation at 10,000 g for 10 min and store at -20 °C.

3.9.15 Characterization of recombinant ASP1-13 enzyme and ASP1-13 without signal peptide enzyme

The protease activity of all crude enzyme and lysed bacteria cell were measured. Then, SDS-PAGE was used to analyses of protein profile. Finally, the aspect protein band were excised, in-gel digestion and analysis by using LC/MS-MS technique.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Identification of bacterial strain

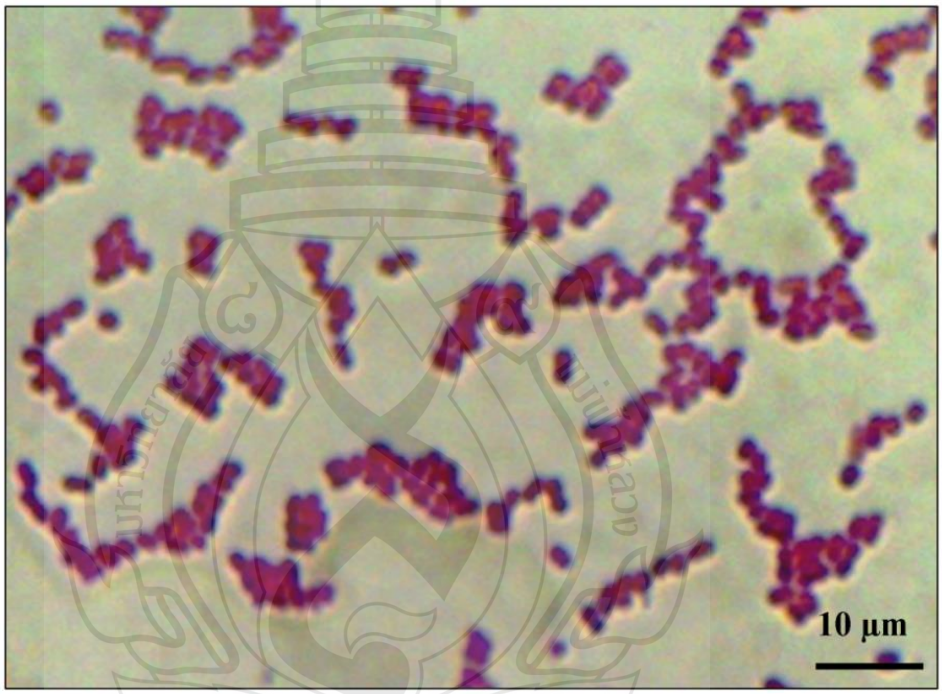


Figure 4.1 *Bacillus* sp. strain S1-13 gram staining (gram positive)

The *Bacillus* sp. strain S1-13 was an aerobic, short rod shape, and gram positive bacteria as shown in figure 4.1. The genomic DNA of strain S1-13 was isolated and used as template for amplification of 16s rRNA gene by PCR technique. The 16s rRNA gene (Accession number: JX441363.1) (figure 4.2), which is 1,256 bp, was compared against all 16s rRNA sequences of bacteria and archaea by using BlastnN program with Genbank database.

LOCUS JX441363 1256 bp DNA linear BCT 09-AUG-2013
DEFINITION Bacillus sp. S1-13 16S ribosomal RNA gene, partial sequence.
ACCESSION JX441363
VERSION JX441363.1 GI:479015895
SOURCE Bacillus sp. S1-13
ORGANISM Bacillus sp. S1-13
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
REFERENCE 1 (bases 1 to 1256)
AUTHORS Yingchutrakul,Y., Waratrujiwong,T., Roytrakul,S., Arfarita,N. and Chukeatirote,E.
TITLE Proteolytic activity of Bacillus sp. S1-13 isolated from terasi
JOURNAL Unpublished
ORIGIN

```
       1 cacgtgggta acctgcctgt aagactggga taactccggg aaaccggggc taataccgga
      61 tggttgtctg aaccgcatgg ttcagacata aaaggtggct tcggctacca cttacagatg
     121 gacccgcggc gcattagcta gttggtgagg taacggctca ccaaggcgac gatgcgtagc
     181 cgacctgaga gggtgatcgg ccacactggg actgagacac ggcccagact cctacgggag
     241 gcagcagtag ggaatcttcc gcaatggacg aaagtctgac ggagcaacgc cgcgtgagtg
     301 atgaaggttt tcggatcgta aagctctgtt gttagggaag aacaagtgcc gttcaaatag
     361 ggcggcacct tgacggtacc taaccagaaa gccacggcta actacgtgcc agcagccgcg
     421 gtaatacgta ggtggcaagc gttgtccgga attattgggc gtaaagggct cgcaggcggt
     481 ttcttaagtc tgatgtgaaa gcccccggct caaccgggga gggtcattgg aaactgggga
     541 acttgagtgc agaagaggag agtggaatcc acgtgagcgt gaaaatgcgt agagatgtgg
     601 aggaacacca gtggcgaagg cgactctctg gtctgtaact gacgctgagg agcgaaagcg
     661 tggggagcga acaggattag ataccctggt agtccacgcc gtaaacgatg agtgctaagt
     721 gttaggggtt ttccgccccc tagtgctgca gctaacgcat taagcactcc gcctggggag
     781 tacggtcgca agactgaaac tcaaaggaat tgacgggggc ccgcacaagc ggtggagcat
     841 gtggtttaat tcgaagcaac gcgaagaacc ttaccaggtc ttgacatcct ctgacaatcc
     901 tagagatagg acgtcccctt cgggggcaga gtgacaggtg gtgcatggtt gtcgtcagct
     961 cgtgtcgtga gatgttgggt taagtcccgc aacgagcgca acccttgatc ttagttgcca
    1021 gcattcagtt gggcactcta aggtgactgc cggtgacaaa ccggaggaag gtggggatga
    1081 cgtcaaatca tcatgccccc tatgacctgg gctacacacg tgctacaatg gacagaacaa
    1141 agggcagcga aaccgcgagg ttaagccaat cccacaaatc tgttctcagt tcggatcgca
    1201 gtctgcaact cgactgcgtg aagctggaat cgctagtaat cgcggatcag catgcc
```

Figure 4.2 The 16s rRNA sequence of *Bacillus amyloliquefaciens* strain S1-13 (Accession number: JX441363.1)

The BlastnN search results of 16s rRNA gene of *Bacillus* strain S1-13 showed high similarity (Max ident > 99%) to *Bacillus amyloliquefaciens, Bacillus atrophaeus, Bacillus subtilis, and Bacillus vallismortis* as shown in table 4.1. Those species was classified in group VI of genus *Bacillus* as shown in figure 2.2 (Shevchenko et al., 2006).

Table 4.1 The BlastN search results of 16s rRNA sequences (bacteria and archaea) and *Bacillus amyloliquefaciens* strain S1-13

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus amyloliquefaciens FZB42 strain FZB42 16S ribosomal RNA, complete sequence	2294	2294	100%	0.0	99%	NR_075005.1
Bacillus amyloliquefaciens strain NBRC 15535 16S ribosomal RNA, partial sequence	2283	2283	100%	0.0	99%	NR_041455.1
Bacillus subtilis subsp. subtilis str. 168 strain 168 16S ribosomal RNA, complete sequence	2278	2278	100%	0.0	99%	NR_102783.1
Bacillus vallismortis strain DSM11031 16S ribosomal RNA, partial sequence	2272	2272	100%	0.0	99%	NR_024696.1
Bacillus subtilis subsp. subtilis strain DSM 10 16S ribosomal RNA, partial sequence	2266	2266	100%	0.0	99%	NR_027552.1
Bacillus atrophaeus 1942 strain 1942 16S ribosomal RNA, complete sequence	2250	2250	100%	0.0	99%	NR_075016.1

Finally, phylogenic tree of all *Bacillus* sp. 16s rRNA gene were constructed with neighbor-joining method, and bootstrap values from 1000 replicates using MEGA (version 5.0) software. *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus subtilis, and Bacillus vallismortis* were also generated as a closely group to *Bacillus amyloliquefaciens* strain S1-13 as shown in figure 4.3. The results showed that *Bacillus* sp. strain S1-13 was most closely associated with *Bacillus amyloliquefaciens*.

Figure 4.3 Phylogenetic dendrogram based on a comparison of complete 16s rRNA sequences for *Bacillus* sp. and *Bacillus amyloliquefaciens* strain S1-13. The numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets

4.2 Optimization of media composition

Protease enzyme was optimized by using nutrient broth (NB) in the present of casein and skim milk as media composition. The 1% of overnight cultured *Bacillus amyloliquefaciens* strain S1-13 was inoculated to 3 different media (NB, NB+1% casein, and NB+1% skim milk) and incubated at 37 °C with sharking at 180 rpm. Normally, protease enzyme production is dependent on the growth condition, media and media composition. The media composition could enhance protease production. Casein and skim milk are an organic nitrogen source that enhances microbial growth. Microbe may produce protease enzyme to hydrolyze casein and skim milk for usage as nitrogen source (Dutt et al., 2009; Shaheen Alishah, Hameed, & Hasan, 2008; Wang, Xu, Wan, & Li, 2013). In this study, figure 4.4 (A) showed that protease activity was found in all culture media. However, maximum protease activity was observed in the presence of skim milk at 30 hr. In presence of casein, the highest protease activity was found at 30 hr. The maximum protease activity was observed in NB alone at 24 hr. The strain S1-13 produced maximum protease activity at the exponential phase when cultures with all optimized media as showed in figure 4.4 (B). Normally, microbial protease enzyme was mostly synthesized at the exponential phase to an early stationary phase. protease enzyme production was decreased because of depletion of nitrogen source and inactivation of protease enzyme by acidic media (Shaheen et al., 2008).

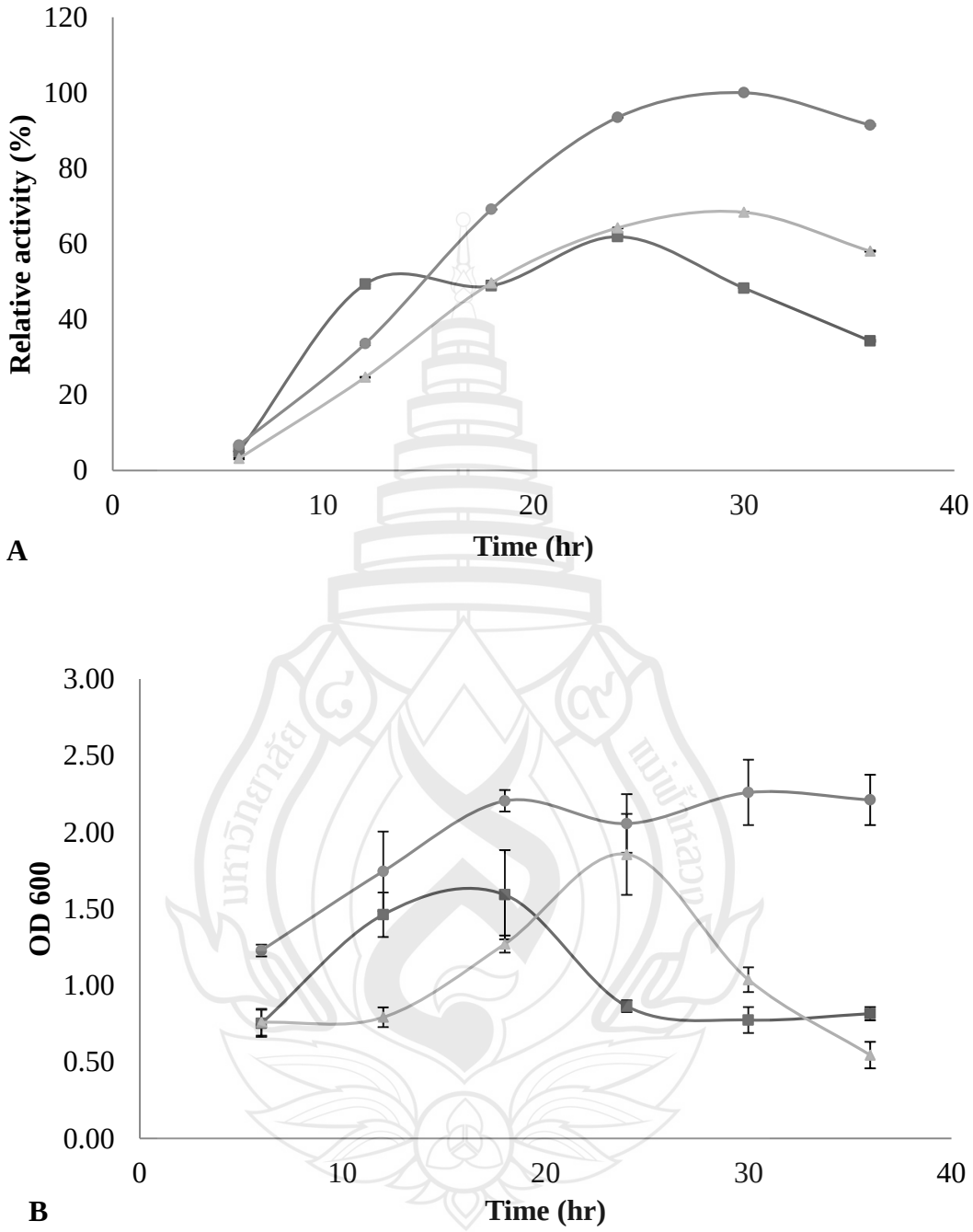


Figure 4.4 Effect of media composition on *Bacillus amyloliquefaciens* strain S1-13 protease enzyme production (A) and growth rate (B); (■-■): nutrient broth, (▲-▲): nutrient broth + 1% casein, (●-●): nutrient broth + 1% skim milk (Mean ± SD, n=3)

4.3 Partial purification steps

The partial purified protease enzyme (ASP1-13) was analyzed for it molecular mass and pI by means of SDS-PAGE, zymogram and isoelectric focusing technique. The molecular weight ~ 40 kDa (figure 4.5A), and isoelectric point ~ 8 (data not showed) were investigated. The crude enzyme (ASP1-13) was purified by acetone precipitation and strong cation exchange chromatography. The purification table and relevant data was summarized and shown in table 4.2. It was purified with 15 fold, specific activity 1324 U/mg, and 2% yield. Several proteins of purified ASP1-13 enzyme were observed on SDS-PAGE. However, only one clear band appeared in the crude enzyme and purified enzyme using zymogram technique as shown in figure 4.5B, (Garcia-Carreno et al., 1993; Wilkesman & Kurz, 2009). In this study, ASP1-13 was further purified with other technique such as Strong anion exchange chromatography (Q-Sepharose), strong hydrophobic exchange chromatography (Sep-Pak® C18 cartridges), weak hydrophobic exchange chromatography (Sep-Pak® C8 cartridges), and gel filtration exchange chromatography (Superose 12 10/300 GL). The purification workflow was shown in figure 4.6.

Table 4.2 Purification of ASP1-13 enzyme from *Bacillus amyloliquefaciens* strain S1-13

Purification step	**Total volume (ml)**	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U/mg)**	**Purification factor (fold)**	**Yield (%)**
Crude enzyme	1000	6641	591667	89	1	100
Acetone	50	92	56750	620	7	10
SP-Sepharose	20	7	9400	1324	15	2

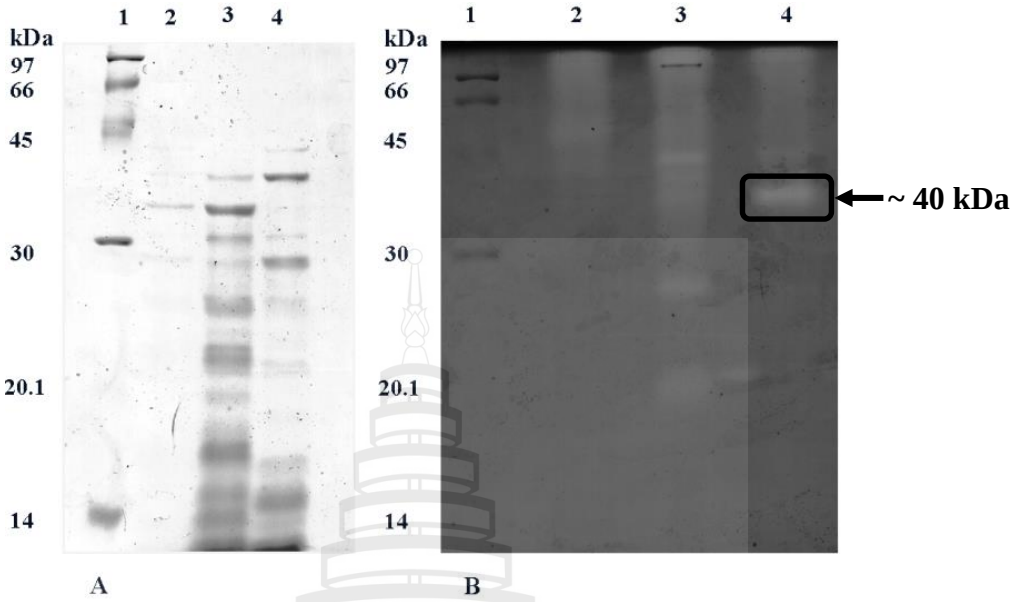


Figure 4.5 SDS-PAGE (A) and zymogram (B) of the crude enzyme and partial purified ASP1-13 enzyme from *Bacillus amyloliquefaciens* strain S1-13; lane 1: protein molecular weight markers, lane 2: crude enzyme, lane 3: acetone precipitation sample, and lane 4: strong cation exchange chromatography sample

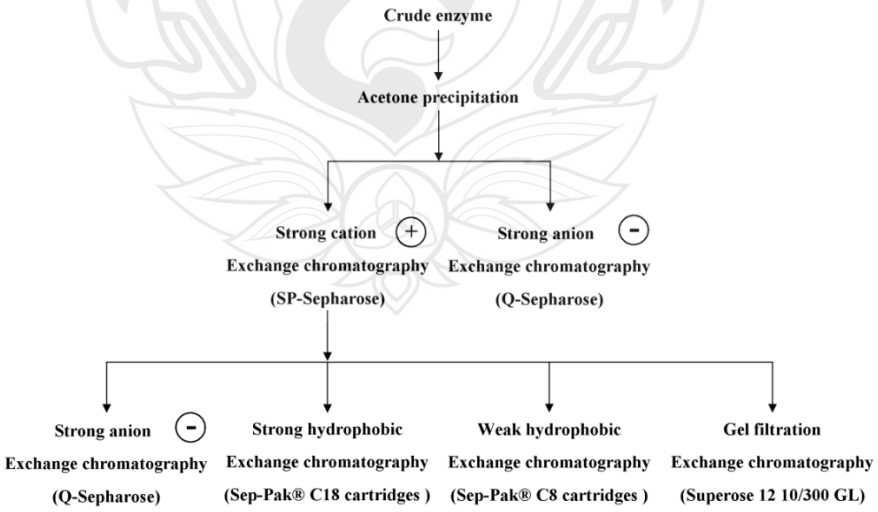


Figure 4.6 Workflow for of ASP1-13 enzyme from *Bacillus amyloliquefaciens* strain S1-13 purification

4.3.1 Acetone precipitation

Different ratios of acetone to crude enzyme were used for partial purification of ASP1-13 enzyme. The maximum protease activity was detected at 2 ratio of acetone solution as shown in figure 4.7. The increasing ratio of acetone to crude enzyme wasn't increased protease activity. Two ratio of acetone solution was used to purify ASP1-13 enzyme. After precipitated, ASP1-13 pellet was dissolved in 10 mM phosphate buffer pH 6.0, dialyzed against the same buffer at 4°C overnight and used as subject for further purification.

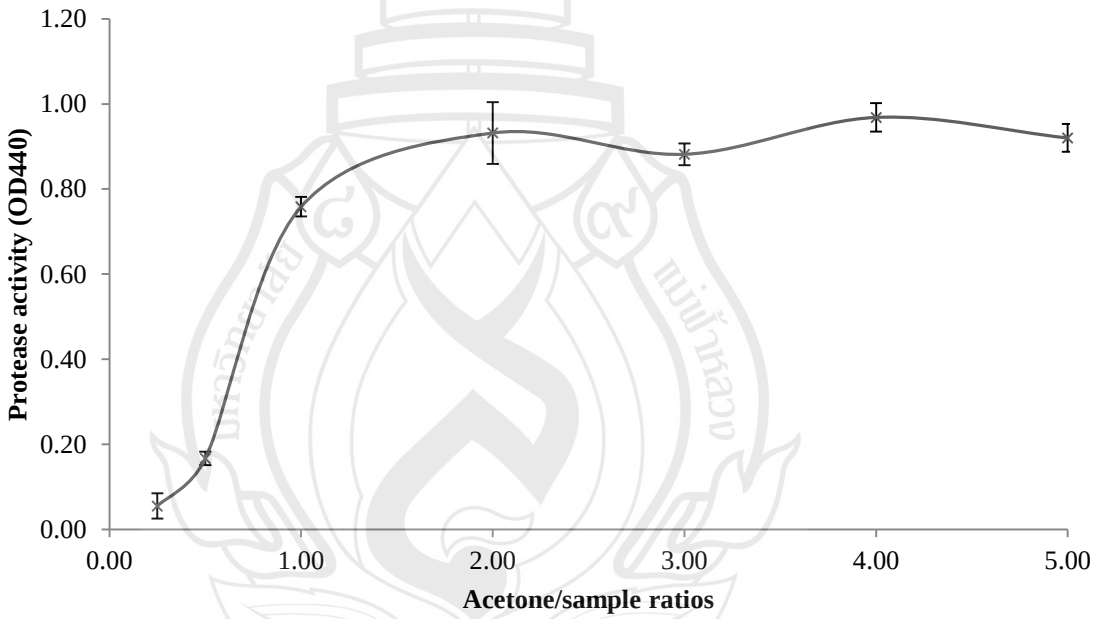


Figure 4.7 Effect of acetone solution on protease activity of ASP1-13 from *Bacillus amyloliquefaciens* strain S1-13 during acetone precipitation with different acetone/sample ratios (Mean ± SD, n=3)

4.3.2 Strong cation exchange chromatography

SP-Sepharose, a strong cation exchange chromatography, was used to partial purify ASP1-13 enzyme. After pre-equilibration with 10mM phosphate buffer pH 6.0,

the dialyzed ASP1-13 enzyme was applied to the SP-Sepharose column. After washing the unbound proteins, the bound proteins were eluded with stepwise 0.1 to 1.0M NaCl in 10 mM phosphate buffer pH 6.0. Total proteins of all fractions were monitored by using optical density at 280 nm (OD_{280}). The protease activity of fractions showed high OD_{280} was measured. The maximum protease activity was detected in fraction 4 to 6 as shown in figure 4.8. The ASP1-13 enzyme was eluted at very lower NaCl concentration indicating that at pH 6.0, the net charge of ASP1-13 protease was quite low.

