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PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE PROTEASE

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บทคัดย่อ

ชัยสิทธิ์ สิทธิเวช: การทำให้บริสุทธิ์และสมบัติบางประการของโปรตีเอสที่เสถียรต่ออุณหภูมิสูง (PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE PROTEASE) อ. ที่ปรึกษา: รศ. นภา ศิวรังสรรค์, อ. ที่ปรึกษาร่วม:, 95 หน้า. ISBN 974-03-0901-1.

แบคทีเรียที่สามารถผลิตโปรตีเอสที่เสถียรต่ออุณหภูมิสูงซึ่งถูกแยกจากดินบริเวณน้ำพุร้อนจาก อำเภอห้วยทรายขาว จังหวัดเชียงราย ได้ถูกนำมาจำแนกสายพันธุ์โดยวิธีการทดสอบสมบัติทางชีวเคมีและการเปรียบเทียบลำดับดีเอ็นเอบริเวณ 16S rRNA gene พบว่าเป็น *Bacillus mycoides* จากการหาภาวะที่เหมาะสมในการวัดแอคติวิตีของเอนไซม์ พบว่าภาวะที่เอนไซม์มีแอคติวิตีสูงสุดคือ 37°C,pH 8.0 (10mM Tris-HCl buffer) เมื่อทำการหาภาวะที่เหมาะสมในการเลี้ยงแบคทีเรียชนิดนี้ในขวดเขย่าขนาด 1 ลิตร โดยใช้ปริมาณอาหารเหลว 300 มล. เพื่อผลิตเอนไซม์พบว่าภาวะที่เหมาะสมคือ การเลี้ยงที่อุณหภูมิ 37°C pH 8.0 ในอาหารสูตรเปปโตนซึ่งใช้เชื้อตั้งต้น 1.0% โดยมีทริปโตนเป็นแหล่งไนโตรเจนและมีปริมาณไนโตรเจนในอาหาร1.4% โปรตีเอสที่เสถียรต่ออุณหภูมิสูงมีการทำงานสูงสุดที่ 60°C และ pH 8.0 ใน 10 mM Tris-HCl buffer

การแยกโปรตีเอสให้บริสุทธิ์ ทำได้โดยตกตะกอนโปรตีนโดยการเติมอะซีโตนลงในน้ำเลี้ยงเชื้อให้มีความเข้มข้นสุทธิ 50% หลังจากนั้นจึงนำโปรตีนที่ได้ไปผ่านการแยกโดย Sephadex G-75 column chromatography ซึ่งได้ผลิตผลโปรตีเอส 0.46% และมีแอคติวิตีจำเพาะ 30.97 units/mg protein ผลการทดลองจาก SDS-PAGE และ Sephadex G-75 column chromatography แสดงว่าน้ำหนักโมเลกุลของโปรตีเอสที่เสถียรต่ออุณหภูมิสูงเท่ากับ 27,600 Da การสูญเสียแอคติวิตีของเอนไซม์โดย PMSF และ EDTA แสดงว่ากรดอะมิโนซีรีนมีความสำคัญในบริเวณเร่งของเอนไซม์และโลหะหนักมีความสำคัญต่อการทำงานของโปรตีเอส การทดสอบความจำเพาะของโปรตีเอสที่เสถียรต่ออุณหภูมิสูงต่อสารตั้งต้น คือ เคซีนและเอโซเคซีน พบว่าค่า Km = 125.00 mg/ml และ 26.13 mg/ ml ตามลำดับ และค่า V_{max} =3.8 Units/mg protein และ 17.00 Units/mg protein ตามลำดับ การประยุกต์ของโปรตีเอสนี้ในการย่อยของเสียที่ได้จากโรงงานผลิตปลาทูน่ากระป๋องนั้นพบว่าโปรตีเอสสามารถย่อยของเสียทั้งสามชนิดได้ดีโดยเฉพาะอย่างยิ่ง ไส้ปลากับเลือดปลาซึ่งพบว่าปริมาณไนโตรเจนเพิ่มขึ้นสูงที่สุด 3.9%

ภาควิชา ชีวเคมี
สาขาวิชา ชีวเคมี
ปีการศึกษา 2544

ลายมือชื่อนิสิต .....
ลายมือชื่ออาจารย์ที่ปรึกษา .....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

ABSTRACT

4272255923 : MAJOR BIOCHEMISTRY

KEY WORD: Thermostable protease / Protease / *Bacillus mycoides* / Purification / Characterization

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Bacteria produced thermostable protease were isolated from soil of hot spring at Amphor Hui Sai Kwao, Chiang Rai province. It was identified by biochemical tests and 16S rRNA gene sequence as *Bacillus mycoides*. The protease production was conducted on optimum condition which consisted of peptone medium at initial pH 8.0, inoculum size of 1.0%, initial nitrogen content of 1.4%, at 37°C and peptone was replaced by tryptone as a nitrogen source. This thermostable protease had optimum activity at 60°C and pH 8.0 in 10mM Tris –HCl buffer.

The thermostable protease was purified by 50% acetone precipitation and Sephadex G-75 column chromatography with 0.46% yield and specific activity of 30.97 units/mg protein. The results from SDS-PAGE and Sephadex G-75 column chromatography indicated that the molecular mass of thermostable protease was 27,600 Da. The loss of activity in the presence of PMSF and EDTA indicated the significant of serine in active site of protease and metal ion had an important on protease activity. Thermostable protease exhibited substrate specificity with the K_m on casein and azocasein 125.00 mg/ml and 26.13 mg/ml while the V_{max} on casein and azocasein were 3.80 Units/mg protein and 17.00 Units/mg protien. The application of this protease towards protein wastes from tuna canned industry was studied. The highest digested product with 3.9% nitrogen content was obtained by protease digestion of the mixture of viscera and blood.

Department/Program Biochemistry Student 's

Field of study Biochemistry Advisor's signature…….. .....

Academic year 2001 Co-advisor's signature……. .....

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ABBREVIATIONS

A	Absorbance
BSA	Bovine serum albumin
cm	centrimeter
^{o}C	Degree Celsius
g	gram
hr	hour
l	liter
min	minute
μl	microlitre
ml	mililitre
mM	milimolar
M	Molar
rpm	Revolution per minute

CHAPTER I

INTRODUCTION

Proteases are proteolytic enzymes catalyzing the cleavage of peptide bonds in proteins. Advances in analytical techniques have demonstrated that proteases conducted highly specific and selective modifications of proteins such as activation of zymogenic form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, including processing and transportation of secretory proteins across the membranes.

The current estimated value for the worldwide sales of industrial enzymes was $1 billion (Godfrey, 1996). Of the industrial enzymes, 75% were hydrolytic enzymes. Proteases were one of the three largest groups of industrial enzymes and accounted for about 60% of the total worldwide sale of enzymes. Distribution of enzyme sales were shown in figure 1.

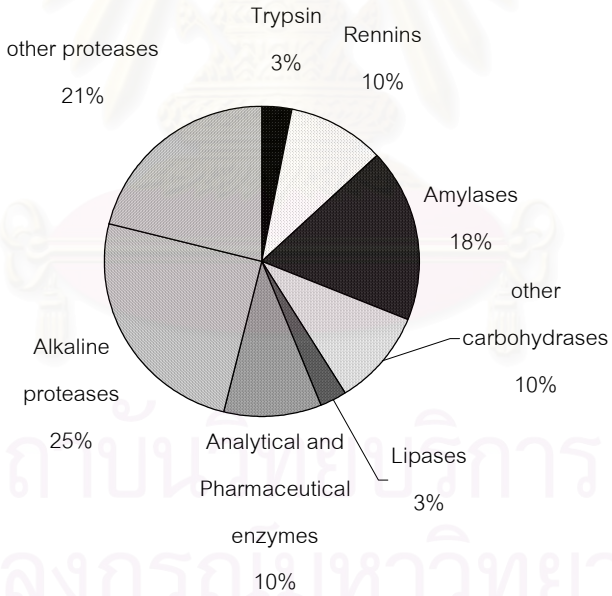

Figure 1.Distribution of enzyme sales. The contribution of different enzymes to the total sale of enzymes were indicated. The shaded portion indicated the total sale of proteases.

1.1 Sources of protease

Since proteases were physiologically necessary for living organisms, they were ubiquitous, and found in a wide diversity of sources such as plants, animals, and microorganisms.

1.1.1 Plant protease

The use of plants as a source of proteases were governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants was a time - consuming process. Papain, bromelain, keratinases, and ficin represented some of well – known proteases of plant origin.

1.1.2 Animal protease

The most familiar proteases of animal origin were pancreatic trypsin, chymotrypsin, pepsin, and rennin (Boyer, 1971). These were prepared in pure form in bulk quantities. However, their productions depended on the availability and agricultural politics.

1.1.3 Microbial protease

The inability of the plant and animal proteases to meet current world demands had led to an increased interest in microbial proteases. Microorganisms represented an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases accounted an approximately 40% of the total worldwide enzyme sales. Protease from microbial source were the most popular among the enzyme from plant and animal sources since they possessed almost all the characteristics desire for their biotechnological applications.

1.2 Classification of protease

According to the nomenclature committee of the International union of biochemistry and molecular biology, proteases were classified in subgroup 4 of group 3 (hydrolase). However, proteases did not comply easily with the general system of enzyme

nomenclature due to their huge diversity of action and structure. Currently, proteases were classified on the base of three major criteria: (1) type of reaction catalyzed, (2) chemical nature of the catalytic site, and (3) evolutionary relationship with reference to structure (Barett, 1994).

Proteases were grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their sites of action. Exopeptidase cleaved the peptide bond proximal to the amino or carboxy termini of the substrate whereas endopeptidase cleaved peptide bonds distant from the termini of the substrate. Base on the functional group presented at the active site, protease were further classified into four prominent groups, i.e,. serine proteases, aspartic proteases, cystein proteases which did not precisely fit into standard classification, e.g., ATP – dependent proteases which required ATP for activity (Menon,1987). Based on their amino acid sequences, protease were classified into different families (Argos, 1987) and further subdivided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Saiga, 1993). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine,cysteine,aspatic,metallo-,or unknown type, respectively.

1.2.1 **Exopeptidase** The exopeptidases acted only near the ends of polypeptide chains. Based on their sites of action at the N or C terminus, they were classified as amino- and carboxypeptidases, respectively.

Aminopeptidase acted at a free N terminus of the polypeptide chain and liberated a single amino acid residue, a dipeptide, or a tripeptide.

Carboxypeptidase acted at C terminals of the polypeptide chain and liberated a single amino acid or a dipeptide. Carboxypeptidase could be divided into three major groups, serine carboxypeptidase, metallocarboxypeptidase, and cysteine carboxypeptidase, based on the nature of the amino acid residues at the active site of the enzyme.

1.2.2 Endopeptidase Endopeptidases were characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group had a negative influence on enzyme activity. The endopeptidases were divided into four sub groups based on their catalytic mechanism, (i) serine proteases, (ii) aspatic proteases, (iii) cysteine proteases,and (iv) metalloproteases. To facilitate quick and unambigous reference to a particular family of peptidases, they have assigned a code letter denoting the catalytic type, i.e., S, A ,C or M, respectively and followed by an arbitrarily assigned number(Rawling,1993).

1.2.2.1 Serine protease Serine protease was characterized by the presence of a serine group in its active site. It was numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that it was vital to the organisms. Serine proteases was found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Base on its structural similarities, serine protease has been grouped into 20 families, and further subdivided into about six clans with common ancestors (Barett, 1994)

1.2.2.1.1 Serine alkaline protease. Servaral bacteria, molds, yeasts, and fungi produced serine alkaline proteases. DFP or a potato protease inhibitor inhibited them but not by tosyl – L – phenylalanine chloromethyl ketone (TPCK) or L-1-Chloro –3- [4-tosyl-amido]-7- amino-2-heptanone-HCl (TLCK). Their substrate specificity were similar to but less stringent than that of chymotrypsin. They hydrolyzed a peptide bond, which consisted of tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimal pH of alkaline protease were around pH 10, and their isoelectric point were around pH 9. Their molecular masses were in the range of 15 to 30 kDa. Although alkaline serine proteases were produced by several bacteria such as *Arthobacter*, *Streptomyces*, and *Flavobacterium* spp. (Bogslawski, 1983), subtilisins produced by *Bacillus* spp. were the best known. Alkaline proteases were also produced by *S. cerevisiae* (Mizuno, 1984) and filamentous fungi such as *Conidiobolus* spp. (Phadatare, 1993), *Aspergillus* and *Neurospora* spp. (Lindberg, 1984).