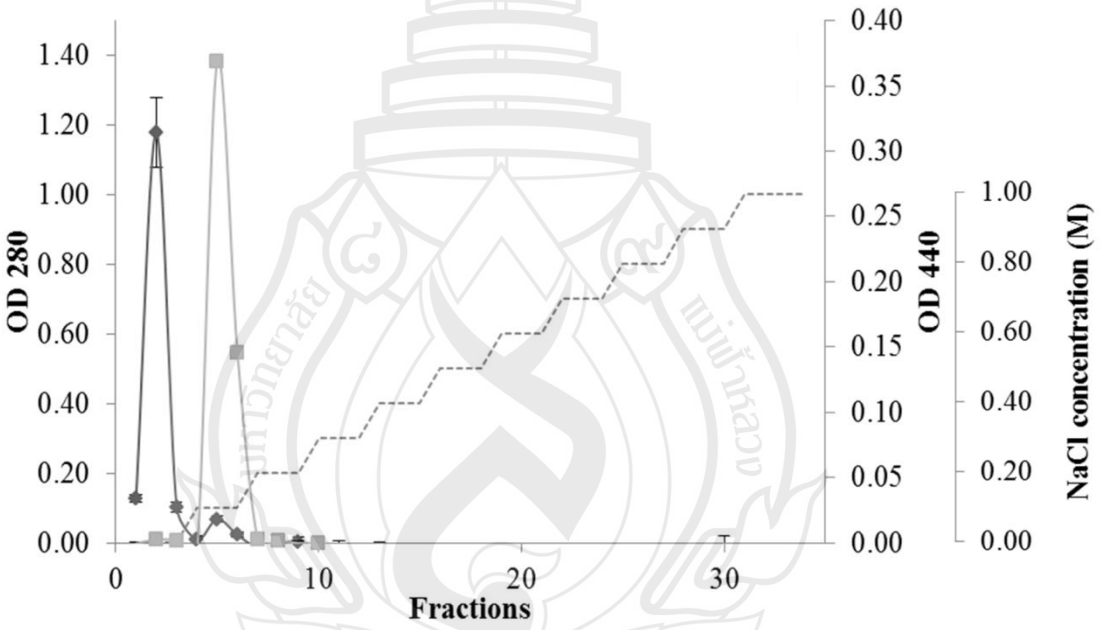


Figure 4.8 Strong cation exchange chromatography (SP-Sepharose) elution profile of dialyzed ASP1-13 enzyme; (♦-♦): total protein, (■-■): protease activity, (---): NaCl concentration (M) (Mean ± SD, n=3)

4.3.3 Strong anion exchange chromatography

Q-Sepharose, a strong anion exchange chromatography, was tried to partial purified ASP1-13 after acetone precipitation and strong cation exchange chromatography step. The dialyzed ASP1-13 enzyme was directly applied to the

Q-Sepharose column and eluted with ten stepwise gradient of 0.1 to 1.0M NaCl in 10 mM phosphate buffer pH 6.0. Total proteins concentration in each fractions were estimated using optical density at 280 nm (OD_{280}). The protease activity of fractions with high OD_{280} was assayed. Surprisingly, protease activity was not detected in all fractions as shown in figure 4.9.

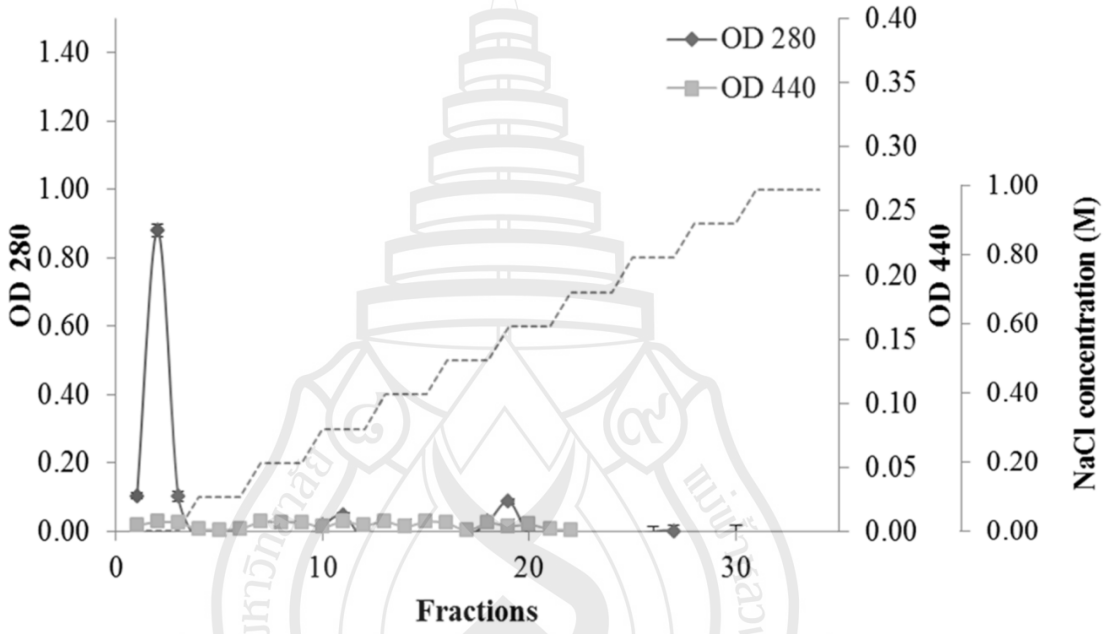


Figure 4.9 Strong anion exchange chromatography (Q-Sepharose) elution profile of dialyzed ASP1-13 enzyme; (♦-♦): total protein, (■-■): protease activity, (---): NaCl concentration (M) (Mean ± SD, n=3)

The purified sample from SP-Sepharose column was applied to the Q-Sepharose column and eluted with stepwise gradient of 0.1 to 1.0M NaCl in 10 mM phosphate buffer pH 6.0. No protease activity was detected in all fractions as shown in figure 4.10. The results showed that ASP1-13 enzyme was not eluted out from the strong anion column. This suggests that ASP1-13 enzyme might contain high content of hydrophobic amino acids causing high affinity to the Q-Sepharose and, therefore, difficult to be eluted from Q-Sepharose column.

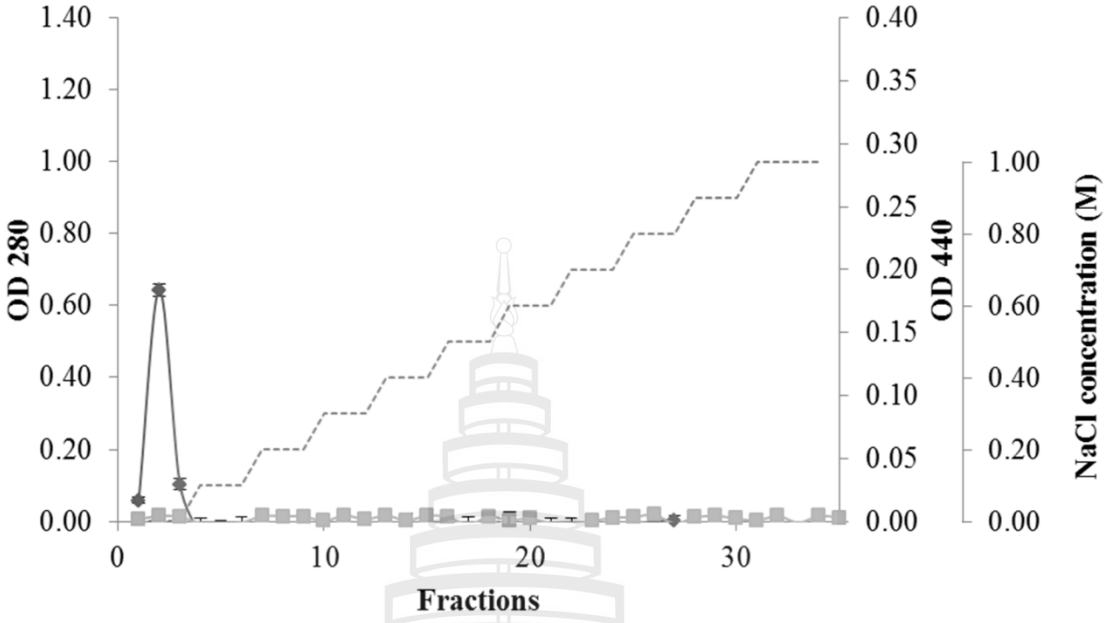


Figure 4.10 Strong anion exchange chromatography (Q-Sepharose) elution profile of strong cation exchange chromatography sample; (♦-♦): total protein, (■-■): protease activity, (---): NaCl concentration (M) (Mean ± SD, n=3)

4.3.4 Hydrophobic exchange chromatography

Sep-Pak® Vac 35cc C18 cartridges (strong hydrophobic exchange chromatography), and Sep-Pak® Vac 35cc C8 cartridges (weak hydrophobic exchange chromatography) were used for further purification of ASP1-13 enzyme. The purified ASP1-13 enzyme from SP-Sepharose column was applied to Sep-Pak® Vac 35cc C18 cartridges (strong hydrophobic exchange chromatography) and eluted with stepwise gradient of 30%, 50%, 70% and 100% acetonitrile containing 0.1% formic acid, 10 mM phosphate buffer pH 6.0. Fractions was measured for total proteins concentration by using optical density at 280 nm (OD_{280}) and protease activity in each fraction was determined under protease assay condition as shown in figure 4.11. No protease activity was detected in all fractions.

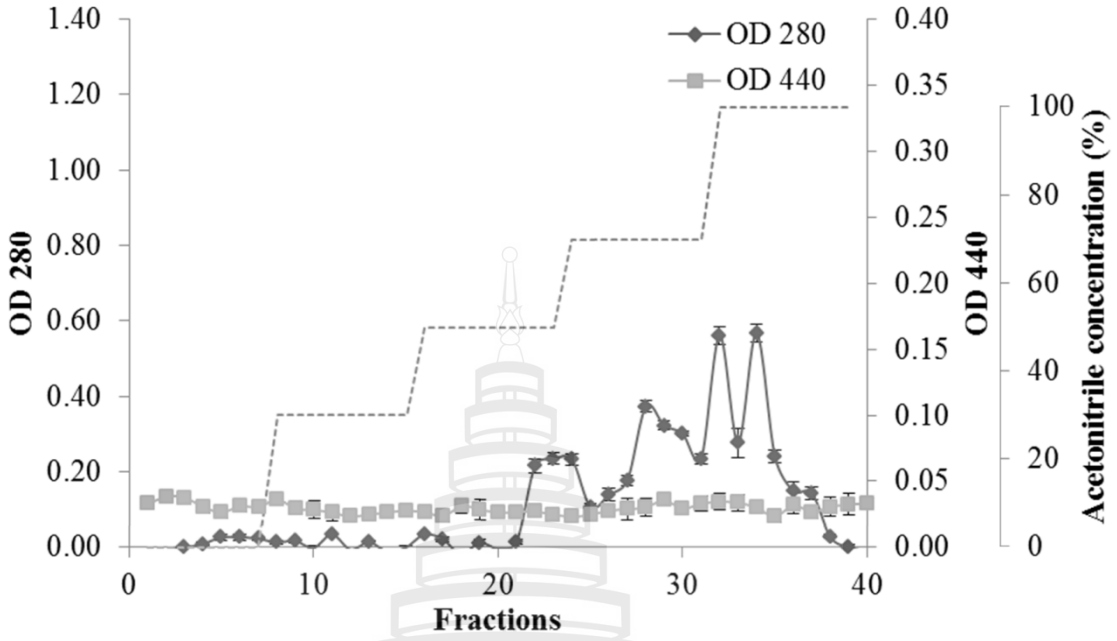


Figure 4.11 Strong hydrophobic exchange chromatography (Sep-Pak® C18) elution profile of strong cation exchange chromatography sample; (♦-♦): total protein, (■-■): protease activity, (---): acetonitrile concentration (%) (Mean ± SD, n=3)

Then, the partial purified ASP1-13 enzyme was applied to Sep-Pak® Vac 35cc C8 cartridges (weak hydrophobic exchange chromatography). The fractions was eluted with stepwise gradient of 30%, 50%, 70% and 100% acetonitrile containing 0.1% formic acid, 10 mM phosphate buffer pH 6.0. Finally, protease activity and total proteins concentration in fractions were measured and the result was shown in figure 4.12. The protease activity was not detected in all fractions. The ASP1-13 enzyme might tightly bind to the strong and weak hydrophobicity column. High concentration of acetonitrile could not eluted ASP1-13 enzyme from the Sep-Pak® Vac 35cc C8 cartridges column. It is noted that ASP1-13 enzyme might had high hydrophobic properties.

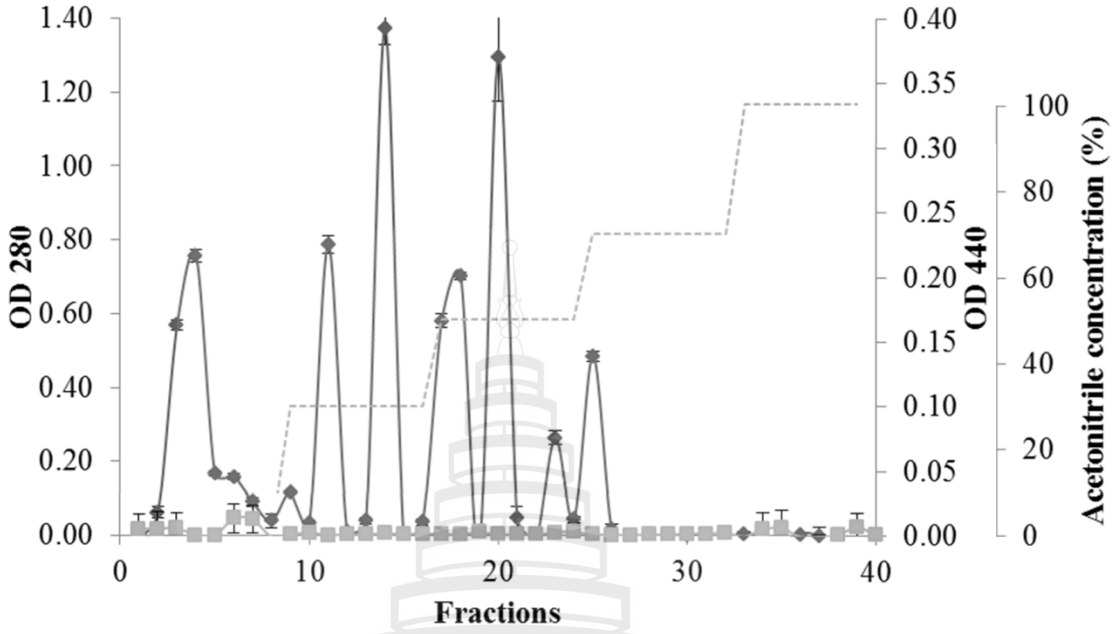


Figure 4.12 Weak hydrophobic exchange chromatography (Sep-Pak® C8) elution profile of strong cation exchange chromatography sample; (♦-♦): total protein, (▪-▪): protease activity, (---): acetonitrile concentration (%)(Mean ± SD, n=3)

4.3.5 Gel filtration exchange chromatography

Superose 12 10/300 GL column was used to partial purified ASP1-13 enzyme after strong cation exchange chromatography step. This column could be used to separate with broad fractionation range of molecules with molecular weights between 1 and 300 kDa (GE Healthcare, USA). The partial purified ASP1-13 enzyme from SP-Sepharose column was applied to Superose 12 10/300 GL column with ÄKTA purifier 10 systems using 10 mM phosphate buffer pH 6.0 as mobile phase at a flow rate of 0.5 ml/min. The sample was aggregated before loaded into Superose 12 10/300 GL column. The aggregation of sample could not separate to a single symmetric peak on a gel filtration elution as shown in Figure 4.13. The protease activity could not be detected in all fractions, showed in figure 4.13. The results imply that ASP1-13 enzyme was tightly bound to Superose 12 10/300 GL column

and could not elute from the column with 10 mM phosphate buffer pH 6.0. High hydrophobicity may cause aggregation via the formation of intermolecular (Reumers, Rousseau, & Schymkowitz, 2009).

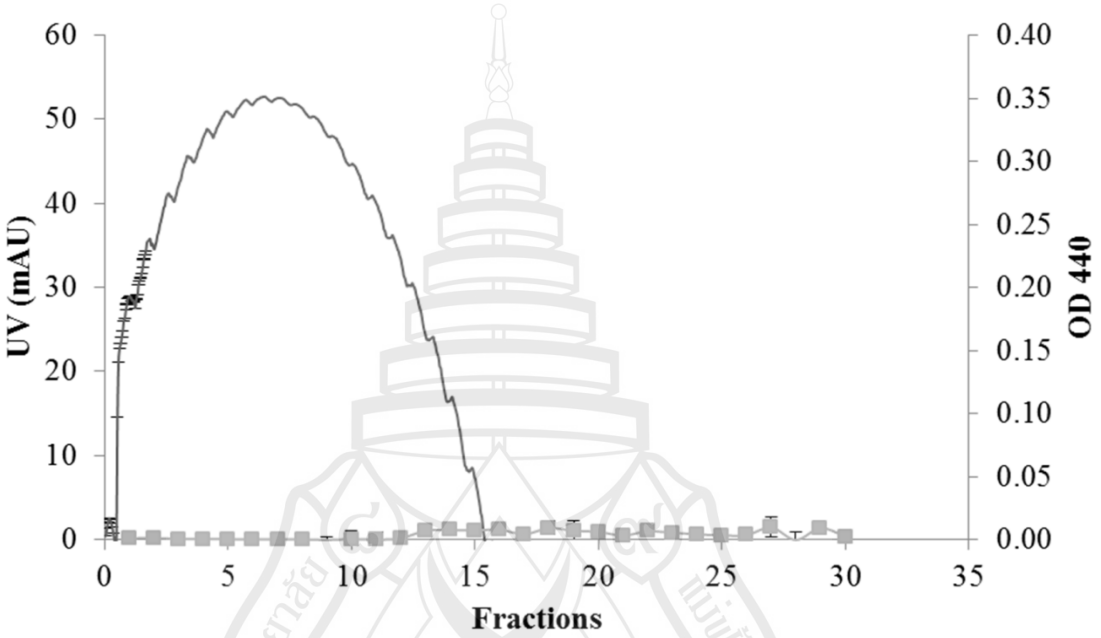


Figure 4.13 Gel filtration exchange chromatography (Superose 12 10/300 GL) elution profile of strong cation exchange chromatography sample; (——): total protein, (▪-▪): protease activity (Mean ± SD, n=3)

4.4 Enzyme characterization

4.4.1 Effect of pH on enzyme activity and stability

The protease activity of ASP1-13 enzyme was not significant decrease between pH 7 to 12. The optimum pH of this protease enzyme was investigated by determination the protease activity of ASP1-13 enzyme in azocasein substrate at various pH buffers (3 to 13). The maximum protease activity at pH 9 was found. The ASP1-13 enzyme was active at pH from 7 to 12 as shown in figure 4.14.

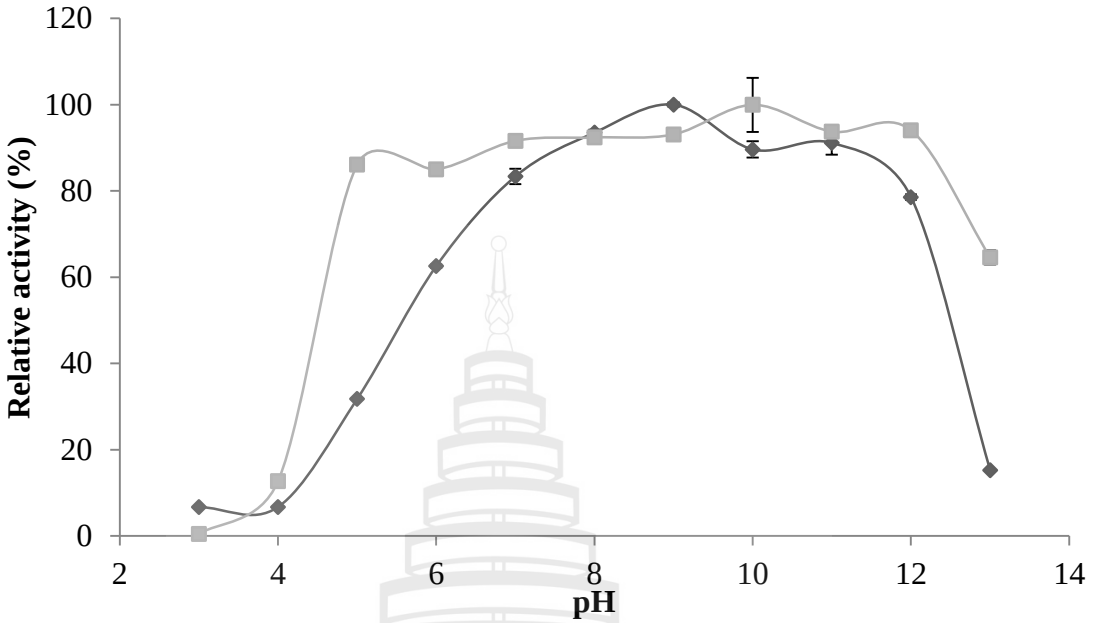


Figure 4.14 Effect of pH on the activity and stability on ASP1-13 enzyme; (♦-♦): effect of pH on enzyme activity, (■-■): pH stability (Mean ± SD, n=3)

For the pH stability, ASP1-13 enzyme was pre-incubated at various pH buffers (3-13) at 4 °C for 12 hr. The results in Figure 4.15 showed that ASP1-13 enzyme was stable between pH 5 to 12 after pre-incubated at 4 °C for 12 hr. The ASP1-13 enzyme was active in an alkaline condition. It might be classified as alkaline protease enzyme. This enzyme will be used as detergent and various industrial applications (Charles et al., 2008; Deng et al., 2010; Schallmey et al., 2004; Shivanand & Jayaraman, 2011).

4.4.2 Effect of temperature on enzyme activity and stability

The optimal temperature for ASP1-13 enzyme was between 50 to 60°C. The optimal temperature was estimated by determining the protease activity of ASP1-13 enzyme at various temperatures using azocasein substrate. The enzyme activity was increased with temperature from 30 to 60 °C and abruptly decreased at 65 °C as shown in figure 4.15.

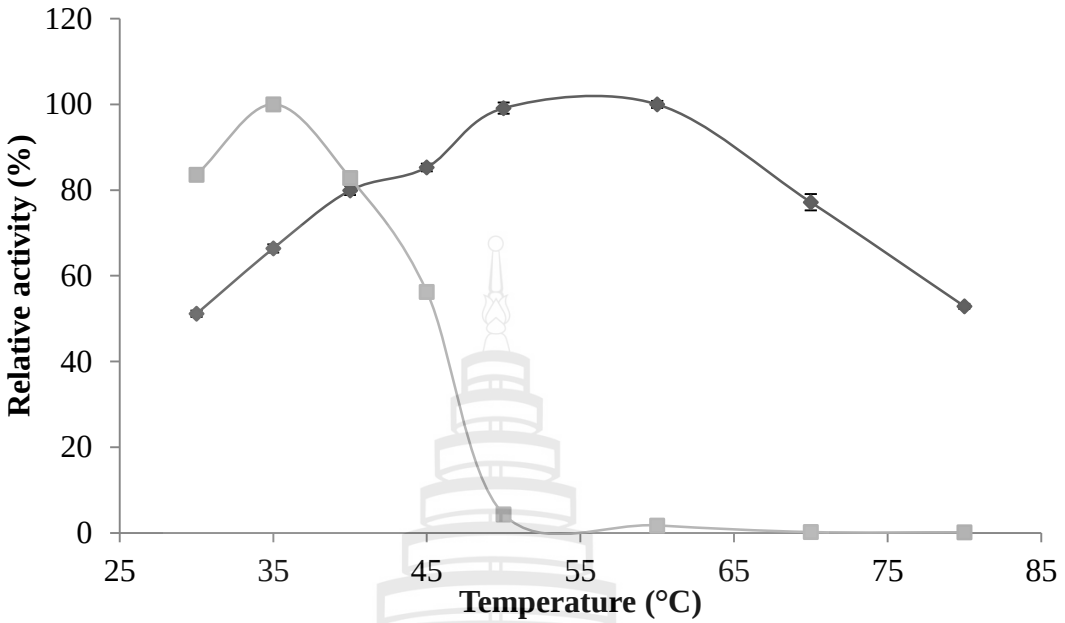


Figure 4.15 Effect of temperature on the activity and stability on ASP1-13 enzyme; (♦-♦): effect of temperature on enzyme activity, (■-■): temperature stability (Mean ± SD, n=3)

For the temperature stability, The ASP1-13 enzyme was pre-incubated at various temperatures (30 to 80°C) for 2 hr and the protease activity was assayed. Finally, the maximum stability of this enzyme was showed at 30 - 40°C for 2 hr as shown in figure 4.15. This property of ASP1-13 enzyme was also used in food and feed processing (Ray, 2012). It could be used active in high temperature that used for prevent the microbial contamination.

4.4.3 Protease inhibitor study

The effect of several protease inhibitors on activity of ASP1-13 enzyme was also studied. The partially purified ASP1-13 enzyme was pre-incubated with various protease inhibitors at 4 °C for 1 hr before standard protease activity assay. Aspartic protease inhibitor (pepstatin), metallo protease inhibitor (DTT) and cysteine protease inhibitor (IAA) were not effect to protease activity of ASP1-13 enzyme while serine

protease inhibitor (PMSF) and metallo protease inhibitor (EDTA) inhibited 100% and 60% protease activity, respectively (figure 4.16).

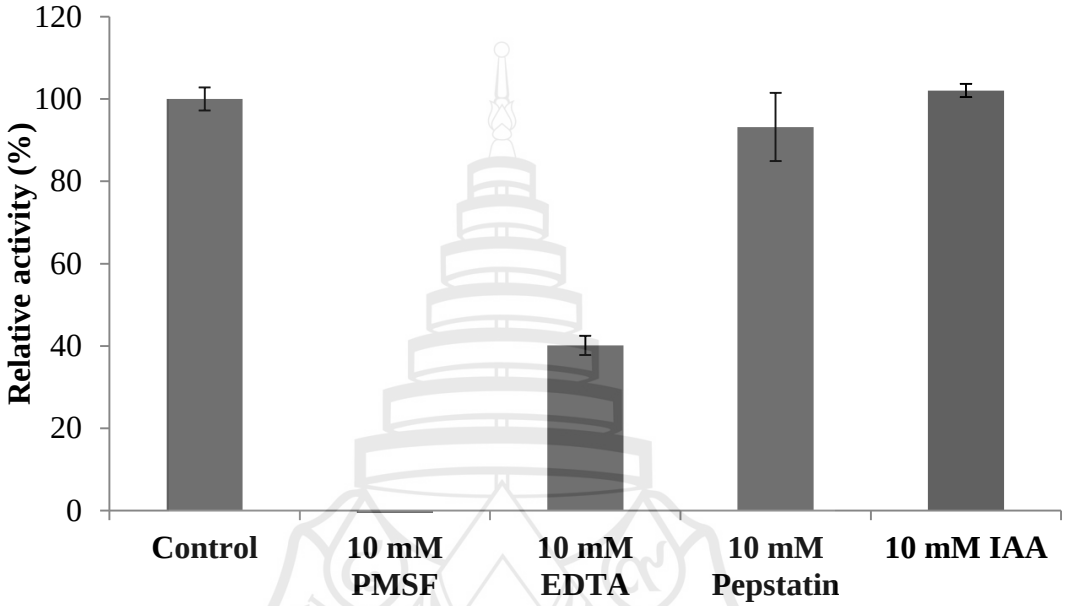


Figure 4.16 Effect of protease inhibitor on ASP1-13 enzyme (Mean ± SD, n=3)

The specific serine protease inhibitor (PMSF) was found to completely inhibit the protease activity of ASP1-13 enzyme. The result suggested that ASP1-13 enzyme was a serine protease type (Deng et al., 2010; Garcia-Carreno, 1992; Phrommao et al., 2011). But, the protease activity of ASP1-13 enzyme was not completely inhibited by EDTA. It was not seem to be a metallo protease type. This result was indicated to a metal ion-dependent serine protease type (Garcia-Carreno, 1992; Shivanand & Jayaraman, 2011).

4.4.4 Effect of surfactant, reducing agent and oxidant on enzyme activity

The effect of surfactant, reducing agent and oxidant agent on the protease activity of ASP1-13 enzyme was studies. The ASP1-13 enzyme was pre-incubated with various reagents at 4 °C for 1 hr before protease activity assay, ASP1-13 enzyme

was exhibited residual activity between 0 – 100 %, in the presence of several substances as shown in figure 4.17.

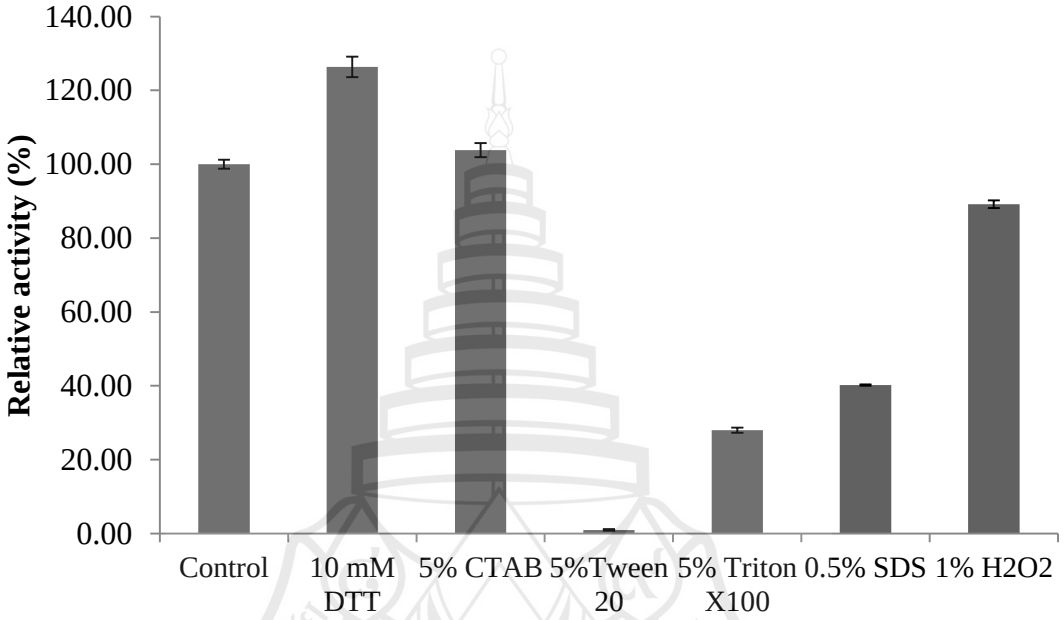


Figure 4.17 Effect of surfactant, reducing and oxidant agent on ASP1-13 enzyme (Mean ± SD, n=3)

These results are consistent with the reports that ionic surfactants and oxidant agent was destabilized most alkaline protease enzyme (Deng et al., 2010; Joo & Chang, 2005; Phrommao et al., 2011). ASP1-13 enzyme activity was retained 80 and 100 % of activity after pre-incubated with 1% H_2O_2 and 5% CTAB, respectively. The activity decreased (1%, 28% and 40% of activity) after pre-incubated with 5% tween 20, 5% triton X100, and 0.5% SDS, respectively. The 126% activity was found in a presence of DTT. Disulfide bonds were not essential for ASP1-13 enzyme. DTT was also enhanced protease activity that required for activity of some protease enzyme. ASP1-13 enzyme could be used in many industrial applications that present of H_2O_2, DTT and CTAB such as detergent application. The present of H_2O_2 and CTAB in reaction was also used as the antimicrobial (Ray, 2012).

4.4.5 Effect of organic solvent on enzyme activity

The effect of organic solvent on the protease activity of ASP1-13 enzyme was measured. The partially purified ASP1-13 enzyme was incubated with various organic solvents before protease activity enzyme assay. The protease activity of ASP1-13 enzyme was increased with 5% concentration of acetone, acetonitrile, ethanol, isopropanol and methanol as shown in figure 4.18.

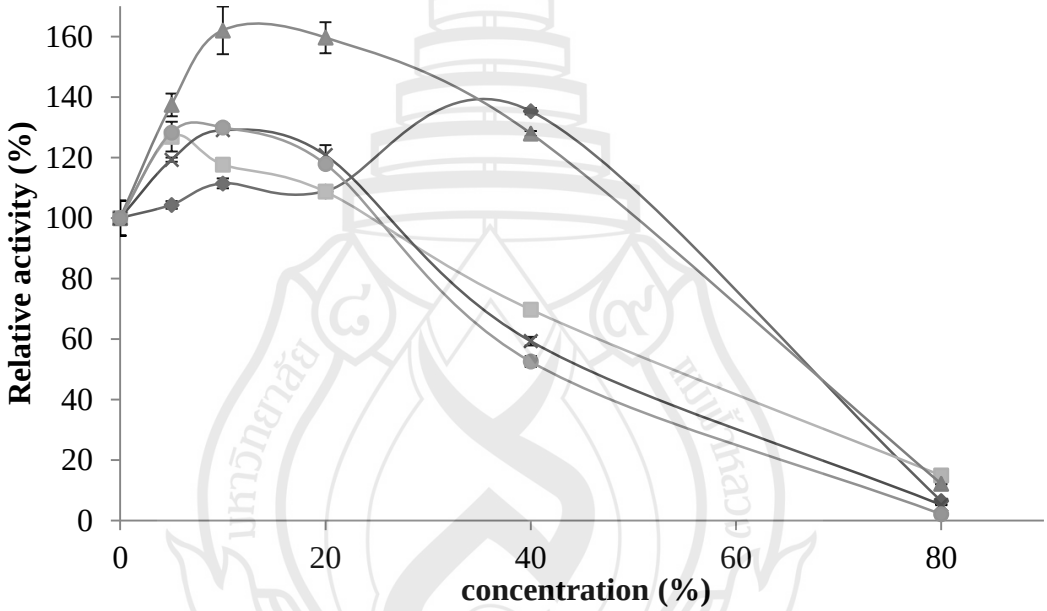


Figure 4.18 Effect of organic solvent on ASP1-13 enzyme; (●-●): acetone, (▲-▲): acetonitrile, (■-■): ethanol, (×-×): isopropanol, (♦-♦): methanol (Mean ± SD, n=3)

At 10-20%, only acetonitrile enhanced activity to 162%. The protease activity of ASP1-13 enzyme was enhanced to 127% and 135% in the presence of > 40% acetonitrile and methanol, respectively. But, the activity was completely inactivated in 80% of all organic solvents. The results are consistent to several reports showing that protease activity could be enhanced by addition of organic solvent (Castro, 1999; Saborowski et al., 2004; Verma & Ghosh, 2010). Its increases the solubility of non-

polar substrates, increase the thermal stability, and decrease water dependent side reaction (Castro, 1999; Ray, 2012; Saborowski et al., 2004).

4.4.6 Effect of metal ion on enzyme activity

The activity of ASP1-13 enzyme was not effect by the addition 10 mM of Ca^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, and Zn^{2}. But, the ASP1-13 enzyme was inhibited in the addition of Cu^{2+} and Fe^{2+} with the resulting in relative activity of 64 as shown in figure 4.19.

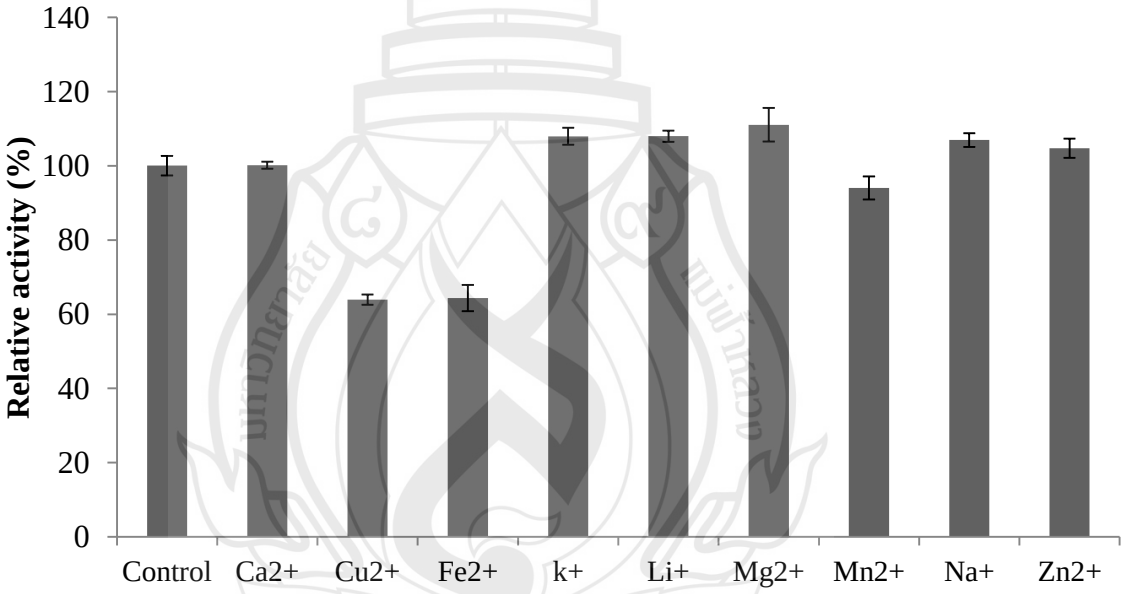


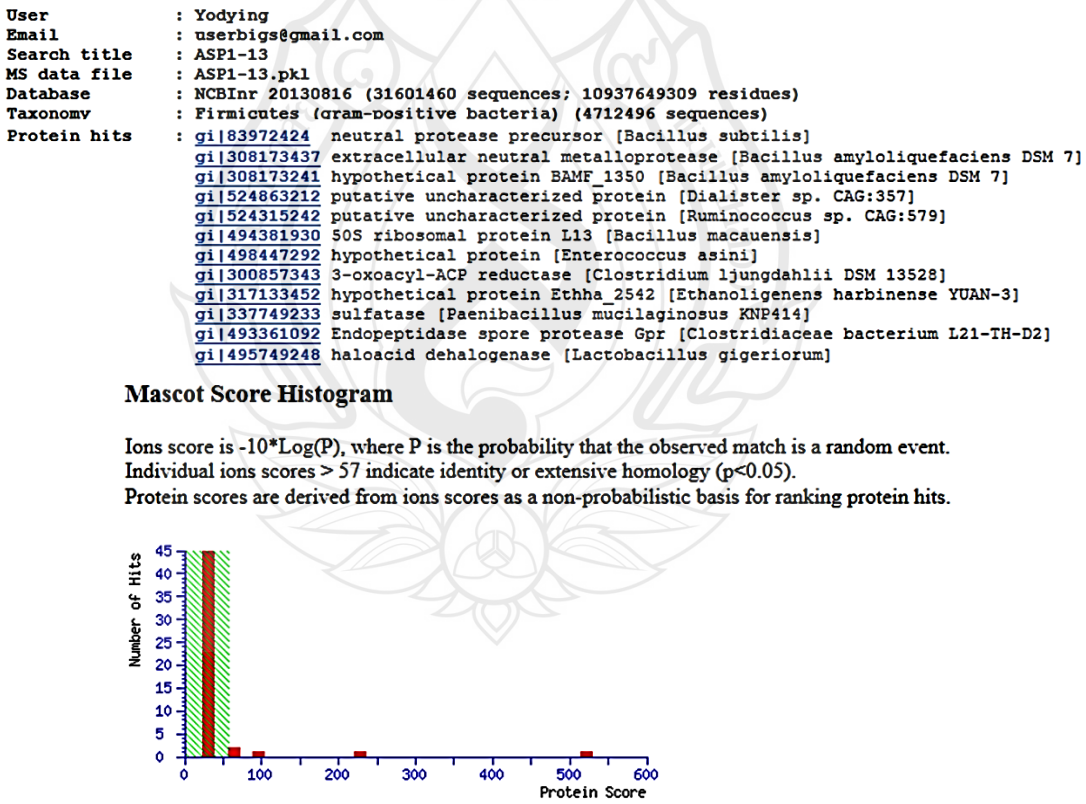
Figure 4.19 Effect of metal ion on ASP1-13 enzyme (Mean ± SD, n=3)

The effect of metal ion on the protease activity of ASP1-13 enzyme was estimated. This enzyme was not effect with a present of Ca^{2+}. But, Cu^{2+}, Mn^{2+}, and Fe^{2+} were inhibited ASP1-13 enzyme activity that according to others alkaline serine protease reports (Deng et al., 2010; In, Oh, & Kim, 2005; Rajkumar, Kothilmozhian, & Ramasamy, 2003; Ruiz & De Castro, 2007) Metal ion is an external factor that affects the protease activity or the stability of the protease enzyme and known to

increase thermal stability that maintaining the active conformation of the protease enzyme (Deng et al., 2010; Phrommao et al., 2011; Shivanand & Jayaraman, 2011).