1.2.2.1.2 Subtilisin. Subtilisins of *Bacillus* origin represented the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Calsberg and subtilisin Novo or bacterial protease Nagase (BPN'), have been identified. Linderstrom, Lang and Ottesen discovered subtilisin Carlsberg produced by *Bacillus licheniformis* in 1947 at the Carlsberg laboratory. Subtilisin Novo or BPN' was produced by *Bacillus amyloliquefaciens*. Subtilisin Carlsberg was widely used in detergents. Its annual production amounts to about 500 tons of pure enzyme protein. Subtilisin BPN' was less commercially important. Both subtilisins had a molecular mass of 27.5 kDa but differed from each other by 58 amino acids. They had similar properties such as an optimal temperature of 60°C and an optimal pH of 10. Both enzymes exhibited a broad substrate specificity and had an active site triad made up of Ser 211, His 64and Asp32. The Carlsberg enzyme had broader substrate specificity and does not depended on Ca^{2+} for its stability. The active site conformation of subtilisin was similar to that of trypsin and chymotrypsin despite the dissimilarity in their over all molecular arrangements. The serine alkaline protease from the fungus *Conidiobolus coronaus* was shown to possess a distinctly different structure from subtilisin Carlsberg in spite of their functional similarities (Phadatare, 1997).

1.2.2.2 Aspartic protease. Aspartic proteases, commonly known as acidic proteases, were the endopeptidases that depended on aspatic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin(A1), retropepsin(A2), and enzymes from pararetroviruses (A3) (Barett, 1995) and have been placed in clan AA. The members of families A1 and A2 were known to be related to each other, while those of family A3 showed some maximal activity at low pH (pH 3 to 4) and had isoelectric points in the range of 30 to 45 kDa. The members of the pepsin family had a biglobal structure with the active site cleft located between the lobes (Sielecki, 1991). The active site aspatic acid residue was situated within the motif Asp- Xaa-Gly, in which Xaa could be Ser or Thr. The aspatic proteases were inhibited by pepstain (Fitzgerald, 1990). They were also sensitive to diazo-ketone compounds such as diazoacetyl – DL- norleucine methyl ester (DAN) and

1,2 epoxy-3-(p-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibited specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which were similar to pepsin, but their action were less stringent than that of pepsin. Microbial aspartic proteases could be broadly divided into two groups, (I) pepsin – like enzymes produced by *Aspergilus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin- like enzymes produced by *Endothia* and *Mucor* spp.

1.2.2.3 Cysteine / Thiol proteases. Cysteine proteases occurred in both prokaryotes and eukaryotes. About 20 families of cysteines proteases have been recognized. The activity of all cysteine protease depended on catalytic dyad consisting of cysteine and histidine. The order of Cys and His (Cys – His or His – Cys) residues differed among the families (Barett, 1994). Generally, cysteine proteases were active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificities, they were broadly divided into four groups: (I) papain – like, (ii) trypsin – like with preference for clevage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain was the best known cysteine protease. They were susceptible to sufhydryl agents such as pCMB but are unaffected by DFP and metal – chelating agent. Clostripain, produced by the anaerobic bacterium *Clostridium histolyticum*, exhibited a stringent specificity for arginyl residues at the carboxyl side of the splitting bond and differed from papain in its obligate requirement for calcium. Sreptopain, the cysteine protease produced *Streptococcus* spp., showed a broader specificity, including oxidized insulin B chain and other synthetic substrates. Clostripain had an isoelectic point of pH 4.9 and a molecular mass of 50 kDa, whereas the isoelectric point and molecular mass of streptopain were pH 8.4 and 32 kDa, respectively.

1.2.2.4. Metalloproteases. Metalloproteases were the most diverse of the catalytic types of proteases (Barett, 1995). They were characterized by the requirement for a divalent metal ion for their activity. They included enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Shanon, 1989) . About 30 families of metalloprotease have been recognized, of which 17 contained only endopeptidases, 12

contained only exopeptidases, and 1 (M3) contained both endo- and exopeptidases. Families of metalloproteases have been grouped into completes clans based on the nature of the amino acid that completed the metal – binding site. For example, clan MA had the sequence His-Glu-X-X-His-Glu and clan MB corresponded to the motif His-Glu-X-X-His-His. In one of the groups, the metal atom bound at a motif other than the usual motif.

Base on the specificity of their actions, metalloproteases could be divided into four groups, (I) neutral, (ii) alkaline, (iii) Myxobacter I, and (iv) Myxobacter II. The neutral proteases showed specificity for hydrophobic amino acids, while the alkaline proteases possessed a very broad specificity. Myxobacter protease I was specific for small amino acid residues on either side of the cleavage bond, whereas Myxobacter protease II was specific for lysine residue on the amino side of the peptide bond. Chelating agent such as EDTA inhibited all of them.

Thermolysin, a neutral protease, was the most thoroughly characterized member of clan M.A.. Histidine residues from the His-Glu-X-X-His motif served as Zn ligands, and Glu had a catalytic function (Weaver, 1977). Thermolysin produced by *B.stearothermophilus* was a single peptide without disulfide bridges and had a molecular mass of 34 kDa. It contained an essential Zn atom embedded in a cleft formed between two folded lobes of the protein and four Ca atoms, which imparted thermostability to the protein. Thermolysin was a very stable protease, with a half – life of 1 hour at 80°C.

Collagenase, another important metalloprotease, was first discovered in the broth of the anaerobic bacterium *Clostridium hystolyticum* as a component of toxic product. Later, it was found to be produced by the aerobic bacterium *Achromobacter tophagus* and other microorganisms including fungi. The action of collagenase was very specific, i.e., it acted only on collagen, gelatin and not any of the other usual protein substrates. Elastase produced by *Pseudomonas aeruginosa* was another important member of the neutral metalloprotease family.

The alkaline metalloprotease produced by *Psudomonas aeruginosa* and *Seratia* spp. were active in the pH range from 7 to 9 and had molecular masses in the region of 48 to 60 kDa. *Myxobacter* proteases I had a pH optimum of 9.0 and a molecular mass of 14 kDa and cell walls of *Arthrobacter crystellopoites*, whereas Myxobacter protease II could not lyse the bacterial cells. Matrix metalloproteases played a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing and might be useful in the treatment of diseases such as cancer and arthritis.

In summary, proteases were broadly classified as endo- or exoenzymes on the basis of their sites of action on protein substrates. They are further categorized as serine proteases, aspatic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanisms. They were also classified into different families and clans depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimum activity, they were also referred to acidic, neutral, or alkaline proteases.

1.3 Applications of proteases

Proteases had a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases were envisaged to have extensive application in leather processing and several bioremediation processes. The worldwide requirement of enzymes for individual application was varied considerably. Proteases were used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases used in the food and detergent industries were prepared in bulk quantities and used as crude preparations, whereas those used in medicine were produced in small amounts but required extensive purification before use.

1.3.1 Detergents

Proteases were one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in laundry detergents accounted for approximately 25% of the total worldwide sales of enzymes. The preparation of the first enzymatic detergent, "Burnus" dated back to 1913; it consisted of sodium carbonate and a crude pancreatic extract. The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name BIO-40. In 1960, Novo industry A/S introduced alcalase, produced by *Bacillus licheniformis*; its commercial name was BIOTEX. Maxatase, a detergent made by Gist – Btoeades, was followed distribution. The biggest market for detergent was in the laundry industry, amounting to a worldwide production of 13 billion tons per year. The ideal detergent protease should possess a broad substrate specificity to facilitate the removal of a large variety of stains due to food, and other body secretions. Activity and stability at high pH and temperature and compatibility with the chelating and oxidizing agents added to the detergent were among the major prerequisites for the use of proteases in detergents. The key parameter for the best performance of a protease in a detergent was its pI. It was known that a protease was the most suitable for this application of its pI coincided with the pH of the detergent solution. Esperase and Savinase T (Novo Industry), produced by alkalophilic *Bacillus* spp., are two commercial preparations with very high isoelectric points (pI 11); hence, they could stand higher pH ranges. Due to the present energy crisis and the awareness for energy conservation, it was desirable to use proteases that were active at lower temperature. A combination of lipase, amylase and cellulase is expected to enhance the performance of protease in laundry detergents.

All detergent protease currently used in the market were serine proteases produced by *Bacillus* strains. Fungal alkaline proteases were advantageous due to the case of downstream processing to prepare a microbe – free enzyme. An alkaline proteases from *Conidiobolus coconatus* was found to be compatible with commercial

detergent used in India and retained 43% of its activity at 50°C for 50 min in the presence of Ca^{2+} (25mM) and glycine (1M) (Bhosale, 1995).

1.3.2 Leather Industry

Leather processing involved several steps such as soaking, dehairing, bating, and tanning. The major building blocks of skin and hair were proteinaceous. The conventional methods of leather processing involved hazardous chemicals such as sodium sulfide, which created problems of pollution and effluent disposal. The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and reducing environmental pollution. Proteases were used for selective hydrolysis of noncollagenous constituents of the skin and for removal of nonfibrillar proteins such as albumins and globulins. The purpose of soaking was to swell the hide. Traditionally, this step was performed with alkali. Currently, microbial alkaline proteases were used to ensure faster absorption of water and reduced the time requirement for soaking. The use of nonionic and, to some extent, anionic surfactant was compatible with the use of enzymes. The conventional method of dehairing and dewooling consisted of development of an extremely alkaline condition followed by treatment with sulfide to solubilize the proteins of the hair root. At present, alkaline proteases with hydrated lime and sodium chloride were used for dehairing, resulting in a significant reduction in the amount of wastewater. Earlier methods of bating were based on the use of animal feaces as the source of proteases; these methods were unpleasant and unreliable and replaced by methods involving pancreatic trypsin. Currently, trypsin was used in combination with other *Bacillus* and *Aspergillus* protease for bating. The selection of the enzyme depended on its specificity for matrix proteins such as elastin and keratin, and the amount of needed enzyme depended on the type of leather (soft or hard) to be produced. Increased usage of enzymes for dehairing and bating not only prevented pollution problems but also was effective in saving energy. Novo Nordisk manufactured three different proteases, Aquaderm, NUE, and Pyrase, for use in soaking, dehairing, and bating, respectively.

1.3.3 Food Industry

The use of proteases in the food industry dated back to antiquity. They have been routinely used for various purposes such as cheese making, baking preparation of soya hydrolysates, and meat tenderization.

Dairy industry. The major application of proteases in the dairy industry was in the manufacture of cheese. The milk-coagulating enzymes fell into three main categories, (I) animal rennets, (ii) microbial milk coagulants, and (iii) genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belonged to a class of acid aspatate proteases and had molecular weights between 30,000 to 40,000 Da. Rennet extracted from the fourth stomach of unveiled calves contained the highest ratio of chymosin (EC 3.4.23.4) to pepsin activity. A world shortage of calf rennet due to the increased demand for cheese production had intensified the search for alternative microbial milk coagulants. The microbial enzymes exhibited two major drawbacks, i.e., (I) the presence of high levels of nonspecific and heat – stable proteases, which led to the development of bitterness in cheese after storage; and (ii) a poor yield. Extensive research in this area has resulted in the production of enzyme that were completely inactivated at normal pasteurization temperatures and contained very low levels of nonspecific proteases. In cheese making, the primary function of proteases was to hydrolyze the specific peptide bond (the Phe 105- Met 106 bond) to generate para - ∝ casein and macropeptide. Chymosin was preferred due to its high specificity for casein, which was responsible for its excellent performance in cheese making. The proteases produced by GRAS (generally regarded as safe) – cleared microbes such as *Mucor michei*,*Bacillus subtilis*, and *Endothia parasitica* were gradually replaced chymosin in cheese making. In 1988, chymosin produced through recombinant DNA technology was first introduced to cheese makers for evaluation. General International manufactures increased the production of chymosin in *Aspergillus niger* var. *awamori* to commercial levels. At present, three recombinant chymosin products were available and awaiting legislative approval for their uses in cheese making (Godfrey, 1996)

Whey was the by- product of cheese manufacture. It contained lactose, proteins, minerals, and lactic acid. The insoluble heat- denatured whey protein was solubilized by treatment with immobilized trypsin.

Baking industry. Wheat flour was a major component of baking processes. It contained an insoluble protein called gluten, which determined the properties of the bakery doughs. Endo and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. Enzymatic treatment of the dough facilitated its handling and machining and permitted the production of a wider range of products. The addition of proteases reduced the mixing time and resulted in increased loaf volumes. Bacterial proteases were used to improve the extensibility and strength of the dough.

Manufacture of soy products. Soybeans served as a rich source of food, due to their high content of good – quality protein. Proteases have been used from ancient times to prepare soy sauce and other soy products. The alkaline and neutral proteases of fungal origin played an important role in the processing of soy sauce. Proteolytic modification of soy proteins helped improve their functional properties. Treatment of soy proteins with alcalase at pH 8 resulted in soluble hydrolysates with high solubility, good protein yield, and low bitterness. The hydrolysate was used in protein – fortified soft drinks and in the formulation of dietetic feeds.