4.4.7 Protein identification

Protein identification of protein enzyme band on SDS-PAGE was analyzed by using LC-MS/MS. The protein band on SDS-PAGE was excised and digested with in-gel digestion technique. Digested peptides were injected and analyzed by LC-MS/MS. The MS/MS data was applied to MASCOT MS/MS ion search software against NCBI data base. The results were shown in figure 4.21. The results were showed this ASP1-13 enzyme has high similarities to neutral protease precursor from *Bacillus subtilis* (accession number: gi|83972424).



User : Yodying
Email : userbigs@gmail.com
Search title : ASP1-13
MS data file : ASP1-13.pkl
Database : NCBInr 20130816 (31601460 sequences; 10937649309 residues)
Taxonomy : Firmicutes (gram-positive bacteria) (4712496 sequences)
Protein hits :
gi|83972424 neutral protease precursor [Bacillus subtilis]
gi|308173437 extracellular neutral metalloprotease [Bacillus amyloliquefaciens DSM 7]
gi|308173241 hypothetical protein BAMF_1350 [Bacillus amyloliquefaciens DSM 7]
gi|524863212 putative uncharacterized protein [Dialister sp. CAG:357]
gi|524315242 putative uncharacterized protein [Ruminococcus sp. CAG:579]
gi|494381930 50S ribosomal protein L13 [Bacillus macauensis]
gi|498447292 hypothetical protein [Enterococcus asini]
gi|300857343 3-oxoacyl-ACP reductase [Clostridium ljungdahlii DSM 13528]
gi|317133452 hypothetical protein Ethha_2542 [Ethanoligenens harbinense YUAN-3]
gi|337749233 sulfatase [Paenibacillus mucilaginosus KNP414]
gi|493361092 Endopeptidase spore protease Gpr [Clostridiaceae bacterium L21-TH-D2]
gi|495749248 haloacid dehalogenase [Lactobacillus gigeriorum]

Figure 4.20 Protein identification of ASP1-13 enzyme by using LC/MS-MS technique

4.5 Amplification of recombinant protease enzyme (ASP1-13)

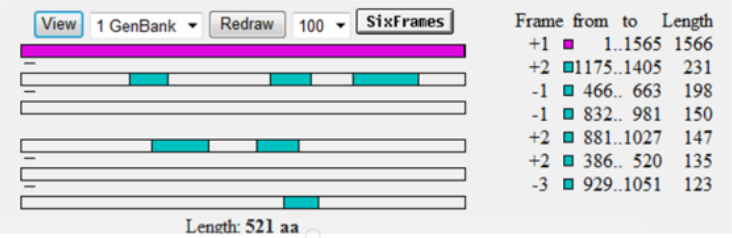
To clone ASP1-13 gene from genomic DNA of *Bacillus amyloliquefaciens* strain S1-13, The protein sequence of neutral protease (accession number: gi|83972424) (figure 3.1) was as DNA template to design specific primer as ASP-13 forward and ASP1-13 reverse as shown in figure 3.2. The PCR products of ASP1-13 gene was eluted from agarose gel, purified and ligated into pTZ57R/T plasmid vector. Then, ligated plasmid (pTZ57R/T_ASP1-13) was transformed in to *E. coli* (DH5α) by using heat shock technique. After that, the transformations containing recombinant plasmid were selected by using colony PCR technique. The plasmid DNA of positive recombinant clone was extracted and sequenced.

The nucleotide sequence of ASP1-13 gene (figure 4.21) were compared against with Genbank database by using BlastN program. The BlastN search result (Table 4.3) were similar to similar to *Bacillus amyloliquefaciens* sub sp. plantarum CAU B946 complete genome, *Bacillus amyloliquefaciens* IT-45 complete genome, *Bacillus subtilis* Npr gene for neutral protease complete cds strain: FP-133, *Bacillus subtilis* B111 neutral protease precursor (npr) gene complete cds and *Bacillus* sp. RH219 extracellular neutral protease precursor (npr) gene partial cds. The open reading frame of ASP1-13 was determined by using open reading frame finder software (http://www.ncbi.nlm.nih.gov/gorf/). The nucleotide sequences of ASP1-13 contains 1,566 bp encoding for a predicted protein of 521 amino acid starting from the first ATG as shown in figure 4.22. Then, the signal peptide of ASP1-13 gene was predicted by using SignalP 4.0 software. The first 28 amino acid of ASP1-13 was determined as signal peptide (figure 4.23). In addition of signal peptide data, it was used as DNA template to design ASP1-13_USN forward (cleaved signal peptide ASP1-13 primer) (figure 3.2). ASP1-13_USN and ASP1-13 reverse primer were used to amplify cleaved signal peptide ASP1-13 gene. After PCR amplification, the target DNA was purified, ligated into pTZ57R/T plasmid vector, and transformed into *E. coli* (DH5α). Finally, the plasmid DNA (pTZ57R/T-ASP1-13_USN gene) of positive recombinant clone was extracted and sequenced.

>ASP1-13_gene

ATGGGTTTAGGTAAGAAATTGTCTGTTGCTGTCGCCGCTTCCTTTATGAGTTTAACCATCAGTCTTCCGGGT
GTTCAGGCAGCTGAGAATCCTCAGCTTAAAGAAAACCTGACGAACTTTGTGCCGAAGCATTCTTTGGTGCAA
TCTGAATTGCCTTCAGTCAGTGACAAAGCAATCAAGCAATACTTGAAACAAAACGGCAAAGTCTTCAAAGGC
AACCCTTCTGAGAGACTGAAGCTGATTGACCACACGACCGATGATCTCGGCTACAAGCACTTCCGTTATGTG
CCTGTCGTTAACGGTGTGCCTGTGAAAGACTCGCAAGTCATTATTCACGTCGATAAATCCAACAATGTCTAT
GCGATTAACGGTGAATTAAACAACGATGCTTCTGCCAAAACGGCAAACAGCAAAAAATTATCTGCAAATCAG
GCGCTGGATCATGCTTTTAAAGCAATCGGCAAATCACCCGAAGCCGTCTCCAACGGCAACGTTGCAAACAAA
AACAAAGCCGAGCTGAAAGCAGCGGCCACAAAAGACGGTAAATACCGACTCGCCTATGATGTAACCATCCGC
TACATCGAACCGGAACCAGCTAACTGGGAAGTAACCGTTGATGCGGAAACAGGGAAAGTCCTGAAAAAGCAA
AACAAAGTGGAGCATGCCGCTGCAACCGGAACAGGTACGACTCTTAAAGGAAAAACGGTCTCATTAAATATT
TCTTCTGAAAACGGCAAATATGTAATGCGTGATCTTTCGAAGCCTACCGGAACACAAATTATTACGTACGAT
CTGCAAAACCGACAATATAACCTGCCGGGCACGCTCGTATCAAGCACTACAAACCAGTTCACAACTTCTTCT
CAGCGCGCTGCCGTTGATGCGCATTACAATCTCGGCAAAGTGTATGATTATTTCTATCAGACGTTTAAACGC
AACAGCTACGACAATAGAGGCGGTAAAATCGTATCTTCCGTTCATTACGGCAGCAGATACAATAACGCGGCC
TGGATCGGCGACCAAATGATTTACGGTGACGGTGACGGCTCATTCTTCTCGCCTCTTTCCGGTTCAATGGAC
GTAACGGCCCATGAAATGACACACGGCGTTACACAGGAAACAGCCAACCTGAACTATGAAAATCAGCCGGGC
GCTTTAAACGAATCCTTCTCCGATGTATTCGGGTACTTCACCGATACTGAGGACTGGGATATCGGTGAGGAT
ATTACGGTCAGCCAGCCGGCTCTCCGCAGCTTATCCAATCCGACAAAATACGGACAGCCAGACCATTACAAA
AATTATCAAAACCTTCCGAACACTGATGCCGGCGACTACGGCGGCGTGCATACAAACAGCGGAATTCCGAAC
AAAGCCGCTTACAACACGATTACAAAAATCGGCGTGAAAAAAGCGGAGCAGATTTACTATCGCGCACTGACG
GTATATCTCACTCCGTCATCAAGCTTTAAAGATGCAAAAGCAGCTTTGATTCAATCAGCGCGGGACCTTTAC
GGCTCTCAAGACGCTGCAAGCGTAGAAGCGGCCTGGAATGCGGTCGGCTTGTAA

Figure 4.21 Nucleotide sequence of ASP1-13 gene from *Bacillus amyloliquefaciens* strain S1-13. The nucleotide encoded signal peptide is also showed with strongly shaded.



MGLGKKLSVAVAASFMSLTISLPGVQAAENPQLKENLTNFVPKHSLVQSELPSVSDKAIKQYLKQNGKVFKGNPSE
RLKLIDHTTDDLGYKHFRYVPVVNGVPVKDSQVIIHVDKSNNVYAINGELNNDASAKTANSKKLSANQALDHAFKA
IGKSPEAVSNGNVANKNKAELKAAATKDGKYRLAYDVTIRYIEPEPANWEVTVDAETGKVLKKQNKVEHAAATGTG
TTLKGKTVSLNISSENGKYVMRDLSKPTGTQIITYDLQNRQYNLPGTLVSSTTNQFTTSSQRAAVDAHYNLGKVYD
YFYQTFKRNSYDNRGGKIVSSVHYGSRYNNAAWIGDQMIYGDGDGSFFSPLSGSMDVTAHEMTHGVTQETANLNYE
NQPGALNESFSDVFGYFTDTEDWDIGEDITVSQPALRSLSNPTKYGQPDHYKNYQNLPNTDAGDYGGVHTNSGIPN
KAAYNTITKIGVKKAEQIYYRALTVYLTPSSSFKDAKAALIQSARDLYGSQDAASVEAAWNAVGL

Figure 4.22 Open reading frame and amino acid encode of ASP1-13 gene

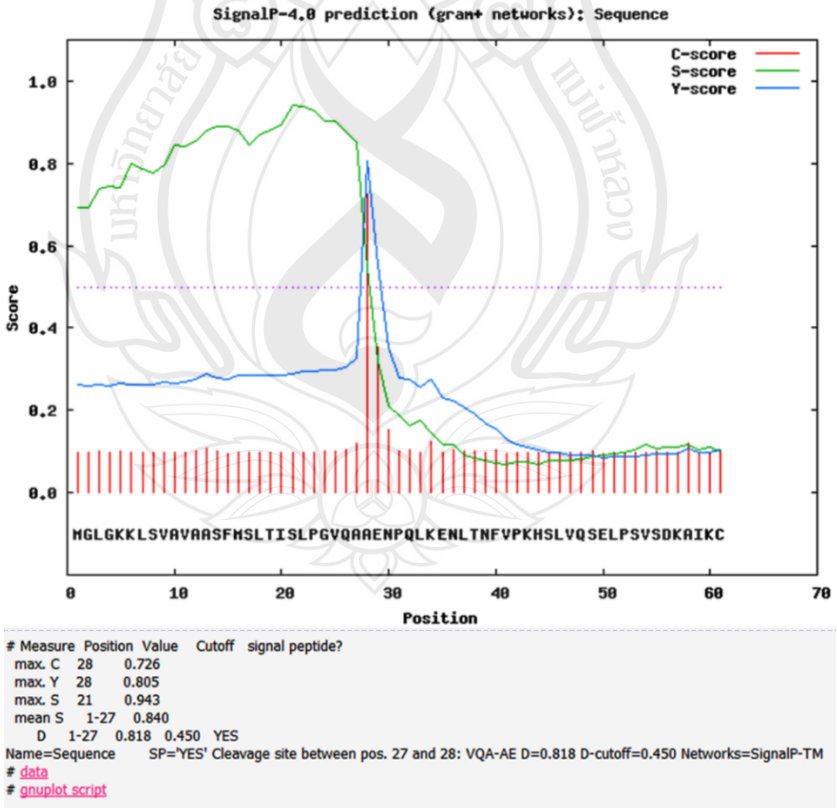


Figure 4.23 Signal peptide of ASP1-13 gene predicted by SignalP 4.0 software

Table 4.3 Alignment of nucleotide sequences of ASP1-13 gene with GenBank database

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus amyloliquefaciens subsp. plantarum CAU B946 complete genome	2,852	2,852	93%	0.0	99%	HE617159
Bacillus amyloliquefaciens IT-45, complete genome	2,848	2,848	93%	0.0	99%	CP004065
Bacillus subtilis Npr gene for neutral protease, complete cds, strain: FP-133	2,848	2,848	93%	0.0	99%	AB568090
Bacillus subtilis B111 neutral protease precursor (npr) gene, complete cds	2,841	2,841	93%	0.0	99%	DQ291130
Bacillus sp. RH219 extracellular neutral protease precursor (npr) gene, partial cds	2,811	2,811	93%	0.0	99%	DQ983789

4.6 Expression of recombinant ASP1-13 and cleaved signal peptide ASP1-13

4.6.1 Construction of pET-17B_ASP1-13, pET-28A_ASP1-13 and pET-17B_ASP1-13_USN recombinant plasmids

The pET-17b expression vector, pET-28a expression vector, recombinant of pTZ57R/T-ASP1-13 plasmid, and recombinant of pTZ57R/T-ASP1-13_USN plasmid were digested with *Nde*I and *Xho*I restriction enzymes. The digested plasmids were analyzed by agarose gel electrophoresis as shown in figure 4.24.

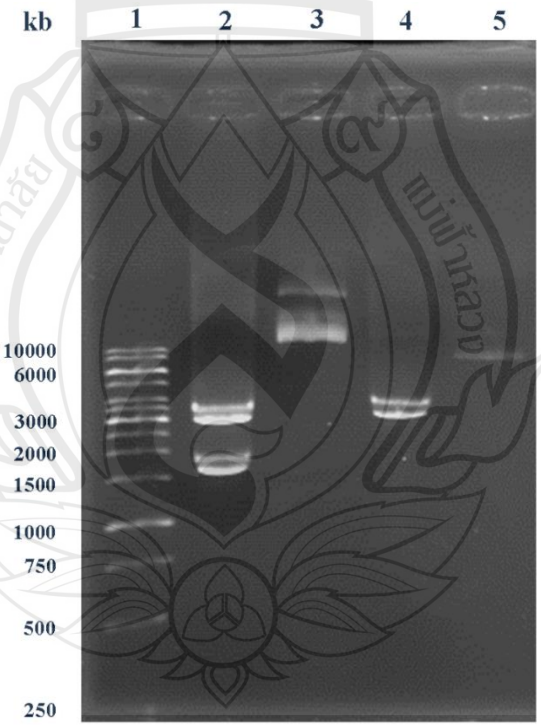


Figure 4.24 Agarose gel electrophoresis of recombinant plasmid digested with *Nde*I and *Xho*I restriction enzymes; lane 1: DNA molecular weight markers, lane 2: digested recombinant of pTZ57R/T-ASP1-13 gene plasmid, lane 3: pTZ57R/T-ASP1-13 gene plasmid, lane 4: digested of pET-17B, lane 5: pET-17B

DNA bands were eluted from agarose and used as DNA insert for ligation reaction. Then, the DNA fragment of ASP1-13 was ligated into digested pET-18a plasmid vector and pET-28a plasmid vector. Furthermore, DNA fragment of ASP1-13_USN genes was ligated into digested pET-18a plasmid vector. The ligated plasmids (pET17B-ASP1-13, pET28A-ASP1-13 and pET17B-ASP1-13_USN) were transformed into *E. coli* (DH5α) by using heat shock technique. After that, the transformed strains were screened for the recombinant clones by using colony PCR technique. The results are shown in figure 4.25. Finally, the plasmid DNA of positive recombinant clone was extracted, sequenced, and transformation to *E. coli* BL21 (DE3).

Figure 4.25 Agarose gel electrophoresis of colony PCR of pET17B-ASP1-13 clones

4.6.2 Isolation of expression strain by using casein agar plate

The positive recombinant plasmid (pET17B-ASP1-13, pET28A-ASP1-13 and pET17B-ASP1-13_USN) isolated from *E. coli* (DH5α) was transformation into *E. coli* BL21 (DE3) by using heat shock technique. Then, the recombinant bacteria were spreaded on LB agar containing 50 ng/ml ampicillin, 1% casein, and 0.5 mM IPTG. After incubated at 37 °C for 18 hr, clear zone producing colony was observed as shown in figure 4.26. ASP1-13 protein might be highly expressed and formed inclusion bodies. Many protein expressed in the *E. coli* was often caused misfolding, segregation into insoluble aggregates known as inclusion bodies. It didn't secrete in to environment (Baneyx, 1999).

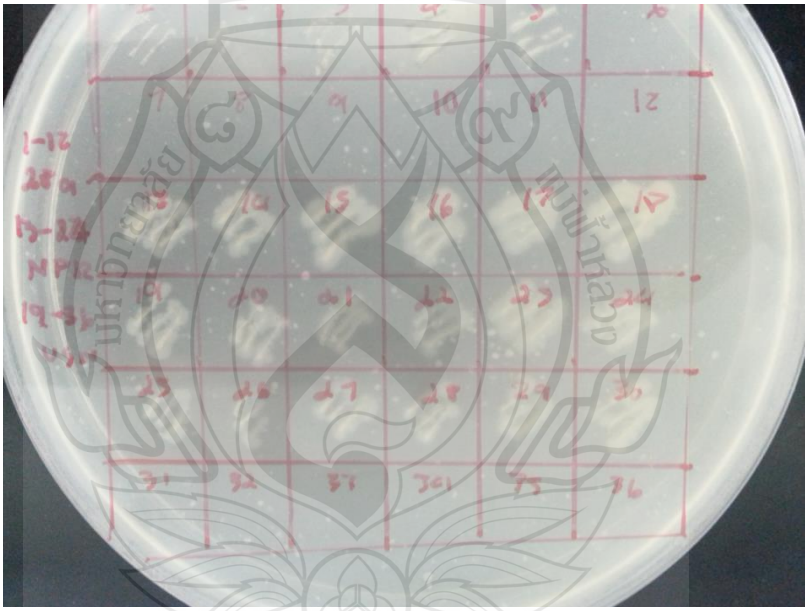


Figure 4.26 Casein agar plate of transformation of expression vector plasmid into *E. coli* BL21 (DE3)

4.6.3 Isolation of expression strain by colony PCR

The recombinant vector (pET17B-ASP1-13, pET28A-ASP1-13 and pET17B-ASP1-13_USN) isolated from *E. coli* (DH5α) was transformation into *E. coli* BL21

(DE3) by using heat shock technique. Then, transformed bacteria were spreaded on LB agar containing 50 ng/ml ampicillin and incubated 37 °C for 18 hr. After that, positive recombinant *E. coli* BL21 (DE3) was determined by using colony PCR technique. The primer pairs, shown in figure 3.2, were used to amplify ASP1-13 and cleaved signal peptide ASP1-13 gene in the clones. The results were shown in figure 4.25. A positive colony was selected for expression of ASP1-13 enzyme and cleaved signal peptide ASP1-13.

4.6.4 Optimization of the expression time for the recombinant ASP1-13 enzyme and ASP1-13 without signal peptide enzyme.

The positive recombinant *E. coli* BL21 (DE3) containing pET17B-ASP1-13, pET28A-ASP1-13 and pET17B-ASP1-13_USN were grown at 37 °C with shaking 180 rpm overnight. The 1% overnight cultures were inoculated into 100 ml of LB broth contained 50 ng/ml ampicillin and incubated at 37 °C with shaking 180 rpm until mid-log phase (OD_{600} ~ 0.4 - 0.6). After that, bacteria cell was induced by adding IPTG to final concentration 1, 3, and 5 mM. The samples were harvested at various times (0, 3, 6, and 24 hr). Culture supernatant was collected and used as crude enzyme. The induced bacterial cells were resuspended in 10 mM phosphate buffer pH 6.0 and lysed by ultrasonic. In this study, no protease activity was observed in all of crude enzyme and induced cells. The SDS-PAGE was used to analysis of the total protein profile of crude enzyme and lysed induced cells. The protein patterns were shown in figure 4.27 - 4.29. The protein band at about 40 kDa was found only in IPTG-induced cells but absence in non-induced cells. The target protein bands at 40 kDa at 0 and 24 hr after induction were excised from SDS-PAGE, in-gel digested and analyzed by LC/MS-MS. Only amino acid sequence of peptides from the protein bands of cleaved signal peptide ASP1-13 enzyme at 24 hr was matched with neutral protease propeptide (gtg start codon) [*Bacillus subtilis*] (figure 4.30). pET17B-ASP1-13 and pET28A-ASP1-13 could not express soluble ASP1-13 protein in the cytoplasm but may express as insoluble aggregates known as inclusion bodies which is inactive form (Baneyx, 1999). The expression of ASP1-13 was then attempted in different expression vector and host strains.