Debittering of protein hydrolysates. Protein hydrolysates had several applications, e.g., as constituents of dietetic and health products, in infant formular and clinical nutrition supplements, and flavoring agents. The bitter taste of protein hydrolysate was a major barrier to their use in food and health care products. The intensity of the bitterness was proportional to the number of hydrophobic amino acids in the hydrolysate. The presence of a proline residue in the center of the peptide also contributed to the bitterness. The peptideses that could cleave hydrophobic amino acids and proline were valuable in debittering protein hydrolysate. Aminopeptidases from lacitic acid bacteria were available under the trade name “Debitrase”. Carboxypeptidase A had a high specificity for hydrophobic amino acids and hence had

a great potential for debittering. A careful combination of an endoprotease for the primary hydrolysis and an aminopeptidase for secondary hydrolysate showed the reduced in bitterness.

Synthesis of aspartame. The Food and Drug Administration had approved the use of aspatame as a noncalorific artificial sweetener. Aspatame was a dipeptide composed of L- aspatic acid and the methyl ester of L-phenylalanine. The L-configuration of the two amino acids was responsible for the sweet taste of aspartame. Maintenance of the sterospecificity was crucial, but it added to the cost of production by chemical methods. Enzymatic synthesis of aspatame was therefore preferred. Although proteases were generally regarded as hydrolytic enzymes, they catalyzed the reverse reaction under certain kinetically controlled conditions. An immobilized preparation of thermolysin from *Bacillus thermoprotyolyticus* was used for the enzymatic synthesis of aspatame. Toya soda (Japan) and DSM (The Netherlands) were the major industrial producers of aspartame.

1.3.4 Pharmaceutical Industry

The wide diversity and specificity of proteases were used to great advantage in developing effective therapeutic agents. Oral administration of proteases from *Aspergillus oryzae* (Luizym and Nortase) has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. Clostridial collagenase or subtilisin was used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. An asparginase isolated from *E.coli* was used to eliminate aspargine from the bloodstream in the various forms of lymphocytic leukemia. Alkaline protease from *Conidiobolus coronatus* was found to be able to replace trypsin in animal cell cultures (Chiplonkar, 1985).

1.3.5 Other Applications

Beside their industrial and medicinal applications, proteases played an importance role in basic research. Their selective peptide bond cleavage was used in the elucidation of structure function relationship, in the synthesis of peptides, and in the sequence of proteins.

In essence, the wide specificity of the hydrolytic action of protease found an extensive application in the food, detergent, leather, and pharmaceutical industries, as well as in the structural elucidation of proteins, whereas their synthetic capacities were used for the synthesis of proteins.

Protease had a large variety of applications, mainly in the detergent and food industry. These included beer chill proofing, meat tenderization, cheese manufacturing, flavor development in fermentation, baking and manufacturing of soy, dietetic, and health products. They were also used extensively in the leather and pharmaceutical industries (Rao, 1998). Thermostable proteases were advantageous in some applications of faster reaction rates, increased in the solubility of nongaseous reactants and products. Protease secreted from Thermophilic bacteria were thus of particular interest and had become increasingly useful in a range of commercial application (Wasserman, 1988).

Thermophilic bacteria, which were widespread in thermal environment, are often good sources of thermostable proteases, such as thermolysin from *Bacillus thermoproteolyticus* (Endo, 1962), aqualysin I from *Thermus aquaticus* YT-1 (Matsuzawa, 1982) and caldolysin from *Thermus aqualyticus* YT-1 (Cowan, 1972). These thermostable proteases were metalloenzymes and has been intensively characterized. To obtain additional potentially useful thermostable proteases, we are successful in isolating a number of thermophillic bacteria produced extracellular protease from a hot spring in Chiang Rai, Thailand. One of the bacterium *Bacillus mycoides*, was isolated from a hot spring and produced a thermostable protease. The purpose of this study is to purification and characterization of extracellular thermostable proteases from *B. mycoides*.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA-30, Hirayama Manufactering Cooperation, Japan

Camera: Model K100, Pentax, Asaki Opt. Co., Japan

Centrifuge: Model J2-21, Beckman Instrument Inc., U.S.A.

Electrophoresis setting: Model Minutesi VE Vertical Electrophoresis system, Pharmacia LKB, Sweden

Fraction collector: Model 2070 LKB Redifrac, Pharmacia LKB, Sweden

Freeze – dryer: Model Flexi – Dry ^{tm} up. Stone Ridge, New York, U.S.A

Orbital incubator Model Gallenkamp, Genway, England

pH meter: Model PHM 95, Radiometer, Pharmacia LKB, Sweden

Power Suply: Model POWER PAC 300, Bio-rad Applied Biosystem company, U.S.A.

Psychrotherm: Model G 760, New Brunswick Scientific Co., U.S.A.

Schimadzu UV-Visible Spectrophotometer: Model UV-240 , Schimadzu, Japan

Shaking waterbath: Model G 76, News Brunswick Scientific Co., U.S.A.

Spectrophotometer: Model 20 D, Beckman, U.S.A.

Ultrafilter: Suprec $^{Tm-01}$, pore size 0.20 μm and 0.22 μm, Takasa Shuzo Co,Ltd., Japan

2.2 Chemicals

Azocasein: Sigma Chemical Co., U.S.A.

Acetone: Merck, Germany

Casein Hammersten: BDH Laboratory Chemicals Division, England

Dialysis tubing 25 mm: Cut off 12,000 Da, Sigma, U.S.A.

Phenylmethylsulfonyl fluoride (PMSF): Sigma Chemical Co., U.S.A.

1,10-Phenanthroline: E.Merck Ag., Germany

Sephadex G-75: Pharmacia Fine Chemicals, Uppsala, Sweden

2.3 Bacteria

Bacillus mycoides, isolated from soil of hot spring water at Amphor Hui-Sai-Kwao, Chiang Rai province, were screened for thermostable and extracellular protease. The method of screening was described below.

2.4 Microorganism screening

Soils and water samples were collected from Thailand hot spring areas. Soils and water samples were collected from eighteen Amphors of 9 provinces in Thailand that had hot spring water (figure.2). Soil samples were collected from hot spring water area.

After collection, samples were saved into the plastic bottles (water samples) and plastic bag (soil samples) in 5 °C cold room. Thermostable protease was screened from 58 samples of hot spring area soil and 18 samples of water from hot spring. Soil samples were dissolved with sterile water 1:10 and suspended with vortex mixture and water samples were used directly to screening. 10μl, 100μl, and 500μl of soil samples solution were added to nutrient agar medium (Meat extract 0.5%, Peptone 1.0%, NaCl 0.5%, Agar 2.0%). Likewise 10μl, 100μl, and 500μl of water samples were also added to medium. Then the medium was incubated at 37°C for 17 hours under aerobic condition. Subsequently the microorganisms were screened for extracellular protease by toothpick technique on skim milk agar plates.

The selected colony were secondary screened by inoculum in nutrient broth medium 5 ml at 37° C, 250 rpm,for 17 hours. Twenty microliter of supernatant was pipetted into the well of skim milk agar plate to check the clear zone diameter in mm^2. Finally, large clear zone – producing microorganism were selected to determine thermostable protease activity by azocasein hydrolysis method.

Figure 2. . Map of hot spring area in Thailand were collected sites in this research.

2.4.1 Determination of enzyme activity

The protease activity was determined using azocasein as substrate compared with subtilisin A as a standard protease. The reaction mixture consisted of 900 μl of 0.1 M Tris-HCl pH 9.0, 1 ml of 0.2% azocasein, 100 μl of enzyme solution. Blank was used 0.1M Tris-HCl pH 9.0 to replace the enzyme solution. The control reaction was stopped reaction before incubation. The mixture was incubated at 60°C for 20 min and stopped reaction and then added 2 ml of cold trichloroacetic acid before centrifugation at 3,500 rpm. Supernatant was collected to measure using spectrophotometer at 440 nm for detecting azo – group of the protein digested products.

One Unit of enzyme was defined as the amount that changed the absorbance at 440 for 0.1 unit at 1 min.

2.4.2. Protein determination

The protein concentration was determined according to the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard.

The reaction mixture 1.1 ml contained 100 μl of protein solution, 100 μl Bradford working reagent (Appendix B.21) was mixed and incubated for 5 min at room temperature. The protein concentration was determined at absorbance of 595 nm using BSA as a standard protein.

2.5 Identification of screened microorganism

The screened bacteria of this study were identified by their biochemical characteristics (*Bacillus subtilis* TISTR25 was used as a standard) and 16S rRNA gene sequences .

2.5.1. Microscopic appearance.

The microscopic appearance of bacterial gram strain could be observed by gram stain method. The bacteria were streaked on nutrient agar plate for 24 hours. Single colony was selected and streaked directly on glass slide. Bacteria on the glass slide was dried with frame and stained with gram's crystal violet solution (Appendix B.1) for 1 min. Secondary stain was washed by gram's iodine solution for 1 min. After that the stained slide was washed by 95% ethanol for 20 seconds and then washed by distilled water to clean ethanol. Finally, the slide was counter stained with gram's safanin O for 30 seconds. The slide was washed by distilled water and observed under microscope to determine the gram strain. Gram positive was blue and gram negative was red.

2.5.2. Biochemical test

2.5.2.1. Production of catalase. The cultures were grown for 1 or 2 days on slants of nutrient agar and flooded with 0.5 ml of 10% hydrogen peroxide. The tube was replaced closure immediately to avoid dispersal of an aerosol and observed for gas production. Production of gas bubbles was indicated to positive test and no gas production was indicated to negative test.

2.5.2.2. Anaerobic growth. Bacteria were inoculated in thioglucolate broth and incubated at 37° C in anaerobic jar for 2 days. Positive was grown on medium while negative was not grown.

2.5.2.3. Voges- Proskauer reaction. Acethyl methyl carbinol production was assayed by inoculation tube of Voges-Proskauer broth in triplicate for 3,5 and 7 days. The 3 ml of 40% (w/v) sodium hydroxide and 0.5-1 mg of creatine were added. The positive production was observed by red color changing in solution for 1 hour at room temperature.

2.5.2.4. Carbohydrate fermentation tests. Carbohydrate fermentation tests were used to determine the ability of microorganism for digestion of specific carbohydrates incorporated in a basal medium and produced acid or acid with visible gas. The bacteria were inoculated on basal medium pH 7.4 for 7-30 days.

In this study various carbohydrates were employed including (1) glucose, (2) lactose, (3) sucrose, (4)mannitol, (5)inositol, (6)sorbitol, (7)arabinose, (8)xylose, (9)galactose, and (10)fructose, respectively. Gas bubble was observed in Durham tube. Positive result was obtained gas and acid productions that changing color of medium to yellow. Likewise negative result was obtained gas and acid production with reddish-pink color which was similar to the original medium color.

2.5.2.5. Utilization of citrate. The plate of citrate utilization medium (Appendix A.10) was inoculated and incubated for 14 days. The production of a red (alkaline) color was indicated the utilization of organic acids.

2.5.2.6. Deamination of phenylalanine. Cultures were innoculated on duplicate plates of phenylalanine agar (Appendix A.12) for 7 days, and then 4 or 5 drops of 10% (w/v) ferric chloride solution were pipetted over colonies on one of duplicate plates. The production of a green color beneath colonies was indicated formation of pyruvic acid from phenylalanine during growth. If the test was negative and then the second culture was repeated after 21 days of incubation to ensure the result.

2.5.2.7. Egg-yolk lecithinase. Culture was inoculated into an egg-yolk broth and a tube of control broth was not consisted of egg-yolk. The incubation was observed on 1,3,5and 14 days for the appearance of a heavy white precipitate in or on the surface of the egg-yolk containing medium.

2.5.2.8. Reduction of nitrate to nitrite. The plate of potassium nitrate medium (Appendix A.14) was inoculated and incubated for 7 days. After incubation, reagent A: 1.0 ml (Appendix A.15) and reagent B: 1.0 ml (Appendix A.16) were added. After the reagent was added within 30 seconds, the sample was observed for red color appearance. If it was negative, there was no color development. Then the sample was added directly a pinch of zinc dusts (Zn) and observed for final interpretation. If red color was occurred within 30 seconds, the result denoted a negative control.

2.5.2.9. Formation of indole. The cultures were incubated for 14 days in indole production medium and added 2 ml of the following test solution: p-dimethylamine benzaldehyde 5g, iso – amyl alcohol 75 ml and concentrated hydrochloric acid 25 ml. The mixtures were shaken vigorously and observed for a pink to red color in the alcohol layer after separation. This indicated the presence of indole.

2.5.2.10. Growth at pH 6.8 and 5.7 (nutrient broth). A small loopful of a broth culture was inoculated into a tube of nutrient broth medium while control flask was uninoculated. Growth or its absence after incubation for 7-14 days should be observed.