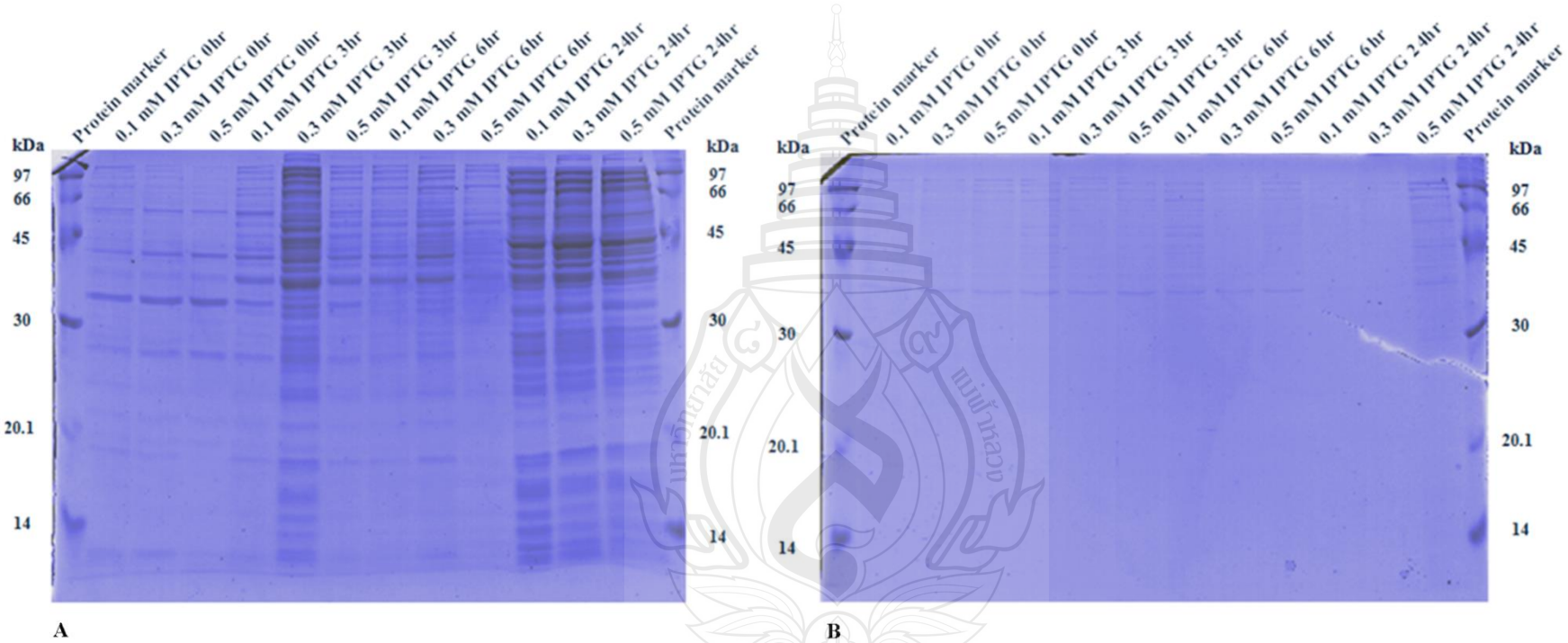


Figure 4.27 SDS-PAGE of recombinant pET17B-ASP1-13 with IPTG concentration (0.1, 0.3 and 0.5 M) and different induced time (0, 3, 6 and 24 hr); lysed induced cell (A), Crude enzyme (B)

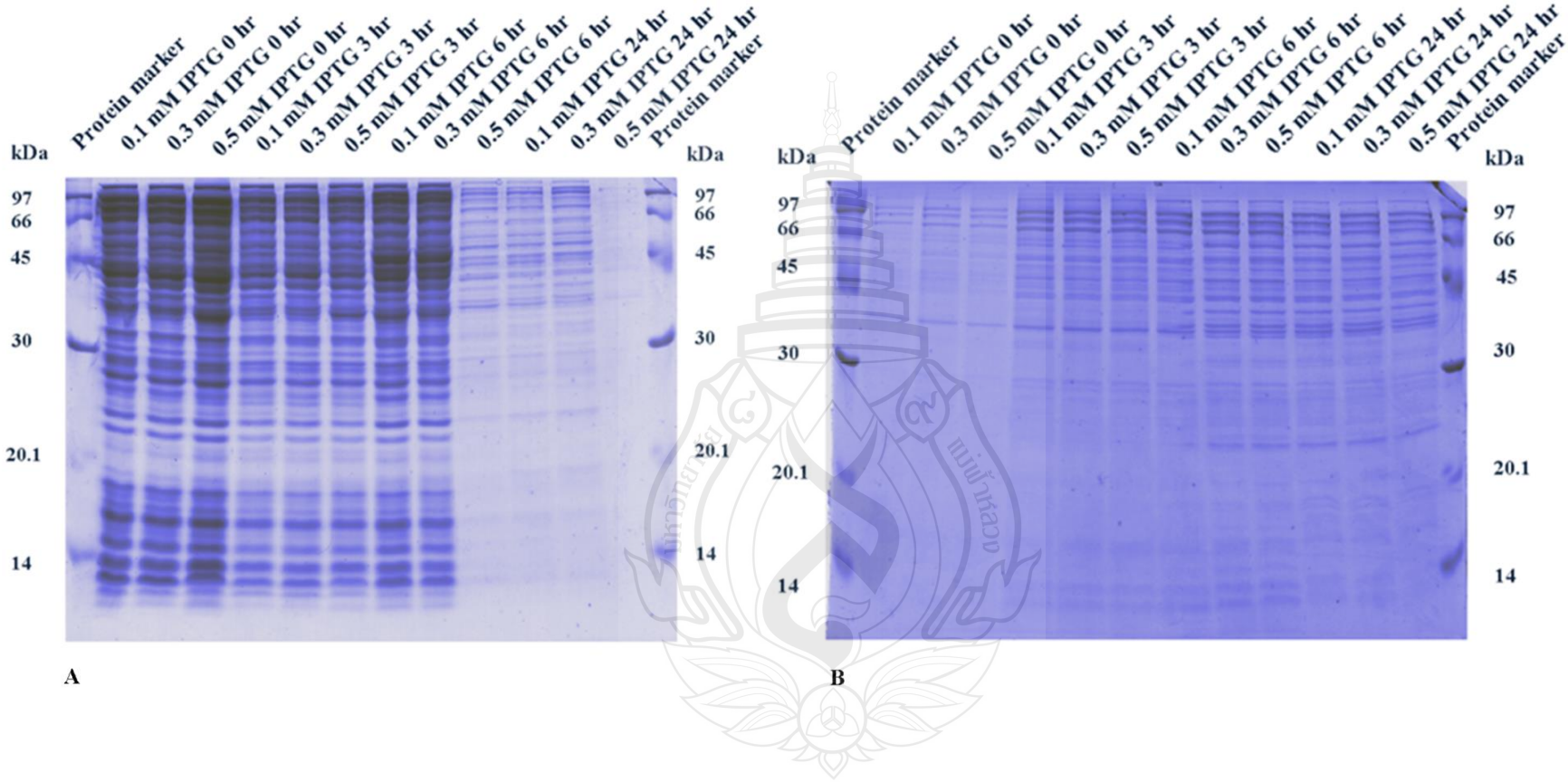


Figure 4.28 SDS-PAGE of recombinant pET28A-ASP1-13 with IPTG concentration (0.1, 0.3 and 0.5 M) and different induced time (0, 3, 6 and 24 hr); lysed induced cell (A), Crude enzyme (B)

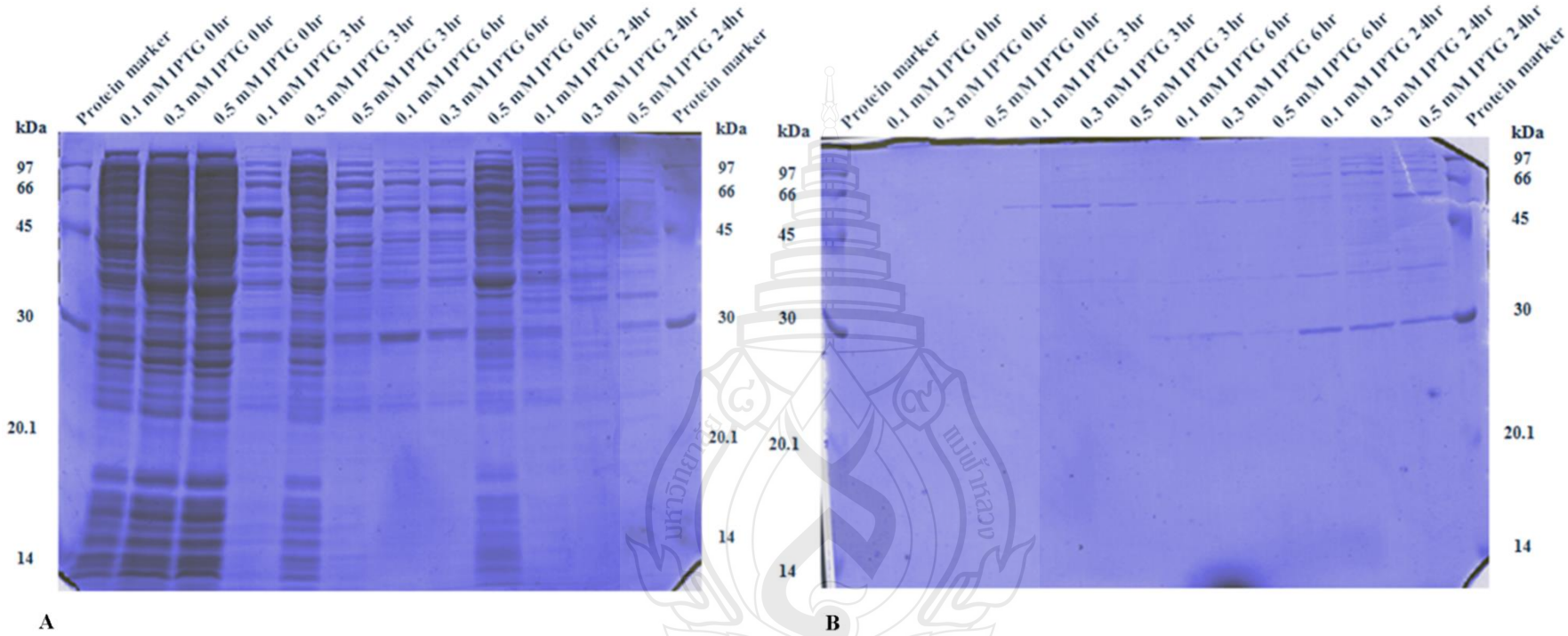


Figure 4.29 SDS-PAGE of recombinant pET17B-ASP1-13_USN with IPTG concentration (0.1, 0.3 and 0.5 M) and different induced time (0, 3, 6 and 24 hr); lysed induced cell (A), Crude enzyme (B)

Figure 4.30 Protein identification of recombinant ASP1-13 without signal peptide enzyme by using LC/MS-MS technique

CHAPTER 5

CONCLUSION

This study was started with molecular identification of *Bacillus* sp. strain S1-13. The *Bacillus* sp. strain S1-13 was to be *Bacillus amyloliquefaciens* identified upon nucleotide sequence of 16s rRNA gene. The result of 1,256 bp was searched for its similarity against the GenBank database by BlastN and performed the phylogenetic analysis using MEGA 5.0 software. Then, the best condition for production of ASP1-13 enzyme from *Bacillus amyloliquefaciens* strain S1-13 was optimized by study the growth curve and ability to secrete protease enzyme when let the bacteria grown in nutrient broth containing 1% skim milk and 1% casein. Highest protease production from *Bacillus amyloliquefaciens* strain S1-13 was observed when it was grown in nutrient broth containing 1% skim milk. The ASP1-13 enzyme was partial purified for about 15 fold with a specific activity of 1,324 U/mg and 2% yield after acetone precipitation and cation chromatography, respectively.

The molecular weight was about 40 kDa with isoelectric point of 8. Partial amino acid sequence of ASP1-13 was similar to neutral protease precursor from *Bacillus subtilis* (accession number: gi|83972424). The results from strong anion and gel filtration chromatographic data showed that ASP1-13 had high hydrophobicity. ASP1-13 was classified as a metal ion-dependent serine protease type, exhibiting a broad range activity at pH 7-12, 50-60 °C, stability to pH 4-12, 30-40°C for 2 hr. The ASP1-13 activity was enhanced by DTT while H_2O_2 and CTAB did not affect its activity. Addition of acetone, acetonitrile, ethanol, isopropanol and methanol at low concentration as 5% was found to increase its activity. Many metal ions including K^+ Li^+, Mg^{2+}, Na^+, and Zn^{2+} enhanced the enzyme activity whereas it was inhibited by the addition of Cu^{2+}, Mn^{2+}, and Fe^{2+}. These properties of ASP1-13 enzyme are suitable for use in the detergent, feed processing, and food processing because of active, stable in high pH value, organic solvent.

The full coding sequence of ASP1-13 gene was successfully cloned. It contained 1,566 bp nucleotide and 521 deduced amino acids with a signal peptide located at first 28 amino acid. ASP1-13 gene and cleaved signal peptide ASP1-13 gene were cloned in pET17b and pET28a expression vector and expressed in *E. coli* BL21 (DE3). However, the protease activity of all recombinant clones could not be detected. Little amount of expressed protein with an apparent mass about 40 kDa on SDS-PAGE can be identified by LC/MS-MS in recombinant clones harbouring cleaved signal peptide ASP1-13 gene. The amino acid sequence of tryptic peptides from that protein band of interest was similarly to neutral protease propeptide from *Bacillus subtilis*

Further experiment, the full coding sequence of ASP1-13 gene will express in other expression vector system such as *Bacillus* system because it is non-pathogenic, does not produce any endotoxins, capable of secreting functional extracellular proteins directly into the culture medium and cheap inducers in the field of fermentation technology. The expression of ASP1-13 enzyme will be characterization. Finally, the expression of ASP1-13 enzyme will be used in industrial application such as detergent application.

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APPENDICES

APPENDIX A

LIST OF CHEMICAL AND REAGENT

Table A1 List of chemical and reagent

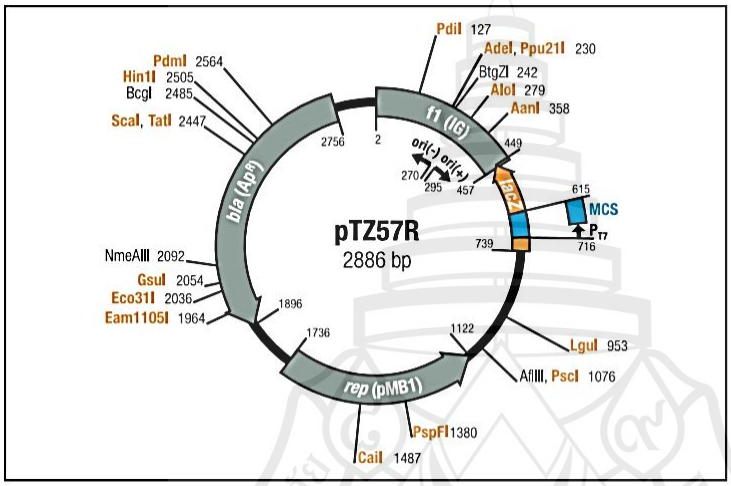
Reagents	**Manufacturer**
Acetone	LAB-SCAN LTD
Acetronitrile	LAB-SCAN LTD
Agarose-ME ultrapure	USB
American bacteriological agar	CONDA
Ammonium chloride	MERCK
Ammonium sulfate	Sigma
Azo-casein	Sigma
Bromophenol blue	USB
Boric acid	MERCK
Calcium chloride dihydrate	Bio Basic INC
Casein	Bio Basic INC
Chloroform	LAB-SCAN LTD
Coomassie brilliant blue R-250	Bio-RAD
Copper (II) sulfate pentahydrate	MERCK
Di-potassium hydrogen orthophosphate	Ajax Fine Chem
Disodium salt, dihydrate	Bio Basic INC
Dimethyl sulfoxide (DMSO)	GE Healthcare
Dithiothreitol (DTT)	Bio Basic INC
EDTA (disodium salt, dihydrate)	MERCK
Ethanol	LAB-SCAN LTD
Ethidium bromide	Applichem
Ferrous Sulfate Heptahydrate	Riedel-deHaen
Folin & Ciocalteu's phenol reagent	MERCK
Formic acid	MERCK
Glacial acetic acid	MERCK
Glycine	Research organics

Table A1 (Continued)

Reagents	Manufacturer
Glycerol 85%	GE Healthcare
Hydrochloric acid	LAB-SCAN LTD
Hydrogen peroxide	Bio-basic
Iodoacetamide (IAA)	GE Healthcare
Manganese chloride tetrahydrate	Bio Basic INC
Magnesium chloride	Bio Basic INC
Magnesium chloride hexahydrate	Bio Basic INC
Magnesium sulfate heptahydrate	MERCK
Manganese chloride tetrahydrate	Bio Basic INC
Meat extract	Bio Basic INC
Methanol	LAB-SCAN LTD
Peptone	Bio Basic INC
Pepstatin A	Sigma
Phenol: chloroform: isoamyl alcohol (25: 24: 1)	USB
phenylmethanesulfonylfluoride (PMSF)	Sigma
Potassium chloride	MERCK
Potassium dihydrogen phosphate	MERCK
Potassium monohydrogen phosphate	Carlo erba
Potassium dihydrogen phosphate	Carlo erba
Skim milk	Bio Basic INC
Sodium acetate	MERCK
Sodium carbonate	Sigma
Sodium chloride	MERCK
Sodium dodecyl sulfate	USB
Sodium hydroxide	MERCK
Sodium molybdate dyhydrate	MERCK
Tatalic acid	Carlo erba
Tetramethylethylenediamine (Temed)	USB
Trichloroacetic acid	Scharlan
Tri-reagent	Research organics
Tris (hydroxymethyl) aminomethane	Research organics
Triton X100	USB
Tryptone powder	Bio Basic INC
Tween 80	MERCK
Yeast extract	Bio Basic INC
Zinc sulfate heptahydrate	Bio Basic INC

APPENDIX B

VECTOR FOR EXPERIMENT



Enzymes which cut pTZ57R DNA once:

AanI	358	HincII*	667
Acc65I*	627	HindIII*	690
AdeI	230	KpnI*	627
AflIII	1076	LguI	953
AlfI*	674	Mph1103I*	639
AloI	279	Mva1269I*	637
ApaI*	661	NmeAIII	2092
BamHI*	654	PaeI*	684
BcgI	2485	PdiI	127
Bsp68I*	633	PdmI	2564
Bsp120I*	661	Ppu21I	230
BtgZI	242	PscI	1076
CaiI	1487	PspFI	1380
Cfr9I*	658	PstI*	672
Eam1105I	1964	SacI*	621
Ecl136II*	621	SalI*	667
Eco31I	2036	ScaI	2447
Eco32I*	648	SmaI*	658
Eco88I*	658	TatI	2447
Eco147I*	678	XbaI*	644
EcoRI*	615	XmiI*	667
GsuI	2054		
Hin1I	2505	* MCS	

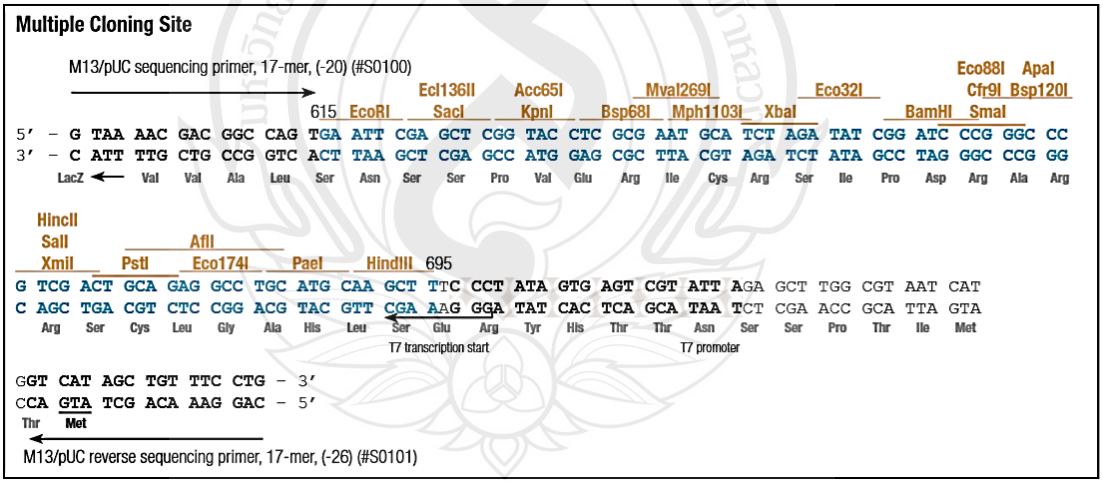


Figure B1 Restriction map and multiple cloning site of pTZ57R/T cloning vector (Thermo scientific, USA)