2.5.2.11. Growth in NaCl 0%, 2%, 5%, 7%, 10%. The flasks of nutrient broth (100 ml/flask) were composed of 0,2,5,7 and 10% (w/v) sodium chloride with a small loop full of a culture grown in nutrient broth. The growths were observed in various concentrations of sodium chloride after 7 and 14 days incubation.

2.5.2.12. Growth at 30°C, 37°C, 40°C, 50°C, 60°C, 70°C. The plates of nutrient agar were inoculated at 30°C, 37°C, 40°C, 50°C, 60°C and 70°C for 3-7 days. The growth in the various temperatures were observed after 24 hours to 7 days incubation.

2.5.2.13. Hydrolysis of starch. The duplicate plates of starch agar were incubated at 37°C. At 3 and at 5 days, the plates were flooded with 5% iodine. After 15-30 min the starch become dark blue after 15-30 min. The clear zone underneath was observed around the growth as an indicator of starch hydrolysis.

2.5.2.14. Decomposition of casein. The plates of milk agar (Appendix A. 3) were incubated with one spot of inoculum and examined at 37°C for 24 hours, 7 days and 14 days. The clear zone on the plate was observed around and below the growth.

2.5.3. 16S rRNA gene sequence comparison.

10 μg of bacterial DNA was subjected to PCR in a total volume of 100μl, with 2.5 units of Taq polymerase, 50 mM KCl, 10mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 100 picomoles of each of the two primers, 200 μM of each dNTP (dATP, dCTP,dGTP, dTTP). The thermal profile involved 36 cycles of denaturation at 95°C for 1 min, primer annealing at 45°C for 2 min., and extension at 72°C for 2 min.

The PCR product of 1,500 bp fragment was separated by agarose gel electrophoresis and recovered by Quigen kit. For sequencing, DNA template (10 pmoles) was annealed to 2.0 pmoles of the sequencing primer (pA,pH,pD). The DNA template was used to DNA sequencing by dideoxy nucleotide method.

The following oligonucleotides were used in the PCR and for sequencing system.

pA : AGA GTT TGA TCC TGG CTC AG

pD : CAG CAG CCG CGG TAA TAC

pH : AAG GAG GTG ATC CAG CCG CA

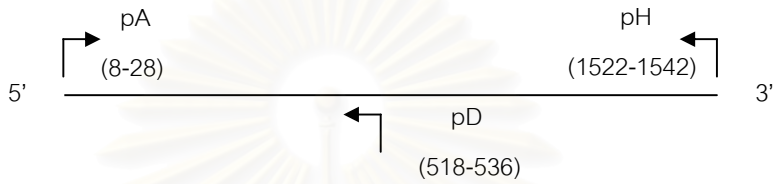

Figure 3. Sequencing map of the primer used with respect to their target sites in the 16S rRNA gene. The nucleotide positions of the synthetic DNA oligomers were corresponded to, *E.coli* 16S rRNA gene as shown in brackets.

2.6 Optimization of protease production

2.6.1. Preparation of starter

A colony of *Bacillus mycoides* from slant agar was grown in 100 ml of starter peptone medium pH 7.0, at 37°C with 250 rpm for 24 hours. After cultivation for 24 hours 1 ml of starter was monitered absorbance at 600 nm in the range of absorbance 0.3-0.7 as a control of cell number.

2.6.2. Comparison between peptone medium (Raja, 1994) and yeast extract medium (Kasem, 1987)

Starter 1 ml was pipetted into 300 ml/flask of each culture at 37°C for 24 hours. The initial pH of the cultivation medium pH 7.0 was performed in 1000 ml conical flask at a horizontal shaker (250rpm) in 300 ml medium. Growth pattern of *Bacillus mycoides* was determined by collection 1 ml of each medium and measured of absorbance at 600 nm every 5 hours. Activity of protease was monitered after centrifugation culture medium. The supernatant was assayed by azocasein hydrolysis method.

2.6.3. Determination on optimum inoculum size

Starter 0.5,1,1.5,2,2.5 and 3 ml were pipetted into 300 ml/flask peptone medium. The conditions of cultivation were described in section 2.5.2.

2.6.4. Determination on optimum temperature of cultivation

From the result of a suitable inoculum size, this experiment was used 1 ml of starter to inoculate peptone medium. The initial pH of the medium was pH 7. Each flask was shaken at 30°C, 37°C, 40°C, 50°C, and 60°C. The growth pattern was monitored as described in section 2.5.2.

2.6.5. Determination on optimum pH of cultivation

From the result of the optimum temperature, this experiment was performed by cultivation at 37°C. The initial pHs of the medium were pH 5,6,7,8,9,10 and 11.

2.6.6. Determination on optimum glucose concentration

The data of Raja (1994) used peptone in medium as a nitrogen source. To improve the production of protease, culture medium were changed various kind of nitrogen sources (peptone, tryptone, yeast extract, beef extract, $(NH_4)_2SO_4$) but the percentage of nitrogen content were analyzed and equilibrated nitrogen content in each nitrogen source into 0.7%.The percentage of nitrogen were analyzed by using Kjeldahl method.

2.6.7. Determination on optimum nitrogen source

The data of Raja(1994) used peptone 1% in the medium as a nitrogen source. To improve the activity production of protease, various nitrogen sources such as peptone, tryptone, yeast extract, beef extract, $(NH_4)_2SO_4$ were analyzed and determined nitrogen content. Then percentages of nitrogen content in each nitrogen source was analyzed by using Kjeldahl method.

2.6.8. Determination on optimum percentage of nitrogen content

The percentage of nitrogen content was analyzed by using Kjeldahl method. Culture mediums were varied percentage of nitrogen content into 0.7,1.4 and 2.8.

2.6.9. Determination on optimum agitation rate

In order to determine a suitable agitation rate, each flask was shaken at 200, 250,and 300 rpm. The growth pattern and enzyme activities were determined at various times as described in section 2.6.2.

2.7 Analysis of nitrogen contents by Kjeldahl method.

This method was used a modification of the Furman's method (1962). The other forms of nitrogen were converted to ammonia and produced as ammonium sulfate by digestion with sulfuric acid. The ammonia was liberated by the addition of sodium hydroxide solution, then distilled and trapped in standard hydrochloric acid. The excess hydrochloric acid was estimated titrimetrically with a standard sodium hydroxide solution.

0.3 g of material was weighed into Kjeldahl digestion flask. 1 ml of concentrated sulfuric acid was added 0.6 ml of a catalyst, and digested in a digestion oven. The heats was raised slowly and boil vigorously. The material has been digested until a clear straw yellow or light green color solution appeared and then stopped the heat. If the mixtures were not clear for a long time, then they were reduced heat. 2 to 3 drops of 30% hydrogen peroxide were carefully added and then continued heating for 5-10 minutes.

After cool the mixture, the tube were distilled by automatic machine. The program of distillation were added 40 ml water, 20 ml of 40% NaOH and distilled by incorporation vapor of boiling water. The reservoir flask were added 1-2 drops of mixing indicators and 10 ml of 10% boric acid. Ammonia from distillation was trapped by boric acid. The solutions were changed from purple to green. The samples were titrated with standard 0.01 N sulfuric acid solution.

$$\%Nitrogen = \frac{(EQ1 - B)xFxF\,2x\,100}{w}$$

EQ1 = Tritration volume of 0.01 N sulfuric acid of sample

B = Tritration volume of 0.01 N sulfuric acid of blank

F = Exactly concentration of standard sulfuric acid

F2 = 0.014

W = Weigh of sample

2.8 Purification of protease

2.8.1. Preparation of protease from *Bacillus mycoides*

A small loop full of a broth culture was inoculated into a flask of 100 ml peptone medium to prepare a starter. The flask was shaken 250 rpm for 24 hours 37°C and measured absorbance of medium with spectrophotometer at 600 nm in the range of absorbance 0.3 -0.7 as a control of cell number. If the absorbance was over 0.7, the stock medium was diluted with aseptic medium into 0.3-0.7.

1 ml of starter was pipetted into 300 ml peptone medium. To prepare 3000 ml of supernatant, 10 flasks of mediums were used. The flasks (1000ml) of peptone medium were shaken at 250 rpm for 24 hours at 37°C. The medium was centrifuged 10,000 rpm to collect supernatant and was stored in the medium at 5°C.

2.8.2. Ultrafiltration of protease.

The ultrafilration was used to concentrate enzyme before next purification step. The filter paper with molecular weight cut off 10,000 Da was used. The nitrogen gas was flown to repel the filtrate out. When the sample level decreased into 300ml, the system was stopped.

2.8.3. Precipitation of protease by acetone.

Organic solvent such as acetone and ethanol was added to precipitate protein from the solution . Concentrations of organic solvents were generally calculated in percentage of added volume in protein solution.

Cold acetone (-20°C) was gently added to 300 ml of sample solution and stirred gently. The volumes of acetone were up to the final concentration. In this study the protein was precipitated by 10%, 20%, 30%, 40%, 50%, 60%, and 70% of acetone. Each step needed to stir for 1 hours to precipitate the protein on ice. The sample was centrifuged at 10,000 rpm, 5°C for 15 minutes. The protein pellet was washed by 10mM phosphate buffer pH7.0 and collected the pellet of each fraction from acetone concentration. The pellet was resuspended by 10 mM Tris – HCl pH 8.0 and gently stirred overnight to evaporate the organic solvent. After that sample was precipitated by acetone. The fraction was further purified by gel permeable Sephadex-G75 column chromatography.

2.8.3 Sephadex gel G-75 column chromatography

Sephadex G-75 was washed thoroughly with deionization water and boiled in water bath. The water was drained and added 10mM Tris-HCl pH8.0. Then the gel was boiled in water bath again. After that the gel was packed into a glass column (1.7x90cm) and equilibrated the gel with 10mM Tris-HCl pH8.0 at a constant flow rate 10ml per 1hour in this system. Automatic fraction collector was used to collect the sample. The collection size was 2.5 ml per tube. In this study sample was eluted by 10 mM Tris-HCl pH8.0. The fractions were determined protein by measuring absorbance with spectrophotometer at 280 nm and monitoring activity by using azocasein hydrolysis method. Protease fractions were pooled and determined purity by native polyacrylamide gel electrophoresis.

2.9 Characterization of protease

2.9.1 Relative molecular mass determination

The relative molecular mass were determined by both sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration.

Gel filtration were carried out in the presence of relative molecular mass standards from Pharmacia(bovine serum albumin, ovalbumin, subtilisin A, lysozyme) in Sephadex G-75 column (1.7x90 cm) and equilibrated with 10 mM Tris –HCl buffer, pH 8.0.

SDS-PAGE was conducted with a vertical slab gel according the method of Laemmli (1970). Phosphorylase b (Mr=97,400), serum albumin (Mr=66,200), ovalbumin (Mr=45,000), carbonic anhydrase (Mr=31,000), trypsin inhibitor (Mr=21,500), lysozyme (Mr=14,400), and aprotinin (Mr=6,500) were used as molecular weight markers (Pharmacia).

2.9.2. Effect of temperature on enzyme activity

The effect of temperature on the protease activity toward azocasein was determined by incubation reaction mixture (100μl enzyme, 0.9 ml of 10mM Tris – HCl pH 8.0 , 0.02% azocasein 1 ml) at 30,37,40,50,60,70,80,90,and 100 °C for 20 min before stop reaction with 2 ml of 10% Trichloroacetic acid . Control of this assay was firstly added 10% trichloroacetic acid 2 ml. The reaction mixture was centrifuged at 3,500 rpm for 15 minutes. The supernatants were measured absorbance at 440 nm.

2.9.3. Effect of pH on enzyme activity

Effect of pH on enzyme activity was examined in the pH range 4.0-11.0. Likewise this experiment was conducted with the same reaction mixture (2.8.2.) 0.9 ml of McIvain buffer (pH=4-6),or Tris-HCl buffer (pH=6-11to replace 10 mM Tris-HCl pH 8.0 in the assayed mixture.

2.9.4. Substrate specificity

The protein substrates (azocasein and casein) were dissolved in 10 mM Tris-HCl, pH 8.0, at concentration of 10 mg, 12mg 14 mg, 16mg, 18mg and 20mg for azocasein and 50mg, 100mg, 150mg and 200mg for casein. The enzyme 100μl was added into the reaction mixture and incubated at 60°C for 20 min. The reaction was terminated with 2 ml of 10%(w/v) TCA at the end of the incubation period. The incubation periods were varied to 5,10,15,20,25,30,35,40,45,50,55,and 60min. After centrifugation, the absorbance of the supernatants were monitored.

2.9.5. Determination on a suitable storage condition.

A suitable storage condition was conducted by adding glycerol, storing with different enzyme forms and keeping in various temperatures (-20°C, 0°C, 5°C and room temperature). Crude enzyme, enzyme precipitation by acetone and purified enzyme from Sephadex G-75 column chromatography were tested by the following description.

Liquid enzyme were added 0%, 10%and 20% glycerol and stored at -20°C, 0°C, 5°C, and room temperature. 100 ml of each enzyme was lyophilized and then stored at –20°C, 0°C, 5°C and room temperature. Activity of protease was monitored every 6 hours for 1 month.

2.9.6. Hydrolysis of protein wastes from Tuna canned industry

The process of tuna canned industry had protein wastes during processing. There were three kind of the wastes, red meat (solid), drained water, viscera and blood. The mixture was blended using a blender at high speed for 1 min. 1g of red meats was adjusted volume to 10 ml by 10mM Tris-HCl pH 8.0 before blending. Drained water was two phases of solid and liquid. The solid wastes 1 g were adjusted volume to 10 ml with liquid wastes before blending. Likewise 1 g of viscera was adjusted volume to 10 ml with blood and blending.

Each protein substrate (0.9 ml) were added into the reaction mixture (0.9 ml 10mM of Tris-HCl pH 8.0, 100 μl enzyme) and incubated at 60°C for 20 min. The reaction was terminated with an equal volume of 10% (w/v) TCA at the end of the incubation period. The control of reaction was firstly stopped reaction. After centrifugation, the %N of supernatants were monitored by Kjeldahl method (Germuth, 1929).

Chapter III

RESULTS

3.1 Screening of protease producing bacteria

Bacteria were isolated from hot spring area and screened for thermostable protease producers. From 58 of soils samples and 18 of water samples were screened on bacterial agar plate as shown on figure 5. Then the colonies were picked on skim milk agar medium and the diameter of clear zone after incubation for 24 hours were measured. After that 80 isolates of bacteria that gave large diameter (larger than 225 mm^2) were inoculated on peptone broth medium and the supernatant were collected by centrifugation. 20 μl of the supernatant was dropped into the well of skim milk agar medium and incubated at 37°C for 17 hours. The clear zone production were measured (table 1.) and 12 strains of isolated bacteria were collected in order to assay for protease activity by azocasein hydrolysis at various temperatures and pHs.The optimum of assayed conditions of strain number 2 was shown in figure 8 and 9.

Figure 4. Location of the hot spring at Amphor Hui Sai Kwao, Chiang Rai province.

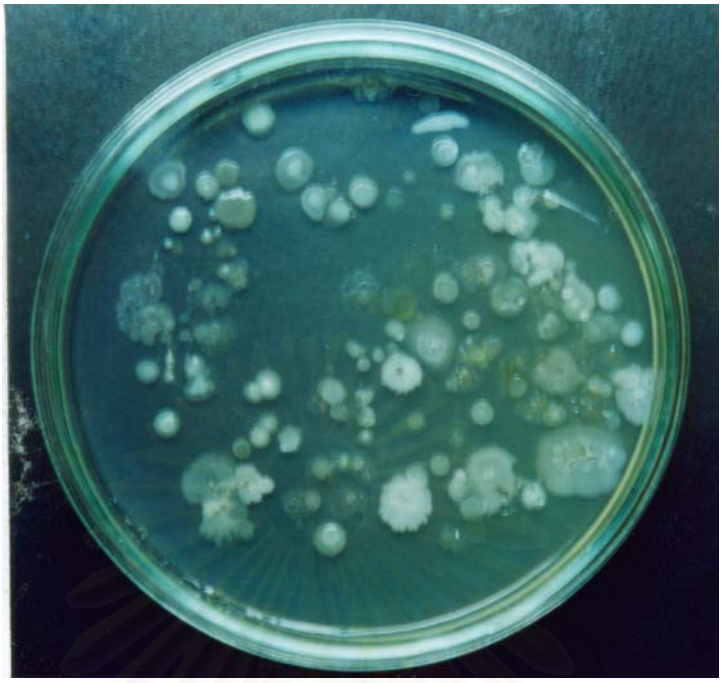

Figure 5. The bacteria on nutrient agar plate (soil sample from Hui Sai Kwao)

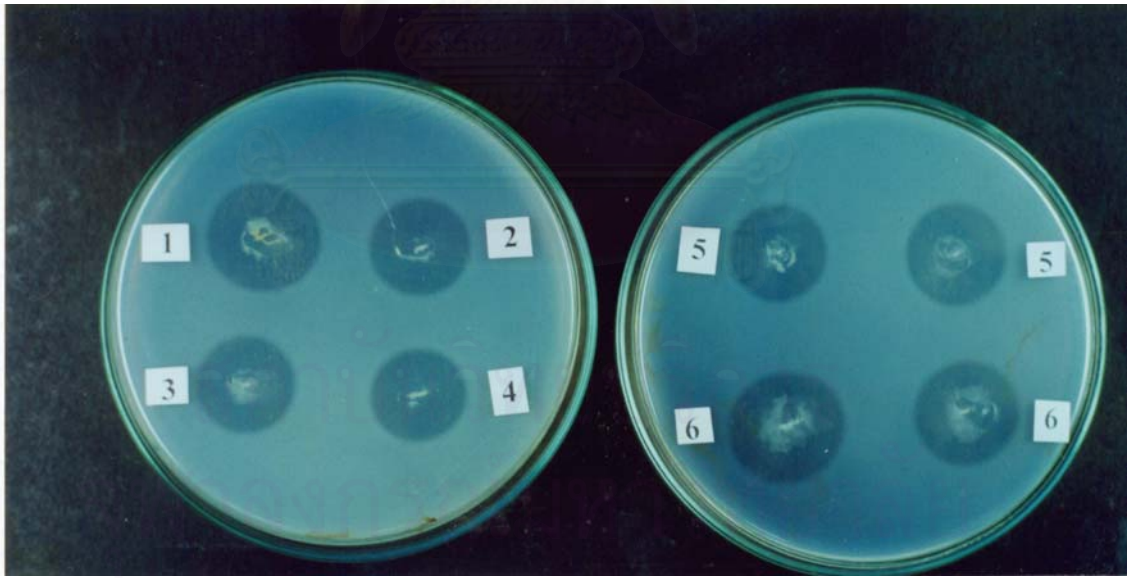

Figure 6. Clear zone of Bacterial strain No.2 by picking the colony of bacteria on skim milk agar medium.

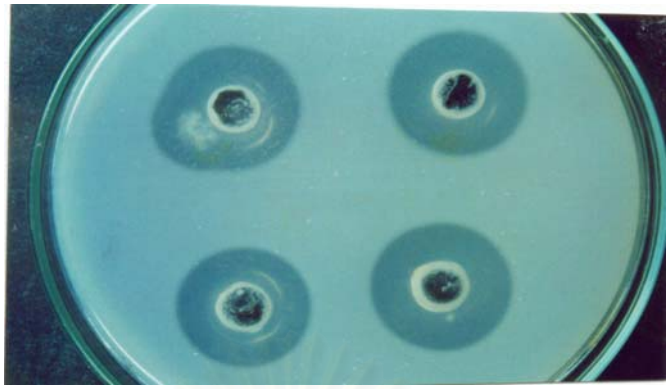

Figure 7. Clear zone of protease activity from Bacterial strain No 2. by well test.

Table 1. The clear zone diameter observed from 12 bacterial strains producing extracellular protease.

Number	Province	Ampher of sample	Clear zone diameter (mm^2)
1.	Chiang Mai	Sankampang	361
2.	Chaing Rai*	Hui Sai Kwao	484*
3.	Krabi	Hui Nam Kwao	361
4.	Krabi	Hui Nam Kwao	420
5.	Ratchaburi	Bor klung	399
6.	Ratchaburi	Bor klung	420
7.	Ratchaburi	Bor klung	380
8.	Ratchaburi	Bor klung	360
9.	Ratchaburi	(Bor Klung)	380
10.	Ratchaburi	(Bor Klung)	380
11.	Ratchaburi	(Bor Klung)	430
12.	Karnchanaburi	(Hin Dard)	420

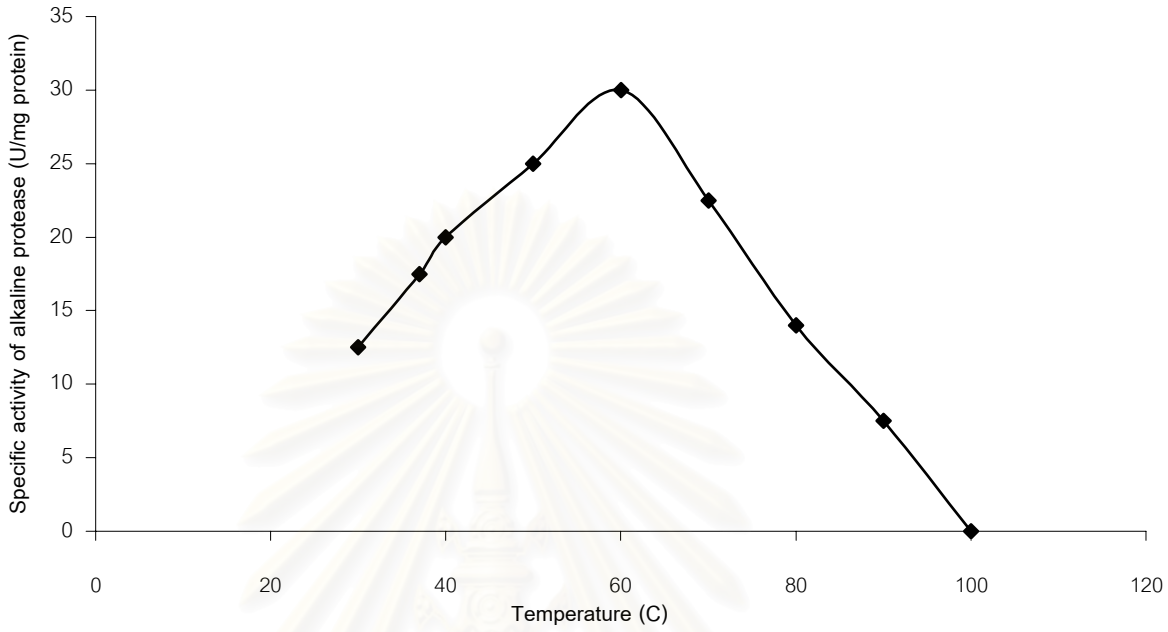

Figure 8. Optimum temperature of protease activity from bacterial strain No.2.

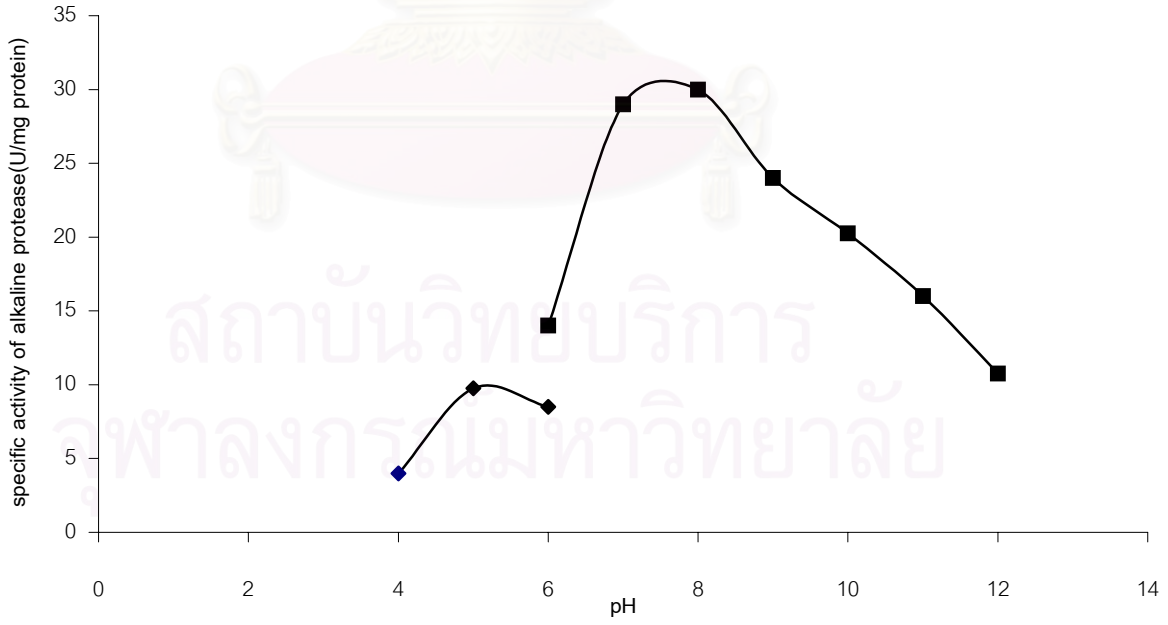

Figure 9. Optimum pH of protease activity from bacterial strain No.2

(Acetate buffer, Tris – HCl buffer)

3.2 Identification of screened microorganism

Bacterial strain No.2 was identified by using biochemical characteristics (*Bacillus subtilis* TISTR25 was used as standard) and 16s rRNA gene for strains comparison.