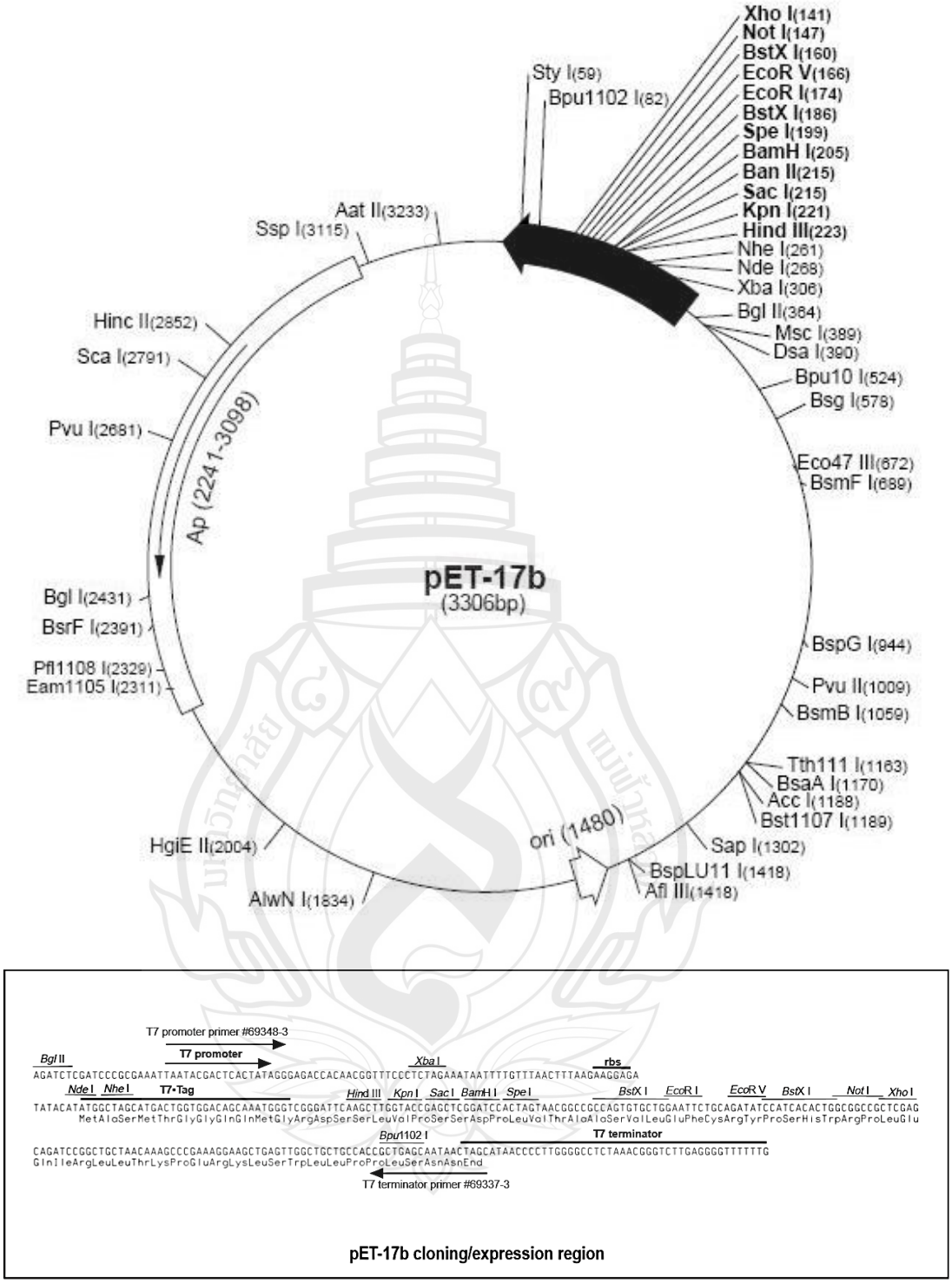


Figure B2 Restriction map and multiple cloning site of pET-17b expression vector (Novagen, USA)

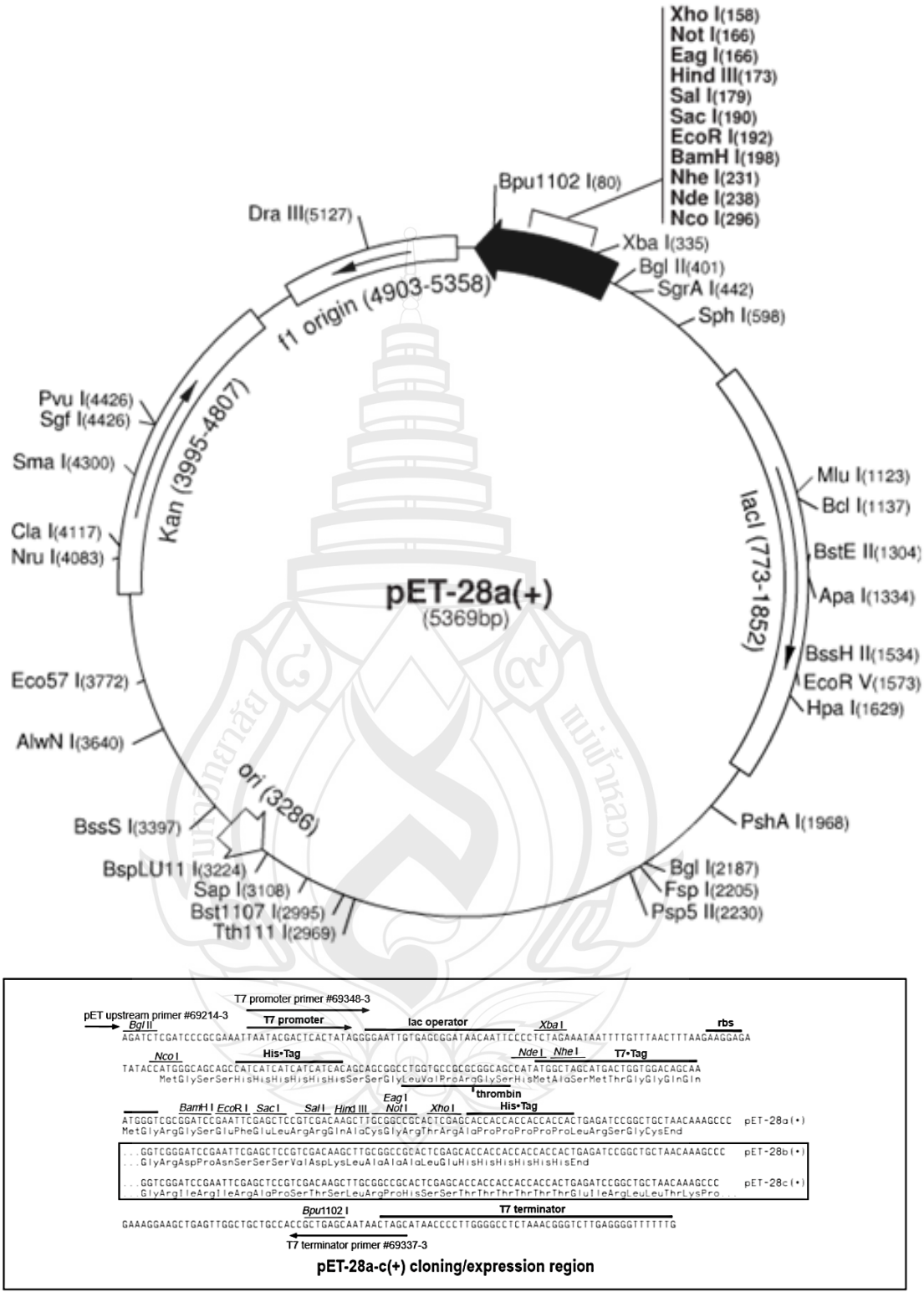


Figure B3 Restriction map and multiple cloning site of pET-28a expression vector (Novagen, USA)

APPENDIX C

PHYLOGENIC TREE OF ALL REFERENCE SEQUENCE OF *Bacillus* sp. AND *Bacillus amyloliquefaciens* STRAIN S1-13

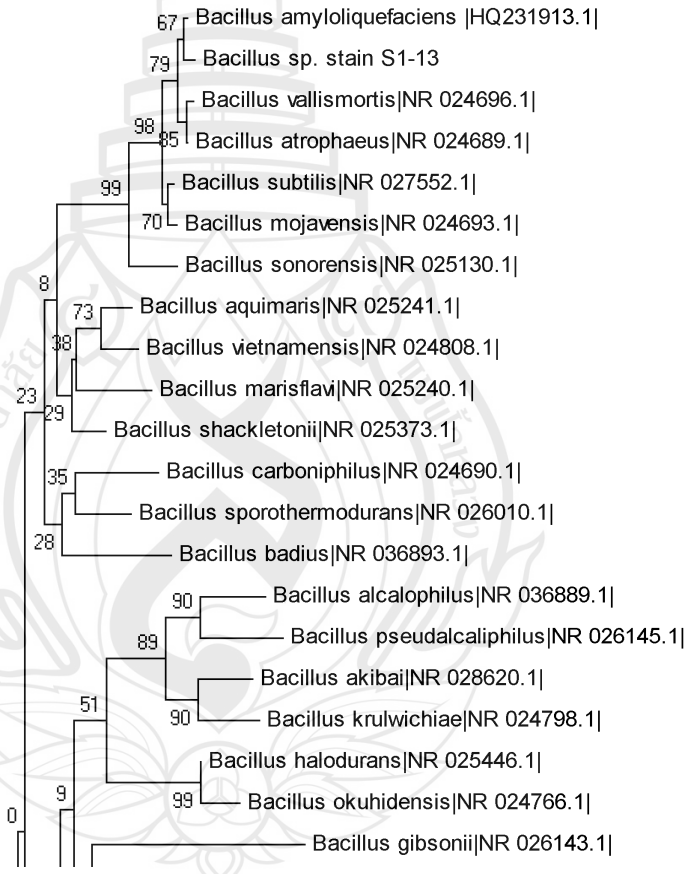


Figure C1 Phylogenetic tree based on 16s rRNA gene of all reference sequence of *Bacillus* sp. and *Bacillus amyloliquefaciens* strain S1-13. The numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets

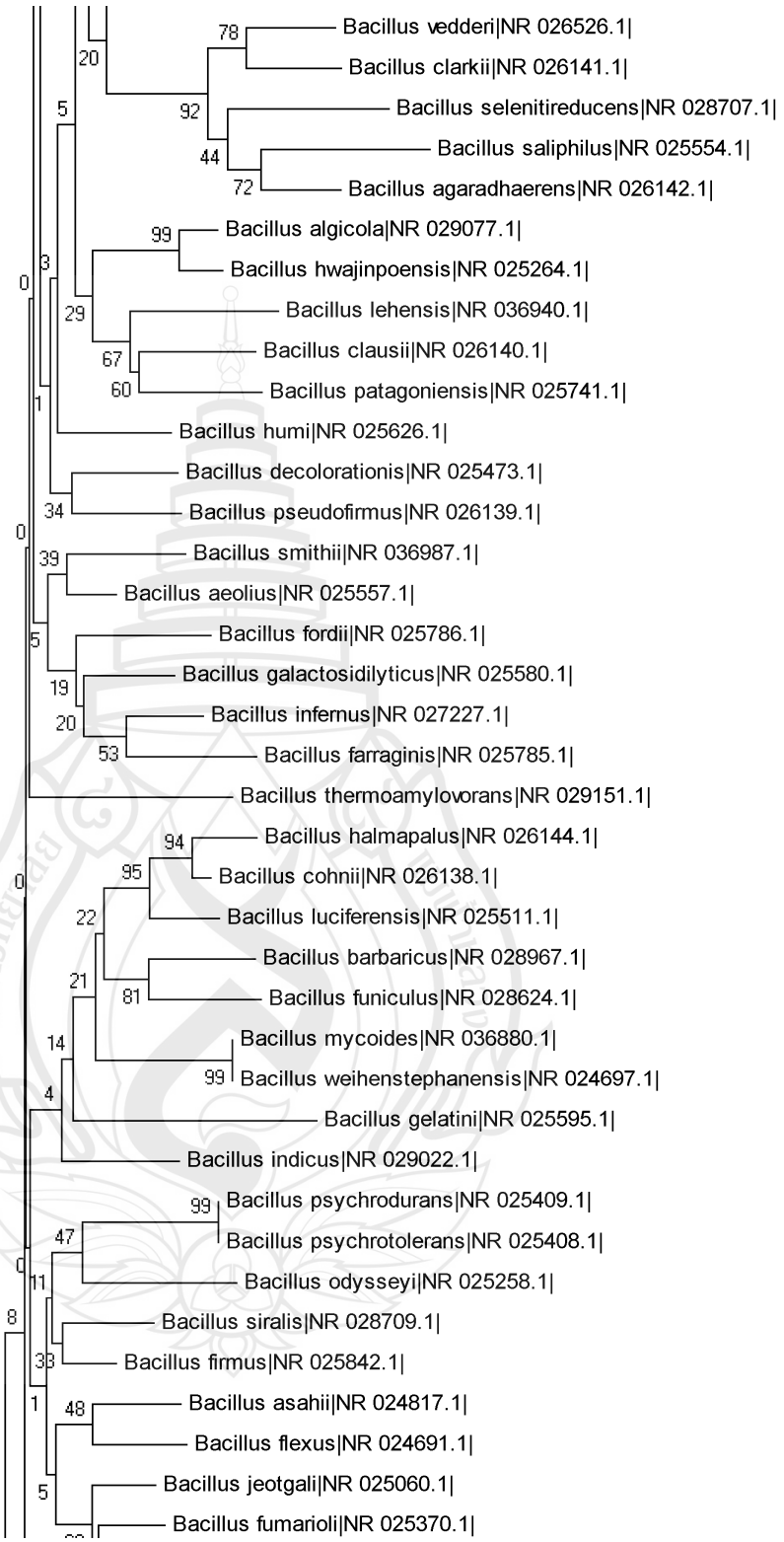


Figure C1 (Continued)

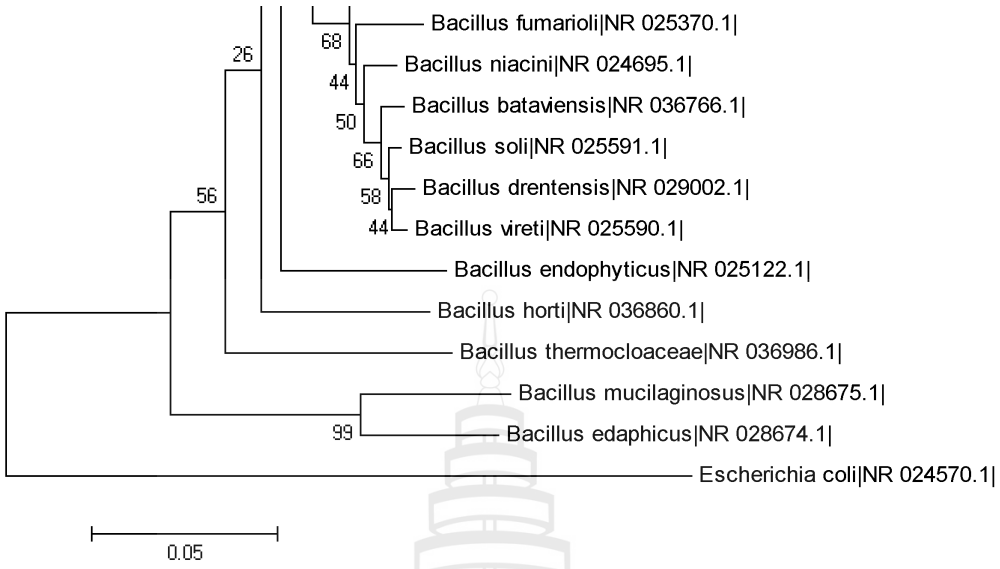


Figure C1 (Continued)

APPENDIX D

STANDARD CURVE AND CALCULATION DATA OF ASP1-13 ENZYME

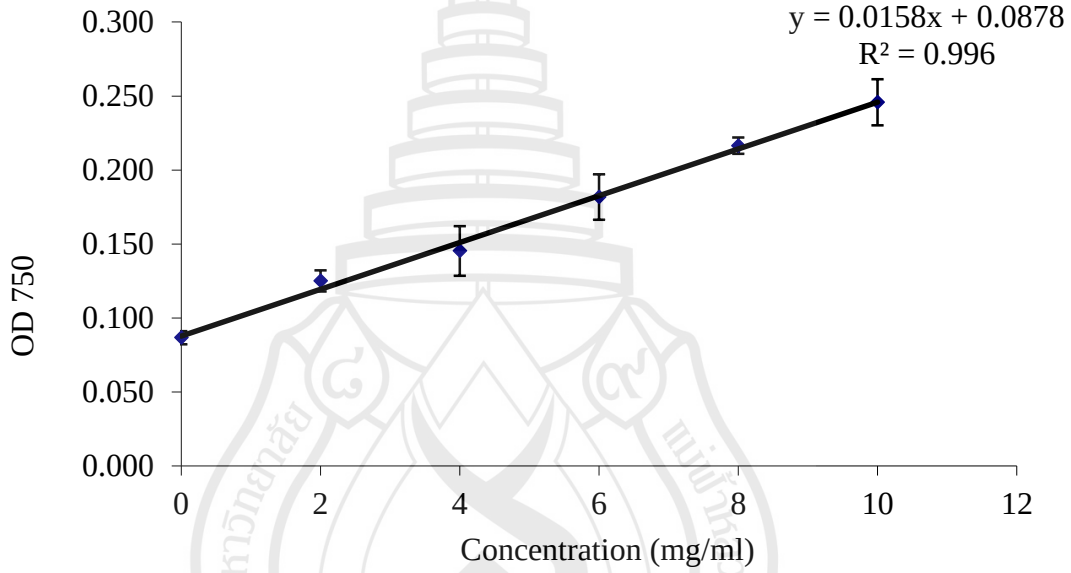


Figure D1 Typical standard curves for bovine serum albumin (BSA) in the Lowry protein assay (Mean ± SD, n=3)

Table D1 Calculation data of typical standard curves for bovine serum albumin (BSA) in the Lowry protein assay

Concentration (mg/ml)	OD 750			Average	S.D.
0	0.087	0.082	0.091	0.087	0.005
2	0.117	0.130	0.129	0.125	0.007
4	0.158	0.152	0.126	0.145	0.017
6	0.167	0.181	0.197	0.182	0.015
8	0.210	0.221	0.218	0.216	0.005
10	0.228	0.254	0.256	0.246	0.016

Table D2 Effect of media composition on *Bacillus amyloliquefaciens* strain S1-13 protease enzyme production

Medium	Time	OD 440			Average	S.D.	Protease activity	Relative activity (%)
NB	6 hr	0.064	0.066	0.065	0.065	0.001	0.008	3
	12 hr	0.114	0.115	0.114	0.114	0.001	0.057	22
	18 hr	0.130	0.129	0.132	0.130	0.002	0.073	29
	24 hr	0.123	0.116	0.114	0.118	0.005	0.061	24
	30 hr	0.111	0.110	0.109	0.110	0.001	0.053	21
	36 hr	0.098	0.105	0.105	0.103	0.004	0.046	18
NB+1% Casein	6 hr	0.070	0.071	0.072	0.071	0.001	0.011	4
	12 hr	0.163	0.164	0.161	0.163	0.002	0.103	40
	18 hr	0.282	0.301	0.278	0.287	0.012	0.227	89
	24 hr	0.317	0.314	0.314	0.315	0.002	0.255	100
	30 hr	0.298	0.297	0.296	0.297	0.001	0.237	93
	36 hr	0.267	0.271	0.265	0.268	0.003	0.208	81
NB+1% skimmilk	6 hr	0.067	0.075	0.071	0.071	0.004	0.023	9
	12 hr	0.147	0.132	0.140	0.140	0.008	0.092	36
	18 hr	0.213	0.210	0.213	0.212	0.002	0.164	64
	24 hr	0.255	0.251	0.251	0.252	0.002	0.204	80
	30 hr	0.260	0.259	0.255	0.258	0.003	0.210	82
	36 hr	0.250	0.256	0.247	0.251	0.005	0.203	80
Blank NB		0.056	0.066	0.049	0.057	0.009		
Blank NB+1% Casein		0.058	0.060	0.061	0.060	0.002		
Blank NB+1% skimmilk		0.048	0.050	0.047	0.048	0.002		

Table D3 Effect of media composition on *Bacillus amyloliquefaciens* strain S1-13 growth rate

Medium	Time		OD 600		Average	S.D.	Dilution	Growth rate	S.D.
NB	6 hr	0.168	0.198	0.199	0.188	0.018	5	0.752	0.088
	12 hr	0.305	0.324	0.362	0.330	0.029	5	1.462	0.145
	18 hr	0.331	0.315	0.423	0.356	0.058	5	1.592	0.291
	24 hr	0.202	0.213	0.217	0.211	0.008	5	0.863	0.039
	30 hr	0.181	0.185	0.212	0.193	0.017	5	0.773	0.084
	36 hr	0.192	0.209	0.202	0.201	0.009	5	0.815	0.043
NB+1% Casein	6 hr	0.324	0.359	0.342	0.342	0.018	5	0.758	0.088
	12 hr	0.336	0.347	0.362	0.348	0.013	5	0.792	0.065
	18 hr	0.432	0.446	0.454	0.444	0.011	5	1.270	0.056
	24 hr	0.561	0.508	0.614	0.561	0.053	5	1.855	0.265
	30 hr	0.390	0.386	0.416	0.397	0.016	5	1.037	0.081
	36 hr	0.290	0.288	0.319	0.299	0.017	5	0.545	0.087
NB+1% skimmilk	6 hr	0.275	0.285	0.290	0.283	0.008	5	1.227	0.038
	12 hr	0.332	0.394	0.435	0.387	0.052	5	1.745	0.259
	18 hr	0.495	0.470	0.472	0.479	0.014	5	2.205	0.069
	24 hr	0.414	0.444	0.490	0.449	0.038	5	2.057	0.191
	30 hr	0.475	0.457	0.538	0.490	0.043	5	2.260	0.213
	36 hr	0.443	0.505	0.493	0.480	0.033	5	2.212	0.164
Blank NB		0.045	0.034	0.034	0.038	0.006			
Blank NB+1% Casein		0.201	0.158	0.211	0.190	0.030			
Blank NB+1% skimmilk		0.326	0.328	0.318	0.324	0.001			

Table D4 Purification of ASP1-13 enzyme from *Bacillus amyloliquefaciens* strain S1-13

Purification step	OD 440	Time (min)	OD/time	Dilution	Reaction volume (µl)	Sample volume (µl)	Enzyme unit (U)	Protein concentration (mg/ml)	Specific activity (U/mg)
Crude enzyme	0.383	30	0.013	100	400	20	25.535	6.641	192
Acetone	0.785	30	0.026	100	400	20	52.327	1.831	1429
SP-Sepharose	0.293	30	0.010	100	400	20	19.536	0.355	2752