Firstly, the morphological characteristic of bacteria was determined using microscope. Then this bacterium was stained with gram's stain method. The microscopic image of the bacteria revealed that this bacterium was gram positive and rod shape (figure 10). To identify bacterial strain, Bergey's manual was used to be a reference method of biochemical test. The methods to determine a genus of bacteria at the first five steps (microscopic appearance and gram strain, facultative anaerobes, sulfate actively reduced to sulfide, catalase, marked acidicity from glucose) of test were conducted. The results of five tests are shown in table 2 which indicated that this bacteria ' s genus is *Bacillus* sp.

The biochemical tests was determined bacterial species. The results were shown in the table 2. The results revealed that the species of this bacterium was *Bacillus mycoides.*

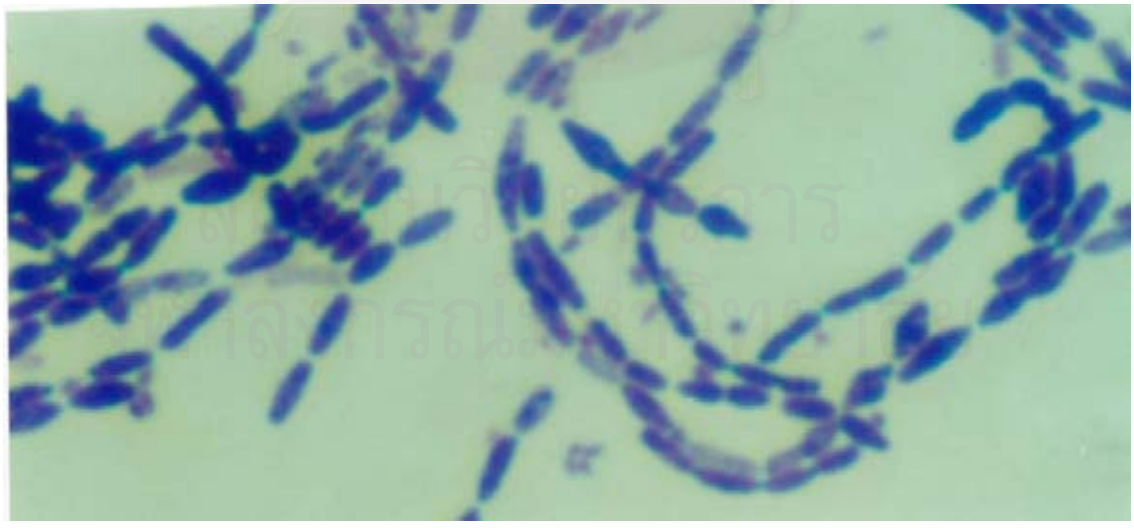

Figure 10. Morphology of bacteria strain No.2 strained by crystal violet and observed under microscopy 100X.

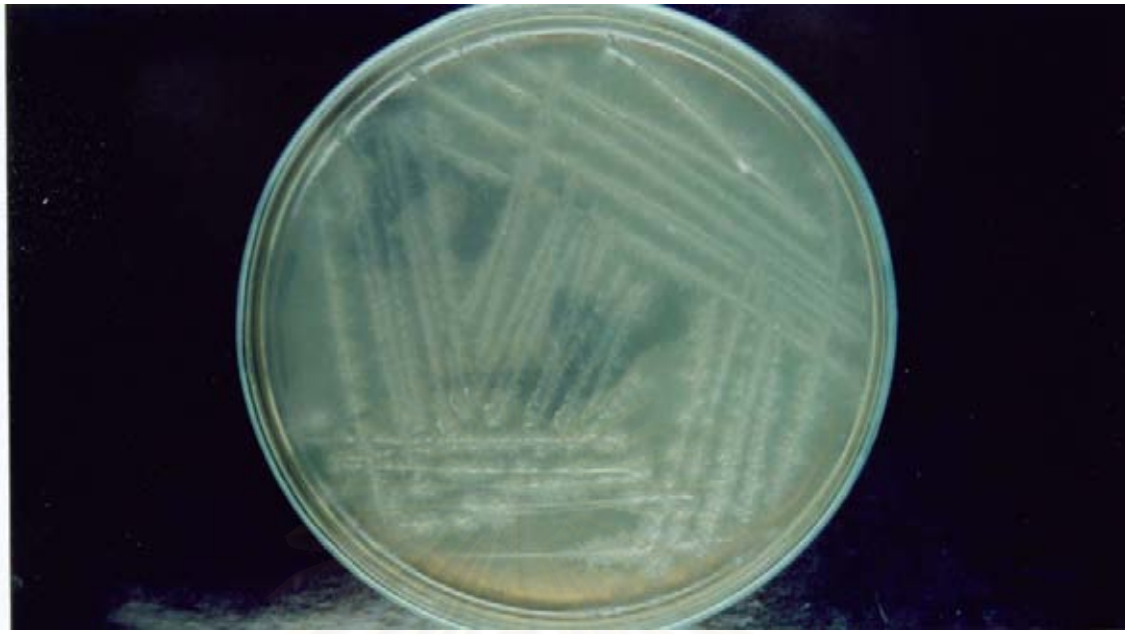

Figure 11. Colony of bacterial strain No.2 on nutrient agar medium

Table 2. Characteristics of the species of the *Bacillus subtilis*TISTR25 *Bacillus mycoides* (Bergey's manual)and ,Sample bacterial strain No.2.

Characteristics	*Bacillus subtilis* TISTR25	*Bacillus mycoides* (Bergey's manual)	Sample bacterial strain No.2
Rod – Shaped	+	+	+
Diameter over 2.5 μm	-	+	+
Motile	+	+	+
Stain Gram – Positive at least in young cultures	+	+	+

Table 2. (Continue)

Characteristics	*Bacillus subtilis* TISTR25	*Bacillus mycoides* (Bergey's manual)	Sample bacterial strain No.2
Facultative anaerobes or microaerophile	-	+	+
Sulfate activity reduced to sulfide	-	-	-
Catalase	+	+	+
Marked acidity from glucose	+	+	+
Nitrate reduced to nitrite	+	+	+
Voges-Proskauer test	+	+	+
. pH in V-P broth			
< 6	-	+	+
>7	-	-	-

Table 2. (Continue)

Characteristics	*Bacillus subtilis* TISTR25	*Bacillus mycoides* (Bergey's manaual)	Sample bacterial strain No.2
Utilization of citrate	+	D	+
Deamination of phenylalanine	-	-	-
Egg-york lecithinase	-	D	+
Formation of indole	-	-	-
Growth in nutrient broth at pH			
6.8	+	+	+
5.7	+	+	-
Growth in NaCl			
2%	+	ND	+
5%	+	ND	+
7%	+	D	-
10%	-	ND	-

Table 2. (Continue)

Characteristics	*Bacillus subtilis* TISTR25	*Bacillus mycoides* (Bergey's manual)	Sample bacterial strain No.2
Growth at			
5°C	-	-	-
30°C	+	+	+
40°C	+	D	+
50°C	-	-	-
60°C	-	-	-
70°C	-	-	-
80°C	-	-	-

ND = not determine

D = different between the same species

To ensure the result of bacterial strain identification, the 16S rRNA gene sequencing was studied. The 1,500 bp fragment of amplified product was used to determine DNA sequencing by dideoxy nucleotide method. The comparison of 16S rRNA gene sequencing was shown in figure 10. The result of comparison 16S rRNA gene sequencing by BLAST program indicated that this bacteria had sequence of 16S rRNA gene similar to *Bacillus mycoides* strain 10206. The homology was about 98%.

CNTTNCTTNCNGGNTNNNNNNNNNNGAGATGAACGCTGGCGGCGTGCCTAATACATG
CAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTA
AGTAACACCTGGGTAAACCTGCCCATAAAGCTGGGATAACTCCGGGAAACCGGGGC
TA ATACATACCGGATA ACATTTTGCA CCGCATGGTG CGAAATTGAA GGCGGCTTCG
GCTGTCACTTATGGATGGACCCGCGGTCGCATTANCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCAACACTGGGACTAA
GACACGGCCCATACTCCTACGGGAGGCAGCAGTTAGGAATCTTCCGAAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTG
TTGTTGGGGAAGAACAAGTGCTAGTTNAATAAGCTGGCACCTTTGACGGTACCTAACC
ANAAAGCCACNGNTAACTACNTGCNANCAGCCGNGGTAATACNGTAGGTGGCAAGC
GTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA
AAGCCCACGGCTAAACCTGGGAGGGTTATTGGAAACTGGGAGACTTGAGTGCAGAA
GAAGGAAAGTGGAATTCCATCTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACAC
CAGTGGCGAAGGCGACTTTCTGGGTTGTAACTGACACTGATGCGCNAAAGCGTGNNG
AAGCNAACNANATTAAATACCCTTNNNTNNCACGCCNTATACNNTNATNGNTAAGTNN
NNGAGNGNTNACAGNCTTTNTTGCTNNNGTNNACTCTTTAANTTCTTCGCNNNNTGAC
TNCNTGGCGCTNGGNTGNTACTCTGNGGANAGCNNNGTNACTNCACTNAANGGCGG
ANTNCTGGTATACACAGAATCAGGGGATAACGCCTGAAACAACATGTGAGCNAAAGG
CCNNCNANGGCCATNAACATGCNGGCGTTCCAATTTGAGTTTCCTTAACTGCCCCCG
GCGGTGTTCGCCACCTCGTACACACAATTAAGCTTCGTTGCGCTTCTGGAATGGTCCA
GAACTGTAGGAGACTGTTGGGATCTCTATCCCGAAAGAGGAAGCCCTCGTCTCACTG
TCACCCACGTACCAACAGCAGTCGAGCACAGCACTCTACAACCCAATTACGGGCGTT
GCTCGGCTTGGAGACTAGAATCAACGGTAGTAATTCAACCCGTGAGATTCCA

Figure 12. The 16S rRNA gene sequence of bacterial strain No.2 DNA was shown. The results of automatic sequencer were 3 fractions of DNA sequence. The underlines were used as the homologous junction of 3 DNA sequences that used to join each DNA sequence into 1 line.

3.3 Optimization of protease production

3.3.1 The comparison between peptone medium (Raja, 1994) and yeast extract medium (Kasem, 1978)

The comparison between peptone medium (Raja, 1994) and yeast extract medium (Kasem, 1987) was performed to find a suitable medium. The result indicated that peptone medium gave the highest protease activity than yeast extract medium. The growth pattern and enzyme production of both mediums were shown in figure 13-15.

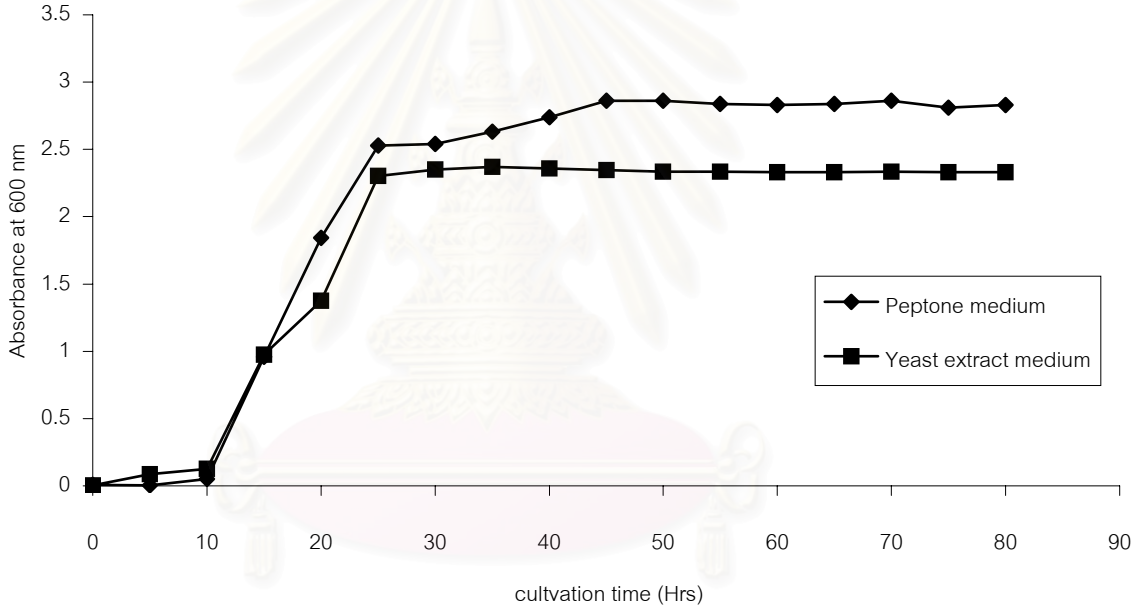

Figure 13. The comparison of growth pattern of *Bacillus mycoides* in peptone medium and yeast extract medium

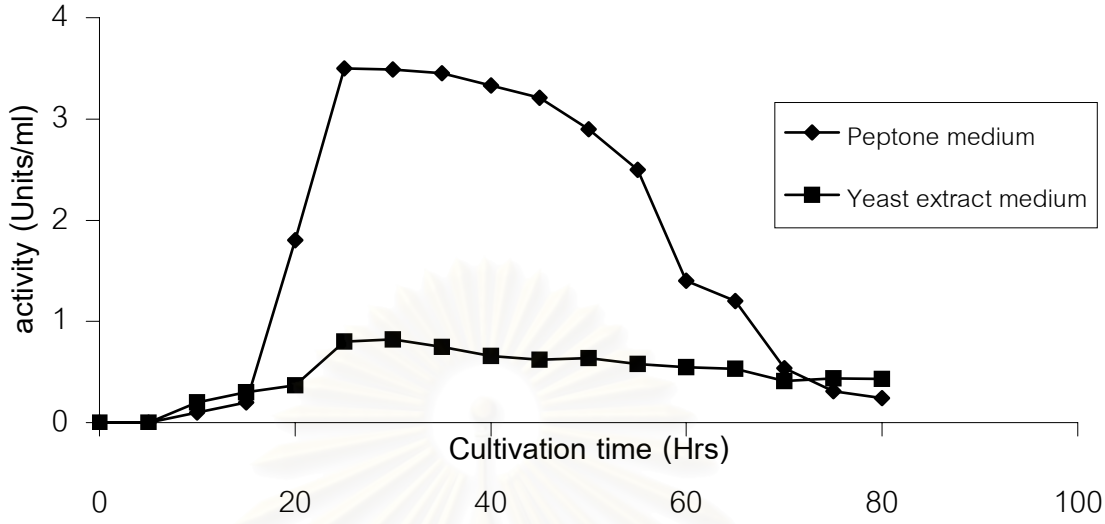

Figure 14. The protease activity profile of *Bacillus mycoides* on yeast extract medium and peptone medium

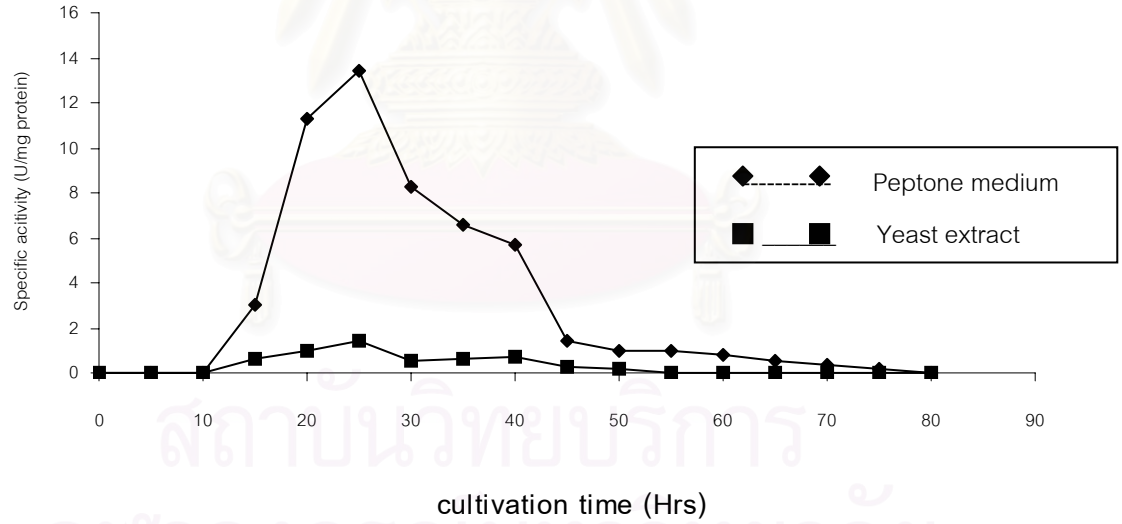

Figure 15. Comparison between activity of protease from peptone medium and yeast extract medium.

3.3.2 The optimum inoculum size

Then the determination of optimum inoculum size was conducted and the growth pattern was shown in figures16-18. The result reveals that 1% of the starter was suitable for inoculation.

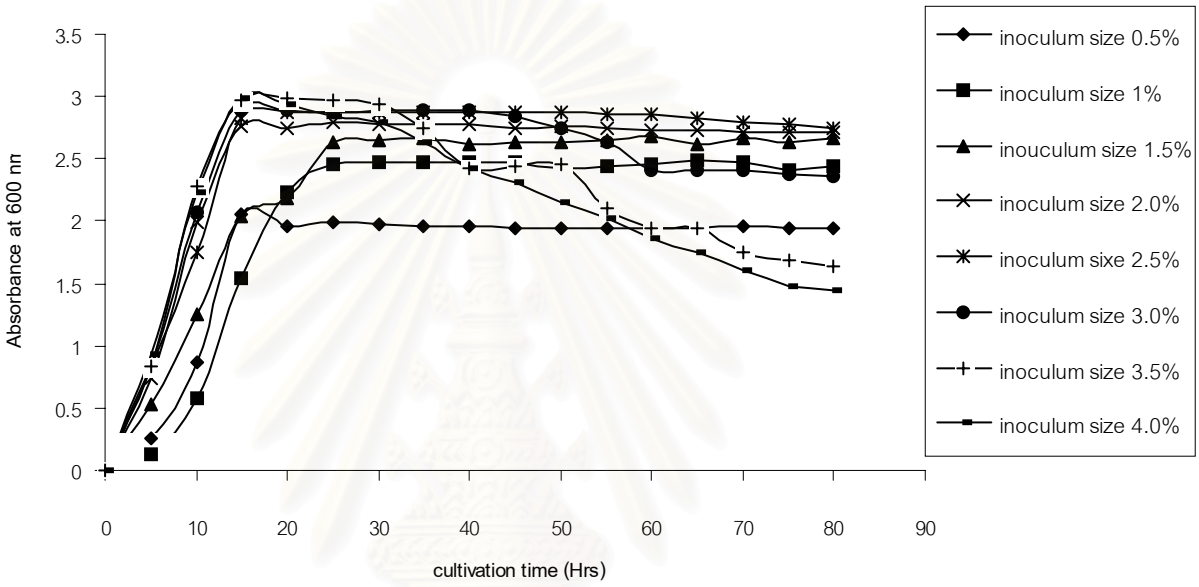

Figure 16. Comparison between growth pattern of *Bacillus mycoides* obtained from various inoculum sizes.

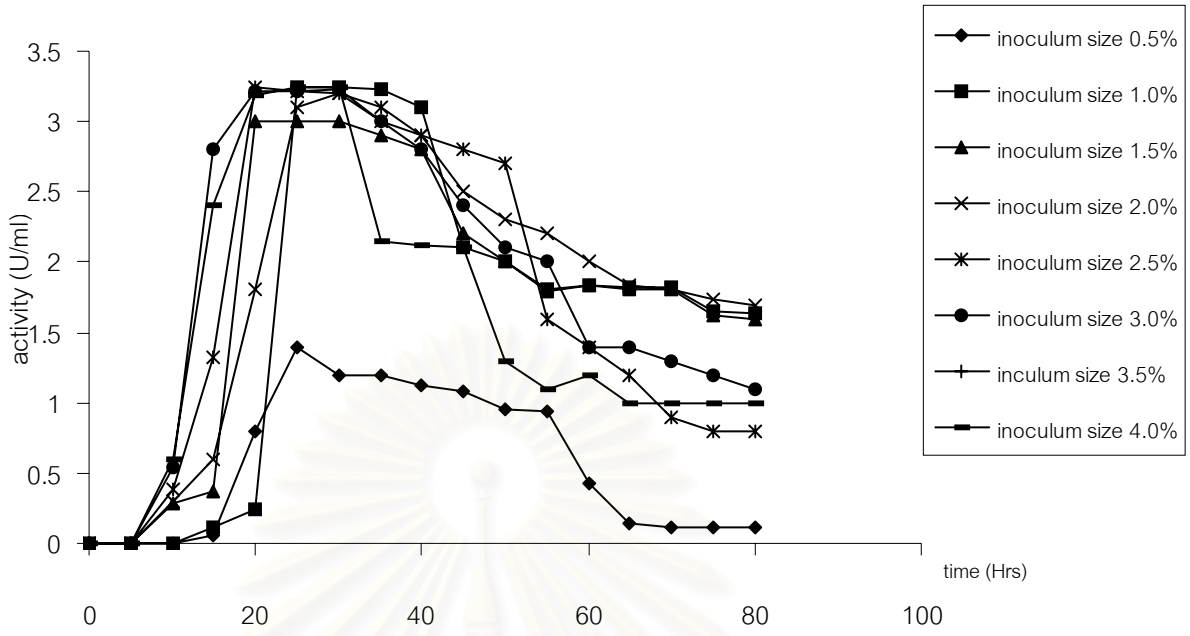

Figure 17. Protease activity profiles of *Bacillus mycoides* obtained from various inoculum sizes.

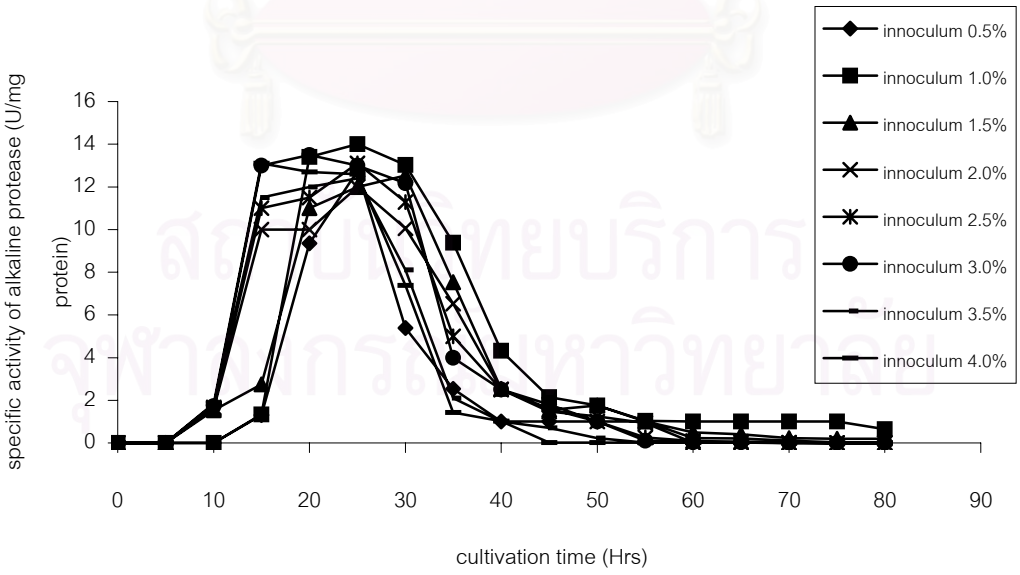

Figure 18. Comparison of specific activity of protease obtained from various inoculum sizes.

3.3.3 Determination on optimum temperature of cultivation

Determination on optimum temperature of cultivation was conducted by using 1 ml of starter to inoculate peptone medium. The initial pH of the medium was pH 7.0. Growth pattern was shown in Figures 19-21. This data revealed that 37°C gave the highest activity. On the other hand, the bacteria could not grow at 50°C and 60°C.

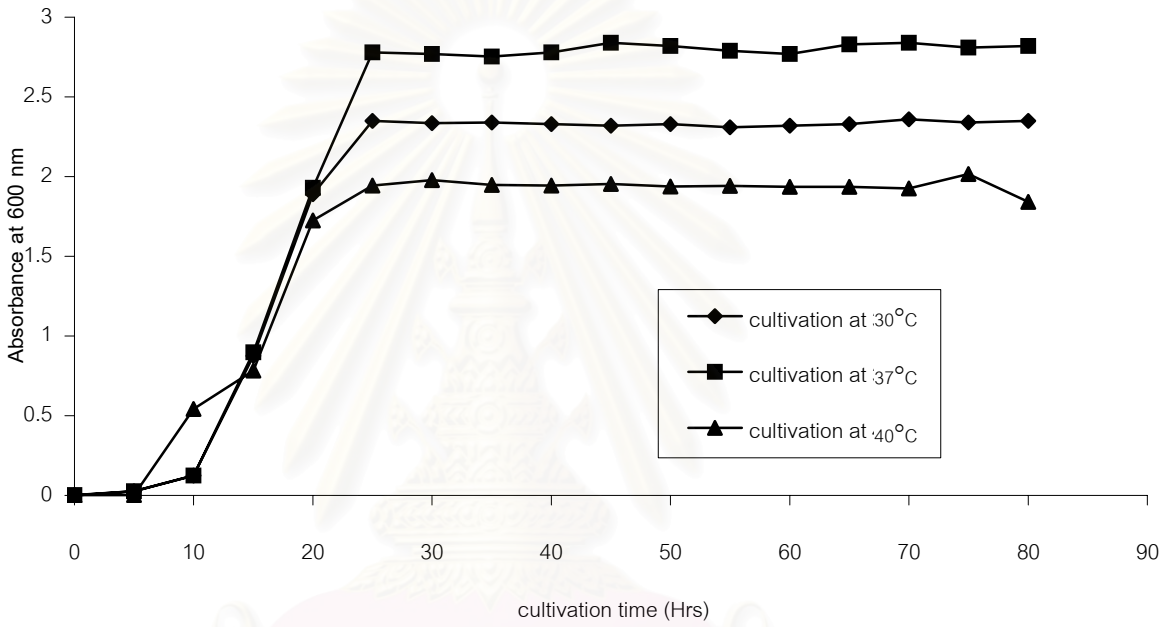

Figure 19.Growth pattern of *Bacillus mycoides* at various temperatures of cultivation.