Table D5 Effect of acetone solution on protease activity ASP1-13 enzyme from *Bacillus amyloliquefaciens* strain S1-13 during acetone precipitation with different acetone/sample ratios

Solvent	Sample ratios		OD 440		Average	S.D.	Dilution	Protease activity
Acetone	0.25	0.058	0.046	0.052	0.052	0.006	5	0.055
	0.50	0.073	0.072	0.078	0.074	0.003	5	0.167
	1.00	0.190	0.190	0.198	0.193	0.005	5	0.758
	2.00	0.213	0.242	0.227	0.227	0.015	5	0.932
	3.00	0.223	0.216	0.213	0.217	0.005	5	0.882
	4.00	0.227	0.239	0.238	0.235	0.007	5	0.968
	5.00	0.218	0.226	0.231	0.225	0.007	5	0.920
Control		**0.108**	**0.118**	**0.110**	**0.112**	**0.005**	**5**	**0.355**
Blank		0.035	0.047	0.042	0.041	0.006		

Table D6 Strong cation exchange chromatography (SP-Sepharose) elution profile of dialyzed ASP1-13 enzyme

Fractions		OD 280		Average	S.D.		OD 440		Average	S.D.	Protease activity
1	0.116	0.137	0.130	0.128	0.011	0.113	0.114	0.114	0.114	0.001	0.000
2	1.099	1.145	1.292	1.179	0.101	0.115	0.117	0.117	0.116	0.001	0.002
3	0.093	0.097	0.120	0.103	0.015	0.114	0.116	0.117	0.116	0.002	0.002
4	-0.005	0.017	0.013	0.008	0.012	0.106	0.106	0.104	0.105	0.001	-0.009
5	0.073	0.073	0.059	0.068	0.008	0.484	0.483	0.481	0.483	0.002	0.369
6	0.026	0.028	0.015	0.023	0.007	0.262	0.260	0.257	0.260	0.003	0.146
7	-0.012	-0.007	-0.020	-0.013	0.007	0.116	0.117	0.116	0.116	0.001	0.002
8	0.010	0.006	-0.017	0.000	0.015	0.115	0.117	0.114	0.115	0.002	0.001
9	-0.011	-0.003	0.018	0.001	0.015	0.114	0.112	0.115	0.114	0.002	0.000
10	-0.019	-0.029	-0.009	-0.019	0.010	0.117	0.113	0.112	0.114	0.003	0.000
11	-0.012	-0.018	-0.022	-0.017	0.005	0.116	0.111	0.108	0.112	0.004	-0.002
12	-0.017	-0.029	-0.037	-0.028	0.010						
13	-0.005	-0.028	-0.004	-0.012	0.014						
14	-0.028	-0.035	-0.014	-0.026	0.011						
15	-0.032	-0.021	-0.016	-0.023	0.008						
16	-0.019	-0.009	-0.026	-0.018	0.009						
17	-0.012	-0.051	-0.022	-0.028	0.020						
18	-0.024	-0.018	-0.004	-0.015	0.010						
19	-0.034	-0.018	-0.025	-0.026	0.008						
20	-0.032	-0.029	-0.025	-0.029	0.004						
21	-0.023	-0.044	-0.043	-0.037	0.012						
22	-0.026	-0.042	-0.042	-0.037	0.009						
23	-0.022	-0.022	-0.046	-0.030	0.014						

Table D6 (Continued)

Fractions		OD 280		Average	S.D.	OD 440	Average	S.D.	Protease activity
24	-0.020	-0.070	-0.021	-0.037	0.029				
25	-0.025	-0.010	-0.015	-0.017	0.008				
26	-0.057	-0.008	-0.032	-0.032	0.025				
27	-0.023	-0.011	-0.018	-0.017	0.006				
28	-0.015	-0.005	-0.009	-0.010	0.005				
29	-0.018	-0.031	-0.024	-0.024	0.007				
30	0.021	-0.034	-0.011	-0.008	0.028				
31	-0.027	-0.013	-0.012	-0.017	0.008				
32	-0.020	-0.025	-0.022	-0.022	0.003				
33	-0.011	-0.033	-0.034	-0.026	0.013				
34	-0.031	-0.018	-0.022	-0.024	0.007				
35	-0.020	-0.012	-0.012	-0.015	0.005				

Table D7 Strong anion exchange chromatography (Q-Sepharose) elution profile of dialyzed ASP1-13 enzyme

Fractions	OD 280			Average	S.D.	OD 440			Average	S.D.	Protease activity
1	0.095	0.112	0.105	0.104	0.009	0.113	0.114	0.114	0.114	0.001	0.005
2	0.882	0.897	0.863	0.881	0.017	0.115	0.117	0.117	0.116	0.001	0.007
3	0.093	0.097	0.120	0.103	0.015	0.114	0.116	0.117	0.116	0.002	0.007
4	-0.022	-0.018	-0.046	-0.029	0.015	0.115	0.109	0.108	0.111	0.004	0.002
5	-0.019	-0.009	-0.026	-0.018	0.009	0.111	0.108	0.112	0.110	0.002	0.001
6	-0.012	-0.024	-0.022	-0.019	0.006	0.106	0.114	0.113	0.111	0.004	0.002
7	-0.032	-0.011	-0.025	-0.023	0.011	0.116	0.117	0.116	0.116	0.001	0.007
8	-0.023	-0.012	-0.043	-0.026	0.016	0.114	0.117	0.117	0.116	0.002	0.007
9	-0.057	-0.022	-0.032	-0.037	0.018	0.115	0.116	0.117	0.116	0.001	0.007
10	0.015	0.021	0.016	0.017	0.003	0.116	0.106	0.115	0.112	0.006	0.003
11	0.043	0.046	0.054	0.048	0.006	0.117	0.117	0.116	0.117	0.001	0.008
12	-0.051	-0.018	-0.015	-0.028	0.020	0.114	0.114	0.113	0.114	0.001	0.005
13	-0.029	-0.009	-0.018	-0.019	0.010	0.114	0.117	0.119	0.117	0.003	0.008
14	-0.044	-0.024	0.021	-0.016	0.033	0.106	0.117	0.115	0.113	0.006	0.004
15	-0.008	-0.011	-0.027	-0.015	0.010	0.118	0.115	0.117	0.117	0.002	0.008
16	-0.034	-0.025	-0.020	-0.026	0.007	0.112	0.118	0.116	0.115	0.003	0.006
17	-0.017	-0.018	-0.011	-0.015	0.004	0.116	0.106	0.107	0.110	0.006	0.001
18	0.023	0.042	0.018	0.028	0.013	0.115	0.115	0.117	0.116	0.001	0.007
19	0.085	0.082	0.096	0.088	0.007	0.106	0.117	0.116	0.113	0.006	0.004
20	-0.007	-0.023	-0.003	-0.011	0.011	0.112	0.112	0.119	0.114	0.004	0.005
21	-0.015	-0.005	-0.009	-0.010	0.005	0.115	0.107	0.110	0.111	0.004	0.002
22	-0.018	-0.031	-0.024	-0.024	0.007	0.114	0.106	0.111	0.110	0.004	0.001

Table D7 (Continued)

Fractions		OD 280		Average	S.D.	OD 440	Average	S.D.	Protease activity
23	-0.023	-0.011	-0.018	-0.017	0.006				
24	-0.020	-0.070	-0.021	-0.037	0.029				
25	-0.012	-0.007	-0.020	-0.013	0.007				
26	0.010	0.006	-0.017	0.000	0.015				
27	-0.011	-0.003	0.018	0.001	0.015				
28	-0.020	-0.033	-0.034	-0.029	0.008				
29	-0.025	-0.018	-0.022	-0.022	0.004				
30	0.021	-0.034	-0.011	-0.008	0.028				
31	-0.027	-0.013	-0.012	-0.017	0.008				
32	-0.011	-0.031	-0.022	-0.021	0.010				
33	-0.018	-0.022	-0.033	-0.024	0.008				
34	-0.018	-0.011	-0.003	-0.011	0.008				
35	-0.020	-0.012	-0.012	-0.015	0.005				

Table D8 Strong anion exchange chromatography (Q-Sepharose) elution profile of strong cation exchange chromatography sample

Fractions		OD 280		Average	S.D.		OD 440		Average	S.D.	Protease activity
1	0.055	0.050	0.071	0.059	0.011	0.113	0.114	0.114	0.114	0.001	0.002
2	0.641	0.608	0.683	0.644	0.038	0.115	0.117	0.117	0.116	0.001	0.004
3	0.093	0.097	0.120	0.103	0.015	0.114	0.116	0.117	0.116	0.002	0.004
4	-0.022	-0.018	-0.046	-0.029	0.015	0.115	0.109	0.108	0.111	0.004	-0.001
5	-0.019	-0.009	-0.026	-0.018	0.009	0.111	0.108	0.112	0.110	0.002	-0.002
6	-0.012	-0.024	-0.022	-0.019	0.006	0.106	0.114	0.113	0.111	0.004	-0.001
7	-0.032	-0.011	-0.025	-0.023	0.011	0.116	0.117	0.116	0.116	0.001	0.004
8	-0.023	-0.012	-0.043	-0.026	0.016	0.114	0.117	0.117	0.116	0.002	0.004
9	-0.057	-0.022	-0.032	-0.037	0.018	0.115	0.116	0.117	0.116	0.001	0.004
10	-0.031	-0.029	-0.009	-0.023	0.012	0.116	0.106	0.115	0.112	0.006	0.000
11	0.043	-0.044	-0.018	-0.006	0.045	0.117	0.117	0.116	0.117	0.001	0.005
12	-0.051	-0.018	-0.015	-0.028	0.020	0.114	0.114	0.113	0.114	0.001	0.002
13	-0.029	-0.009	-0.018	-0.019	0.010	0.114	0.117	0.119	0.117	0.003	0.005
14	-0.044	-0.024	0.021	-0.016	0.033	0.106	0.117	0.115	0.113	0.006	0.001
15	-0.008	-0.011	-0.027	-0.015	0.010	0.118	0.115	0.117	0.117	0.002	0.005
16	-0.034	-0.003	-0.020	-0.019	0.016	0.112	0.118	0.116	0.115	0.003	0.003
17	-0.017	-0.033	-0.011	-0.020	0.011	0.116	0.106	0.107	0.110	0.006	-0.002
18	-0.044	-0.022	-0.027	-0.031	0.012	0.115	0.115	0.117	0.116	0.001	0.004
19	-0.008	0.001	-0.020	-0.009	0.011	0.106	0.117	0.116	0.113	0.006	0.001
20	-0.007	-0.023	-0.003	-0.011	0.011	0.112	0.112	0.119	0.114	0.004	0.002
21	-0.015	-0.005	-0.009	-0.010	0.005	0.115	0.107	0.110	0.111	0.004	-0.001
22	-0.018	-0.031	-0.024	-0.024	0.007	0.114	0.106	0.111	0.110	0.004	-0.002

Table D8 (Continued)

Fractions		OD 280		Average	S.D.		OD 440		Average	S.D.	Protease activity
23	-0.023	-0.011	-0.018	-0.017	0.006	0.118	0.106	0.113	0.112	0.006	0.000
24	-0.020	-0.070	-0.021	-0.037	0.029	0.112	0.117	0.116	0.115	0.003	0.003
25	-0.012	-0.007	-0.020	-0.013	0.007	0.115	0.114	0.117	0.115	0.002	0.003
26	0.010	0.006	-0.017	0.000	0.015	0.118	0.117	0.117	0.117	0.001	0.005
27	-0.011	-0.003	0.018	0.001	0.015	0.106	0.117	0.108	0.110	0.006	-0.002
28	-0.020	-0.033	-0.034	-0.029	0.008	0.115	0.117	0.114	0.115	0.002	0.003
29	-0.025	-0.018	-0.022	-0.022	0.004	0.118	0.115	0.116	0.116	0.002	0.004
30	0.021	-0.034	-0.011	-0.008	0.028	0.106	0.118	0.119	0.114	0.007	0.002
31	-0.027	-0.013	-0.012	-0.017	0.008	0.106	0.115	0.117	0.113	0.006	0.001
32	-0.011	-0.031	-0.022	-0.021	0.010	0.117	0.116	0.116	0.116	0.001	0.004
33	-0.018	-0.022	-0.033	-0.024	0.008	0.114	0.113	0.106	0.111	0.004	-0.001
34	-0.018	-0.011	-0.003	-0.011	0.008	0.116	0.117	0.116	0.116	0.001	0.004
35	-0.020	-0.012	-0.012	-0.015	0.005	0.114	0.114	0.117	0.115	0.002	0.003

Table D9 Strong hydrophobic exchange chromatography (Sep-Pak® C18) elution profile of strong cation exchange chromatography sample

Fractions		OD 280		Average	S.D.		OD 440		Average	S.D.	Protease activity
1	-0.005	0.001	-0.019	-0.008	0.010	0.182	0.188	0.185	0.185	0.003	0.033
2	-0.010	-0.011	-0.002	-0.008	0.005	0.190	0.190	0.190	0.190	0.000	0.038
3	-0.004	0.003	0.001	0.000	0.004	0.190	0.188	0.190	0.189	0.001	0.037
4	0.006	0.014	0.008	0.009	0.004	0.187	0.182	0.178	0.182	0.004	0.030
5	0.021	0.021	0.038	0.027	0.010	0.173	0.180	0.182	0.178	0.005	0.026
6	0.025	0.018	0.037	0.027	0.010	0.178	0.183	0.188	0.183	0.005	0.031
7	0.027	0.018	0.025	0.023	0.005	0.181	0.183	0.183	0.182	0.001	0.030
8	0.017	0.014	0.009	0.013	0.004	0.188	0.187	0.189	0.188	0.001	0.036
9	0.013	0.016	0.023	0.017	0.005	0.183	0.182	0.179	0.181	0.002	0.029
10	-0.008	0.001	0.003	-0.001	0.006	0.174	0.187	0.179	0.180	0.007	0.028
11	0.037	0.029	0.038	0.035	0.005	0.172	0.184	0.178	0.178	0.006	0.026
12	-0.011	0.001	-0.007	-0.006	0.006	0.175	0.177	0.173	0.175	0.002	0.023
13	0.019	0.008	0.015	0.014	0.006	0.176	0.180	0.174	0.177	0.003	0.025
14	-0.005	-0.010	-0.017	-0.011	0.006	0.175	0.183	0.177	0.178	0.004	0.026
15	-0.002	-0.003	0.004	0.000	0.004	0.179	0.181	0.179	0.180	0.001	0.027
16	0.035	0.038	0.031	0.035	0.004	0.177	0.180	0.177	0.178	0.002	0.026
17	0.028	0.023	0.014	0.022	0.007	0.170	0.180	0.177	0.176	0.005	0.024
18	-0.024	-0.019	-0.011	-0.018	0.007	0.188	0.177	0.183	0.183	0.006	0.031
19	0.024	0.004	0.010	0.013	0.010	0.175	0.177	0.189	0.181	0.008	0.029
20	-0.009	-0.001	0.000	-0.003	0.005	0.176	0.180	0.179	0.178	0.002	0.026
21	0.009	0.014	0.020	0.014	0.006	0.175	0.183	0.179	0.179	0.004	0.027

Table D9 (Continued)

Fractions		OD 280		Average	S.D.		OD 440		Average	S.D.	Protease activity
22	0.212	0.199	0.237	0.216	0.019	0.179	0.181	0.178	0.179	0.002	0.027
23	0.244	0.242	0.218	0.235	0.014	0.177	0.180	0.173	0.177	0.004	0.025
24	0.215	0.245	0.237	0.232	0.016	0.175	0.177	0.173	0.175	0.002	0.023
25	0.116	0.100	0.104	0.107	0.008	0.176	0.180	0.174	0.177	0.003	0.025
26	0.125	0.158	0.134	0.139	0.017	0.180	0.182	0.177	0.180	0.002	0.028
27	0.185	0.162	0.184	0.177	0.013	0.183	0.188	0.172	0.181	0.008	0.029
28	0.388	0.357	0.374	0.373	0.016	0.188	0.183	0.175	0.182	0.006	0.030
29	0.335	0.310	0.324	0.323	0.013	0.190	0.189	0.183	0.188	0.004	0.036
30	0.294	0.309	0.300	0.301	0.008	0.182	0.179	0.183	0.181	0.002	0.029
31	0.221	0.247	0.236	0.235	0.013	0.187	0.179	0.189	0.185	0.006	0.033
32	0.584	0.563	0.537	0.561	0.024	0.190	0.190	0.179	0.187	0.007	0.034
33	0.234	0.310	0.286	0.277	0.039	0.190	0.188	0.179	0.186	0.006	0.034
34	0.583	0.542	0.578	0.568	0.022	0.187	0.182	0.178	0.182	0.004	0.030
35	0.221	0.243	0.254	0.239	0.017	0.173	0.180	0.173	0.175	0.004	0.023
36	0.155	0.168	0.124	0.149	0.023	0.178	0.183	0.190	0.184	0.006	0.032
37	0.124	0.154	0.153	0.144	0.017	0.181	0.183	0.173	0.179	0.005	0.027
38	0.024	0.034	0.027	0.028	0.005	0.182	0.176	0.190	0.183	0.007	0.031
39	0.007	-0.005	-0.001	0.000	0.006	0.188	0.175	0.190	0.185	0.008	0.032
40	-0.017	-0.013	0.001	-0.010	0.009	0.190	0.182	0.183	0.185	0.005	0.033

Table D10 Weak hydrophobic exchange chromatography (Sep-Pak® C8) elution profile of strong cation exchange chromatography sample

Fractions		OD 280		Average	S.D.		OD 440		Average	S.D.	Protease activity
1	-0.017	-0.013	0.001	-0.010	0.009	0.043	0.063	0.043	0.050	0.011	0.005
2	0.079	0.050	0.059	0.063	0.015	0.042	0.065	0.043	0.050	0.013	0.005
3	0.574	0.552	0.581	0.569	0.015	0.044	0.064	0.045	0.051	0.012	0.006
4	0.738	0.763	0.770	0.757	0.017	0.044	0.045	0.046	0.045	0.001	0.000
5	0.174	0.158	0.167	0.166	0.008	0.045	0.045	0.045	0.045	0.000	0.000
6	0.167	0.155	0.152	0.158	0.008	0.064	0.065	0.045	0.058	0.011	0.013
7	0.080	0.090	0.102	0.091	0.011	0.045	0.064	0.064	0.058	0.011	0.013
8	0.020	0.038	0.059	0.039	0.020	0.043	0.044	0.046	0.044	0.001	-0.001
9	0.114	0.113	0.119	0.115	0.003	0.047	0.045	0.046	0.046	0.001	0.001
10	0.024	0.041	0.032	0.032	0.009	0.045	0.045	0.052	0.047	0.004	0.002
11	0.809	0.762	0.791	0.787	0.024	0.044	0.046	0.046	0.045	0.001	0.000
12	0.018	0.013	0.002	0.011	0.008	0.045	0.046	0.046	0.045	0.001	0.000
13	0.047	0.031	0.043	0.040	0.008	0.045	0.047	0.046	0.046	0.001	0.001
14	1.352	1.342	1.424	1.373	0.045	0.045	0.047	0.047	0.046	0.001	0.001
15	-0.008	0.000	0.002	-0.002	0.005	0.046	0.046	0.046	0.046	0.000	0.001
16	0.044	0.026	0.039	0.036	0.009	0.046	0.047	0.046	0.046	0.001	0.001
17	0.581	0.561	0.598	0.580	0.019	0.046	0.047	0.046	0.046	0.001	0.001
18	0.700	0.693	0.712	0.702	0.010	0.046	0.045	0.046	0.046	0.000	0.001
19	-0.012	-0.055	-0.039	-0.035	0.022	0.050	0.046	0.047	0.047	0.002	0.002
20	1.363	1.362	1.158	1.294	0.118	0.046	0.045	0.046	0.046	0.000	0.001
21	0.030	0.084	0.027	0.047	0.032	0.046	0.047	0.046	0.046	0.000	0.001
22	-0.022	0.001	-0.007	-0.009	0.012	0.046	0.045	0.046	0.046	0.000	0.001

Table D10 (Continued)

Fractions		OD 280		Average	S.D.		OD 440		Average	S.D.	Protease activity
23	0.253	0.251	0.287	0.264	0.020	0.048	0.047	0.047	0.047	0.000	0.002
24	0.034	0.042	0.051	0.042	0.009	0.049	0.047	0.048	0.048	0.001	0.003
25	0.473	0.481	0.500	0.485	0.014	0.046	0.046	0.045	0.046	0.000	0.001
26	0.028	0.019	0.005	0.017	0.012	0.045	0.045	0.045	0.045	0.000	0.000
27	-0.003	-0.005	-0.001	-0.003	0.002	0.045	0.045	0.045	0.045	0.000	0.000
28	-0.006	0.003	-0.010	-0.004	0.007	0.045	0.046	0.046	0.046	0.000	0.001
29	-0.010	-0.017	-0.004	-0.010	0.007	0.046	0.045	0.046	0.046	0.000	0.001
30	-0.003	0.004	-0.001	0.000	0.004	0.046	0.046	0.047	0.046	0.001	0.001
31	-0.005	-0.008	-0.010	-0.008	0.003	0.046	0.048	0.045	0.046	0.001	0.001
32	-0.017	-0.003	-0.007	-0.009	0.007	0.048	0.046	0.049	0.047	0.001	0.002
33	0.001	0.003	0.010	0.005	0.005	0.042	0.045	0.044	0.044	0.001	-0.001
34	-0.034	0.009	0.001	-0.008	0.023	0.043	0.064	0.043	0.050	0.012	0.005
35	-0.106	-0.055	-0.042	-0.068	0.034	0.042	0.066	0.043	0.050	0.014	0.005
36	0.005	0.004	0.003	0.004	0.001	0.044	0.045	0.044	0.044	0.000	-0.001
37	-0.016	-0.008	0.028	0.001	0.023	0.046	0.044	0.044	0.044	0.001	-0.001
38	-0.023	-0.003	-0.003	-0.010	0.012	0.046	0.046	0.047	0.046	0.001	0.001
39	-0.037	-0.003	0.005	-0.012	0.022	0.046	0.064	0.046	0.052	0.011	0.007
40	-0.031	-0.005	-0.001	-0.012	0.016	0.045	0.046	0.046	0.045	0.001	0.000
41	-0.021	-0.016	-0.010	-0.016	0.006	0.043	0.045	0.046	0.045	0.002	0.000
42	-0.001	-0.023	-0.004	-0.009	0.012	0.044	0.047	0.046	0.046	0.001	0.001

Table D11 Gel filtration exchange chromatography (Superose 12 10/300 GL) elution profile of strong cation exchange chromatography sample

Fractions	UV (mAU)		OD 440		Average	S.D.	Protease activity
1	0.438	0.044	0.044	0.044	0.044	0.000	0.001
2	28.965	0.045	0.044	0.044	0.044	0.000	0.001
3	34.557	0.044	0.044	0.044	0.044	0.000	0.001
4	42.517	0.044	0.044	0.044	0.044	0.000	0.001
5	47.971	0.043	0.044	0.043	0.043	0.000	0.000
6	50.661	0.044	0.043	0.043	0.043	0.000	0.000
7	51.815	0.044	0.043	0.043	0.043	0.000	0.000
8	52.535	0.043	0.044	0.043	0.043	0.000	0.000
9	51.239	0.043	0.043	0.043	0.043	0.000	0.000
10	48.11	0.044	0.044	0.044	0.044	0.000	0.001
11	44.727	0.043	0.043	0.043	0.043	0.000	0.000
12	39.936	0.043	0.046	0.043	0.044	0.001	0.001
13	32.76	0.052	0.046	0.052	0.050	0.004	0.007
14	24.09	0.053	0.046	0.053	0.051	0.004	0.008
15	16.862	0.053	0.046	0.053	0.050	0.004	0.007
16	7.159	0.054	0.046	0.053	0.051	0.004	0.008
17	-5.747	0.053	0.046	0.043	0.047	0.005	0.004
18	-19.498	0.052	0.052	0.052	0.052	0.000	0.009
19	-30.501	0.054	0.044	0.053	0.050	0.006	0.007
20	-42.335	0.052	0.043	0.052	0.049	0.005	0.006

Table D11 (Continued)

Fractions	UV (mAU)		OD 440		Average	S.D.	Protease activity
21	-56.407	0.052	0.044	0.043	0.046	0.005	0.003
22	-79.643	0.053	0.053	0.043	0.050	0.006	0.007
23	-94.357	0.052	0.051	0.043	0.049	0.005	0.006
24	-109.637	0.046	0.053	0.044	0.047	0.005	0.004
25	-123.4	0.043	0.043	0.053	0.046	0.006	0.003
26	-136.796	0.044	0.043	0.053	0.047	0.005	0.004
27	-145.024	0.053	0.052	0.053	0.053	0.001	0.010
28	-158.33	0.043	0.043	0.043	0.043	0.000	0.000
29	-174.139	0.052	0.052	0.052	0.052	0.000	0.009
30	-186.334	0.044	0.046	0.047	0.046	0.002	0.003

Table D12 Effect of pH on the activity of ASP1-13 enzyme

Samples	pH		OD 440		Average	S.D.	Protease activity	Relative activity (%)	S.D.
	3	0.042	0.041	0.041	0.041	0.000	0.001	7	0
	4	0.042	0.041	0.041	0.041	0.000	0.002	7	0
	5	0.196	0.193	0.196	0.195	0.001	0.150	32	0
	6	0.382	0.384	0.387	0.384	0.003	0.342	63	0
	7	0.499	0.516	0.520	0.512	0.011	0.468	85	2
ASP1-13	8	0.570	0.578	0.575	0.574	0.004	0.529	94	1
	9	0.613	0.611	0.617	0.614	0.003	0.570	100	0
	10	0.558	0.556	0.537	0.550	0.012	0.507	87	2
	11	0.541	0.566	0.572	0.560	0.017	0.515	93	3
	12	0.478	0.486	0.483	0.482	0.004	0.439	79	1
	13	0.094	0.093	0.094	0.094	0.001	0.046	15	0
	3	0.040	0.040	0.040	0.040	0.000			
	4	0.039	0.039	0.039	0.039	0.000			
	5	0.046	0.046	0.045	0.045	0.000			
	6	0.043	0.042	0.042	0.042	0.000			
	7	0.044	0.043	0.043	0.043	0.000			
Blank	8	0.045	0.045	0.045	0.045	0.000			
	9	0.044	0.044	0.044	0.044	0.000			
	10	0.044	0.044	0.044	0.044	0.000			
	11	0.045	0.045	0.045	0.045	0.000			
	12	0.044	0.044	0.044	0.044	0.000			
	13	0.047	0.048	0.048	0.048	0.000			

Table D12 (Continued)

Samples	pH	OD 440			Average	S.D.	Protease activity	Relative activity (%)	S.D.
	3	0.045	0.045	0.045	0.045	0.000	0.002	1	0
	4	0.099	0.101	0.100	0.100	0.001	0.057	13	0
	5	0.427	0.424	0.428	0.426	0.002	0.384	86	0
	6	0.425	0.422	0.421	0.423	0.002	0.379	85	0
	7	0.454	0.448	0.452	0.451	0.003	0.409	92	1
ASP1-13	8	0.458	0.451	0.455	0.455	0.003	0.412	92	1
	9	0.459	0.458	0.460	0.459	0.001	0.415	93	0
	10	0.477	0.469	0.521	0.489	0.028	0.446	100	6
	11	0.464	0.464	0.457	0.462	0.004	0.418	94	1
	12	0.460	0.463	0.466	0.463	0.003	0.419	94	1
	13	0.323	0.335	0.336	0.331	0.007	0.288	65	2
	3	0.043	0.043	0.043	0.043	0.000			
	4	0.043	0.044	0.043	0.043	0.000			
	5	0.042	0.042	0.043	0.042	0.000			
	6	0.044	0.043	0.044	0.044	0.000			
	7	0.042	0.043	0.042	0.043	0.000			
Blank	8	0.043	0.043	0.043	0.043	0.000			
	9	0.044	0.044	0.044	0.044	0.000			
	10	0.043	0.043	0.044	0.043	0.000			
	11	0.044	0.043	0.044	0.044	0.001			
	12	0.044	0.043	0.043	0.043	0.000			
	13	0.043	0.043	0.044	0.043	0.001			

Table D13 Effect of temperature on the activity of ASP1-13 enzyme

Samples	Temperatures (°C)	OD 440			Average	S.D.	Protease activity	Relative activity (%)	S.D.
ASP1-13	30	0.172	0.174	0.177	0.174	0.002	0.309	51	1
	35	0.217	0.222	0.223	0.221	0.003	0.401	66	1
	40	0.264	0.270	0.267	0.267	0.003	0.483	80	1
	45	0.275	0.281	0.279	0.278	0.003	0.515	85	1
	50	0.315	0.320	0.323	0.319	0.004	0.599	99	1
	60	0.323	0.328	0.317	0.323	0.006	0.604	100	2
	70	0.250	0.256	0.256	0.254	0.003	0.466	77	1
	80	0.180	0.181	0.182	0.181	0.001	0.319	53	0
Blank	30	0.040	0.040	0.040	0.040	0.000			
	35	0.042	0.040	0.040	0.041	0.001			
	40	0.052	0.052	0.051	0.052	0.000			
	45	0.041	0.041	0.041	0.041	0.000			
	50	0.040	0.040	0.041	0.040	0.000			
	60	0.041	0.042	0.041	0.041	0.000			
	70	0.042	0.042	0.042	0.042	0.000			
	80	0.042	0.043	0.043	0.043	0.000			

Table D14 Effect of temperature on the stability of ASP1-13 enzyme

Samples	Temperatures (°C)	OD 440			Average	S.D.	Protease activity	Relative activity (%)	S.D.
ASP1-13	30	0.384	0.392	0.389	0.388	0.004	0.344	84	1
	35	0.457	0.455	0.456	0.456	0.001	0.412	100	0
	40	0.382	0.386	0.388	0.385	0.003	0.341	83	1
	45	0.274	0.277	0.276	0.276	0.002	0.232	56	0
	50	0.062	0.062	0.062	0.062	0.000	0.018	4	0
	60	0.053	0.050	0.050	0.051	0.002	0.007	2	0
	70	0.044	0.045	0.045	0.045	0.000	0.001	0	0
	80	0.044	0.044	0.045	0.044	0.001	0.000	0	0
Blank		0.044	0.044	0.044	0.044	0.000			

Table D15 Effect of protease inhibitor on ASP1-13 enzyme

Samples	Reagents	OD 440			Average	S.D.	Protease activity	Relative activity (%)	S.D.
ASP1-13	10 mM PMSF	0.448	0.447	0.448	0.447	0.000	-0.008	-4	0.23
	10 mM EDTA	0.576	0.571	0.580	0.576	0.005	0.084	40	2.33
	10 mM Pepstatin	0.637	0.666	0.668	0.657	0.017	0.194	93	8.30
	10 mM IAA	0.666	0.659	0.663	0.663	0.003	0.213	106	1.60
	Control	**0.663**	**0.664**	**0.675**	**0.668**	**0.007**	**0.201**	**100**	**3.13**
Blank	10 mM PMSF	0.450	0.457	0.458	0.455	0.004			
	10 mM EDTA	0.478	0.498	0.500	0.492	0.012			
	10 mM Pepstatin	0.462	0.460	0.468	0.463	0.004			
	10 mM IAA	0.447	0.450	0.454	0.450	0.004			
	Control	**0.461**	**0.464**	**0.476**	**0.467**	**0.008**			

Table D16 Effect of surfactant, reducing and oxidant agent on ASP1-13 enzyme

Samples	Reagents		OD 440		Average	S.D.	Protease activity	Relative activity (%)	S.D.
	10 mM DTT	0.708	0.707	0.717	0.711	0.006	0.567	126	3
	5% CTAB	0.535	0.541	0.550	0.542	0.008	0.409	104	2
	5%Tween 20	0.149	0.148	0.150	0.149	0.001	0.004	1	0
ASP1-13	5% Triton X100	0.228	0.229	0.233	0.230	0.003	0.110	28	1
	0.5% SDS	0.301	0.301	0.302	0.302	0.001	0.158	40	0
	1% H2O2	0.519	0.525	0.526	0.523	0.004	0.351	89	1
	Control	**0.6264**	**0.6187**	**0.6272**	**0.624**	**0.005**	**0.394**	**100**	**1**
	10 mM DTT	0.447	0.445	0.449	0.447	0.002			
	5% CTAB	0.133	0.132	0.134	0.133	0.001			
	5%Tween 20	0.144	0.148	0.143	0.145	0.002			
Blank	0.5% SDS	0.145	0.143	0.143	0.143	0.001			
	5% Triton X100	0.118	0.120	0.121	0.120	0.001			
	1% H2O2	0.172	0.173	0.171	0.172	0.001			
	Control	**0.164**	**0.1649**	**0.165**	**0.165**	**0.001**			

Table D17 Effect of methanol and ethanol on ASP1-13 enzyme

Organic solvents	Samples	Concentration (%)	OD 440			Average	S.D.	Protease activity	Relative activity (%)	S.D.
Methanol	ASP1-13	5	0.220	0.221	0.224	0.222	0.002	0.171	104	1.25
		10	0.223	0.227	0.227	0.226	0.003	0.182	111	1.65
		20	0.223	0.225	0.228	0.225	0.003	0.178	108	1.73
		40	0.264	0.268	0.267	0.266	0.002	0.221	135	1.08
		80	0.058	0.058	0.058	0.058	0.000	0.011	6	0.28
	Blank	5	0.051	0.051	0.050	0.051	0.001			
		10	0.043	0.043	0.044	0.043	0.000			
		20	0.047	0.047	0.047	0.047	0.000			
		40	0.045	0.045	0.044	0.045	0.000			
		80	0.047	0.048	0.047	0.047	0.000			
Ethanol	ASP1-13	5	0.249	0.248	0.262	0.253	0.008	0.208	126	4.93
		10	0.235	0.235	0.237	0.236	0.001	0.193	117	0.72
		20	0.223	0.220	0.225	0.222	0.002	0.178	108	1.38
		40	0.155	0.157	0.160	0.158	0.003	0.114	69	1.56
		80	0.076	0.075	0.076	0.076	0.001	0.025	14	0.40
	Blank	5	0.045	0.045	0.046	0.045	0.000			
		10	0.043	0.043	0.043	0.043	0.000			
		20	0.045	0.044	0.045	0.045	0.000			
		40	0.043	0.044	0.043	0.043	0.000			
		80	0.050	0.052	0.052	0.051	0.001			
Control			**0.517**	**0.524**	**0.530**	**0.524**	**0.006**	**0.164**	**100**	**5.77**

Table D18 Effect of acetonitrile and iso-propanal on ASP1-13 enzyme

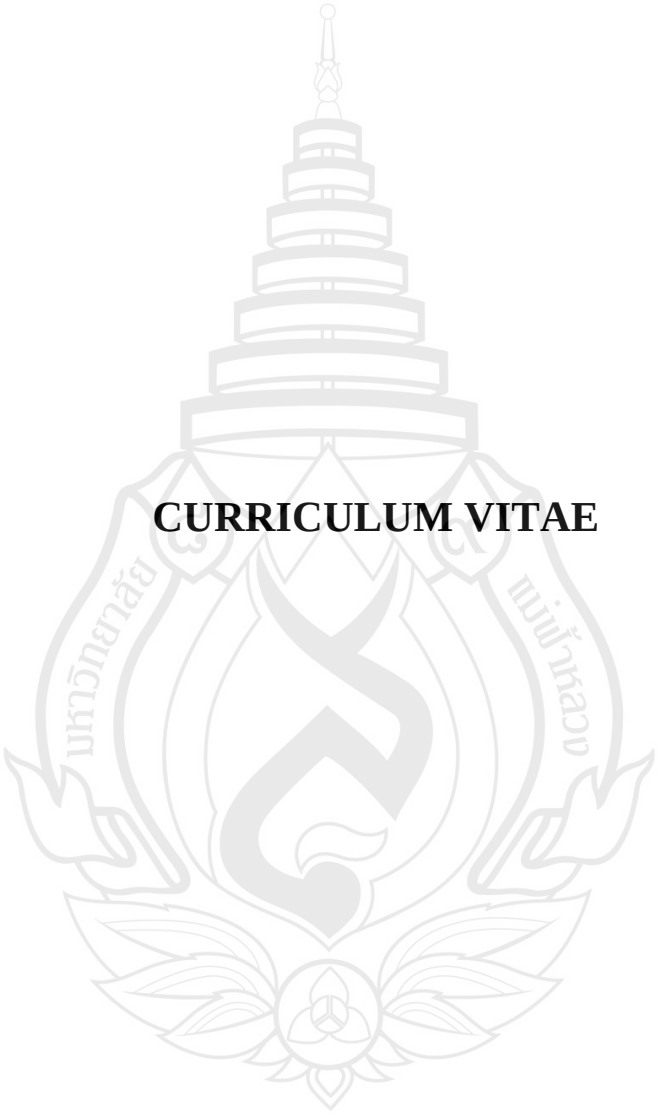
Organic solvents	Samples	Concentration (%)		OD 440		Average	S.D.	Protease activity	Relative activity (%)	S.D.
Acetonitrile	ASP1-13	5	0.268	0.269	0.279	0.272	0.006	0.225	137	3.76
		10	0.327	0.303	0.307	0.312	0.013	0.265	162	7.88
		20	0.304	0.310	0.321	0.312	0.008	0.261	160	5.14
		40	0.265	0.262	0.263	0.263	0.001	0.209	128	0.86
		80	0.088	0.088	0.089	0.088	0.000	0.020	12	0.19
	Blank	5	0.045	0.051	0.046	0.047	0.003			
		10	0.047	0.047	0.047	0.047	0.000			
		20	0.050	0.049	0.050	0.050	0.001			
		40	0.054	0.053	0.054	0.054	0.001			
		80	0.068	0.068	0.069	0.069	0.000			
Iso-propanal	ASP1-13	5	0.241	0.239	0.241	0.241	0.001	0.195	119	0.66
		10	0.254	0.256	0.256	0.255	0.001	0.211	129	0.70
		20	0.237	0.248	0.245	0.243	0.005	0.198	121	3.32
		40	0.149	0.148	0.152	0.150	0.002	0.097	59	1.42
		80	0.063	0.063	0.063	0.063	0.000	0.009	5	0.12
	Blank	5	0.046	0.046	0.045	0.045	0.000			
		10	0.044	0.044	0.044	0.044	0.000			
		20	0.046	0.045	0.046	0.046	0.001			
		40	0.053	0.053	0.053	0.053	0.000			
		80	0.054	0.055	0.054	0.054	0.000			
	Control		**0.517**	**0.524**	**0.530**	**0.524**	**0.006**	**0.164**	**100**	**5.77**

Table D19 Effect of acetone on ASP1-13 enzyme

Organic solvents	Samples	Concentration (%)	OD 440			Average	S.D.	Protease activity	Relative activity (%)	S.D.
Acetone	ASP1-13	5	0.253	0.253	0.252	0.253	0.001	0.210	128	0.32
		10	0.256	0.254	0.257	0.256	0.001	0.213	130	0.86
		20	0.237	0.237	0.239	0.238	0.001	0.193	118	0.83
		40	0.131	0.136	0.137	0.135	0.003	0.086	53	1.80
		80	0.055	0.055	0.056	0.055	0.000	0.004	2	0.13
	Blank	5	0.043	0.043	0.043	0.043	0.000			
		10	0.043	0.043	0.043	0.043	0.000			
		20	0.045	0.045	0.045	0.045	0.000			
		40	0.049	0.048	0.049	0.049	0.000			
		80	0.052	0.052	0.051	0.052	0.000			
	Control		**0.517**	**0.524**	**0.530**	**0.524**	**0.006**	**0.164**	**100**	**5.77**

Table D20 Effect of metal ion on ASP1-13 enzyme

Sample	Metal ion		OD 440		Average	S.D.	Protease activity	Relative activity (%)	S.D.
ASP1-13	10 mM Ca^{2+}	0.319	0.318	0.331	0.323	0.007	0.278	100	3
	10 mM Cu^{2+}	0.224	0.222	0.228	0.225	0.003	0.178	64	1
	10 mM Fe^{2+}	0.258	0.261	0.265	0.261	0.004	0.179	64	1
	10 mM k^{+}	0.336	0.350	0.355	0.347	0.010	0.300	108	4
	10 mM Li^{+}	0.341	0.345	0.354	0.347	0.006	0.300	108	2
	10 mM Mg^{2+}	0.349	0.355	0.357	0.353	0.004	0.309	111	2
	10 mM Mn^{2+}	0.440	0.459	0.464	0.454	0.013	0.262	94	5
	10 mM Na^{+}	0.335	0.346	0.352	0.344	0.009	0.297	107	3
	10 mM Zn^{2+}	0.333	0.333	0.342	0.336	0.005	0.291	105	2
Control		**0.316**	**0.329**	**0.328**	**0.324**	**0.007**	**0.278**	**100**	**3**
Blank	10 mM Ca^{2+}	0.047	0.043	0.044	0.045	0.002			
	10 mM Cu^{2+}	0.047	0.047	0.047	0.047	0.000			
	10 mM Fe^{2+}	0.081	0.083	0.083	0.082	0.001			
	10 mM k^{+}	0.047	0.047	0.047	0.047	0.000			
	10 mM Li^{+}	0.046	0.046	0.046	0.046	0.000			
	10 mM Mg^{2+}	0.044	0.044	0.045	0.045	0.000			
	10 mM Mn^{2+}	0.198	0.179	0.202	0.193	0.012			
	10 mM Na^{+}	0.046	0.046	0.049	0.047	0.002			
	10 mM Zn^{2+}	0.044	0.044	0.045	0.044	0.000			
Control		**0.046**	**0.046**	**0.046**	**0.046**	**0.000**			



CURRICULUM VITAE

CURRICULUM VITAE

NAME Mr. Yodying Yingchutrakul

DATE OF BIRTH 27 April 1988

ADDRESS 68 Tunvera Rd, Betong District,
Yala Province, Thailand, 95110

EDUCATIONAL BACKGROUND

2010 Bachelor of Science
Biotechnology, Mae Fah Luang University

WORK EXPERIENCE

2013 Research assistant in preparation of bioactive thermostable peptides from defatted rice bran project (ARDA)

PUBLICATION

Y., Yingchutrakul, K., Saharat, N., Daechacupt, S., Kittisenachai, A., Paemanee, E., Chukeatirote, S., Roytrakul, T., Waratrujiwong. (2011). Identification of protease enzyme from *Bacillus* sp. strain S1-13. Paper presented at The 6th international symposium of the protein society of Thailand, Thailand. 141-145.