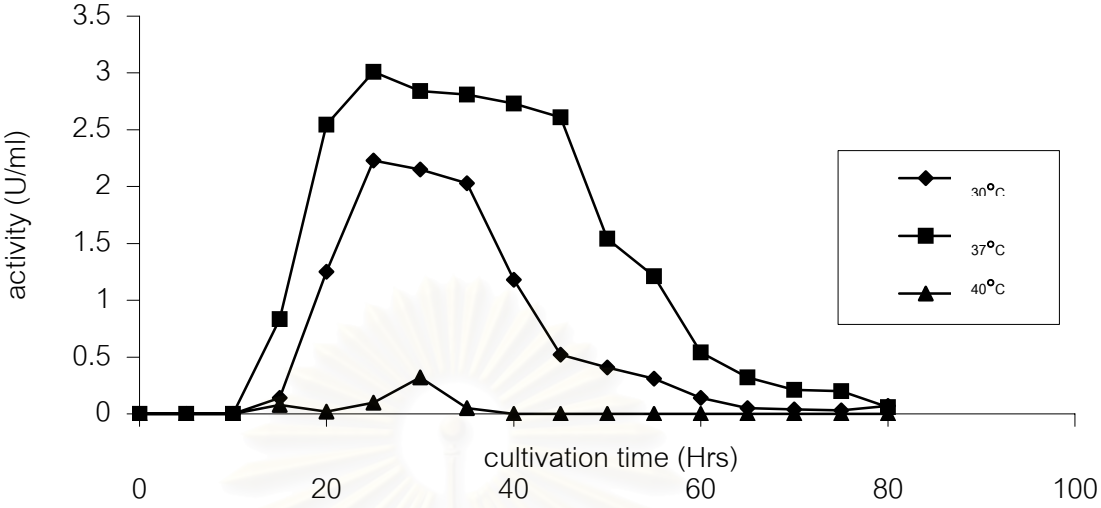

Figure 20. Activity profile of protease from *Bacillus mycoides* at various temperatures of cultivation.

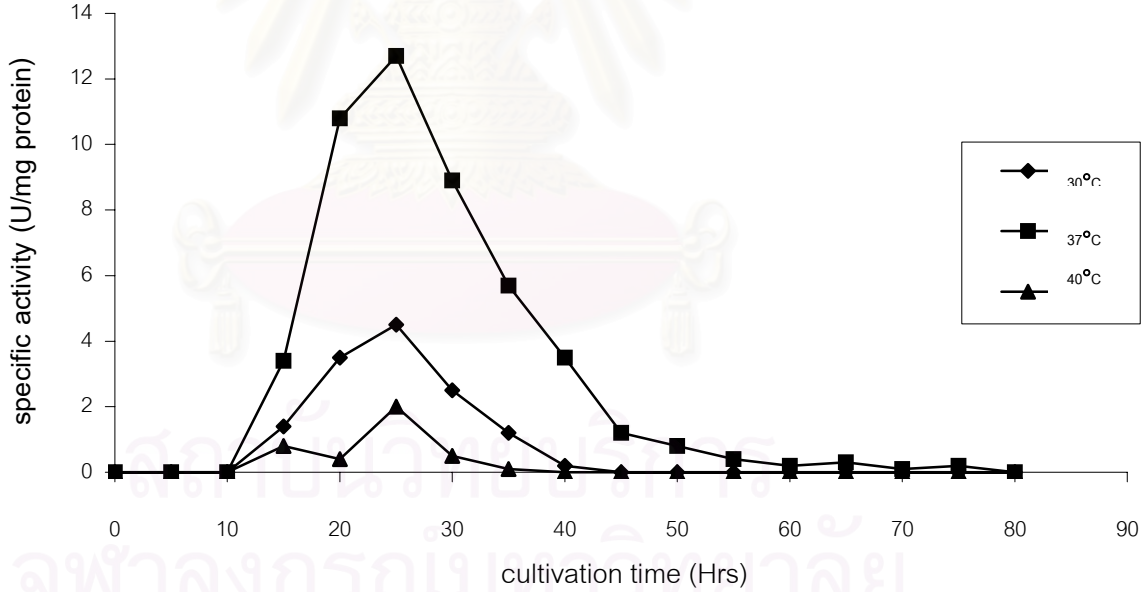

Figure 21. The comparison of specific activity of protease at various temperatures of cultivation.

3.3.4. Determination on optimum initial pH of cultivation

The cultivation at 37°C was performed at different initial pHs of the medium (pH 5,6,7,8,9,10 and 11). The inability of bacteria to grow at pH 3,4,5 and 11 indicated that this bacterium could grow at neutral and slightly alkaline medium. Cultivation at pH 8 in peptone medium gave the highest protease activity.

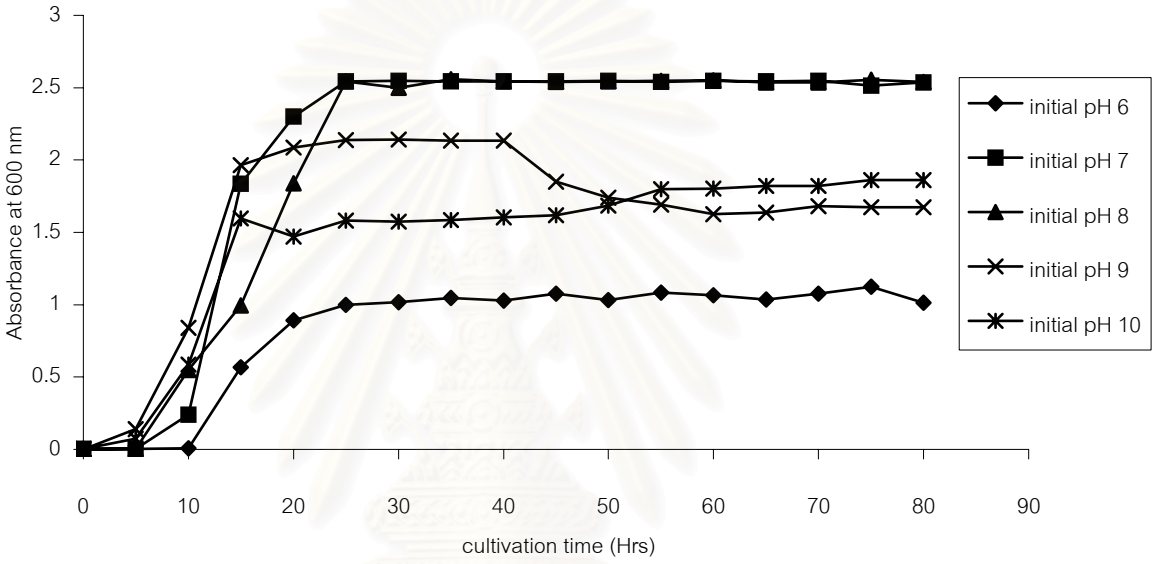

Figure 22. Growth pattern of *Bacillus mycoides* at various initial pHs of cultivation.

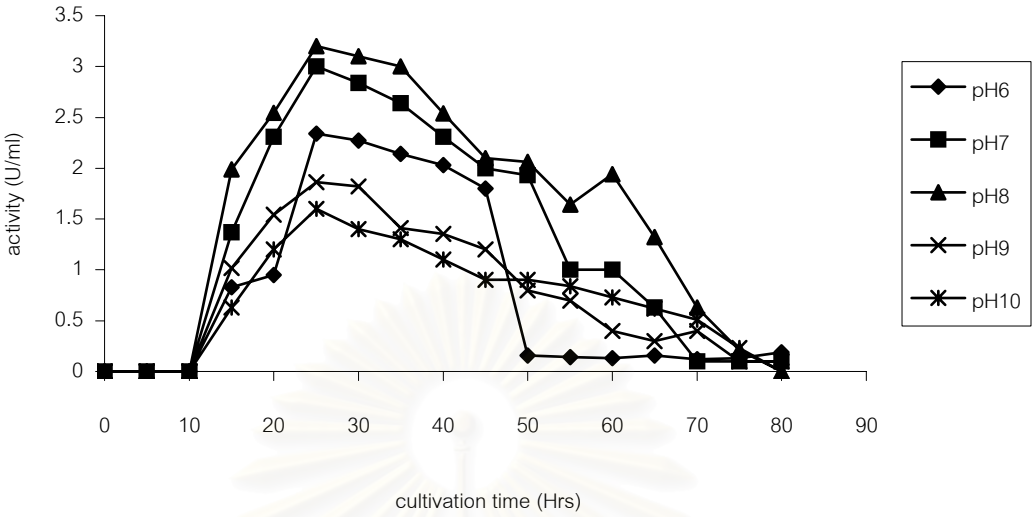

Figure 23. Activity profile of protease from *Bacillus mycoides* at various initial pHs of cultivation.

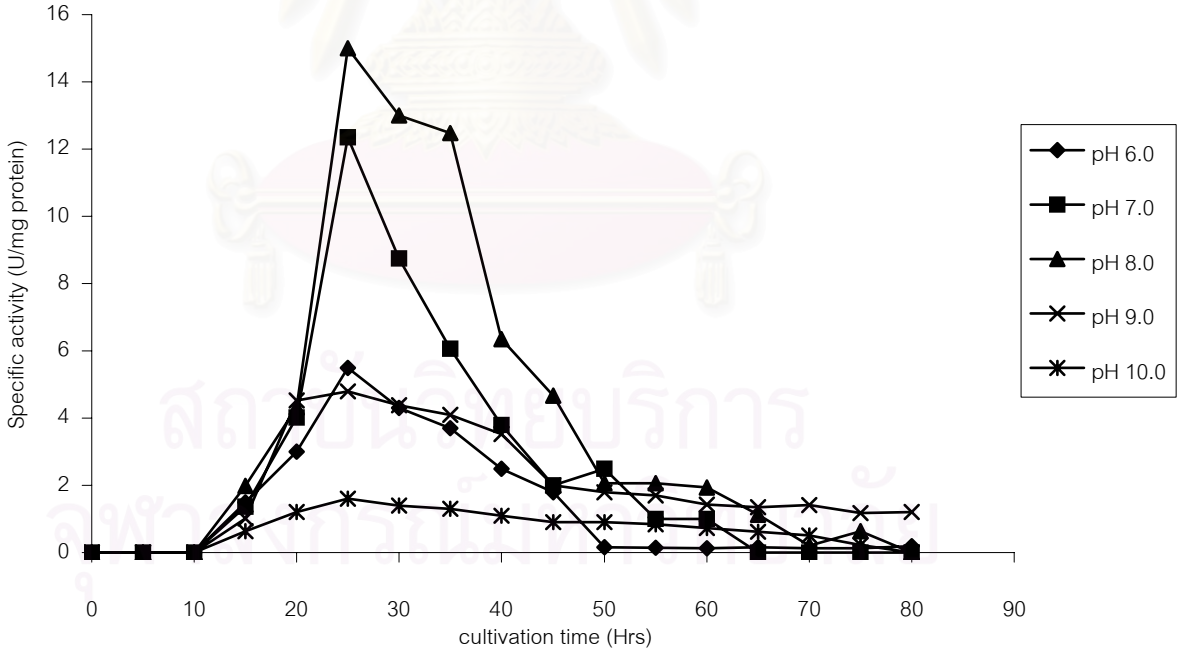

Figure 24. Comparison of protease activity at various initial pHs of cultivation.

3.3.5 Determination on optimum glucose concentration

Peptone medium (Raja, 1994) was used to cultivate at 37 °C, pH 7, 250 rpm. The work of Raja, (1994) did not use glucose or any sugar with no reason. To study the effect of glucose ,this experiment was conducted by using control without glucose and experiment with various glucose concentration. The result revealed that the control gave the highest protease activity and decreasing activity was observed in the presence of glucose. The increase in glucose concentration in the medium decreased protease activity. Growth and activity patterns of this bacterium were shown in figures 25-27.

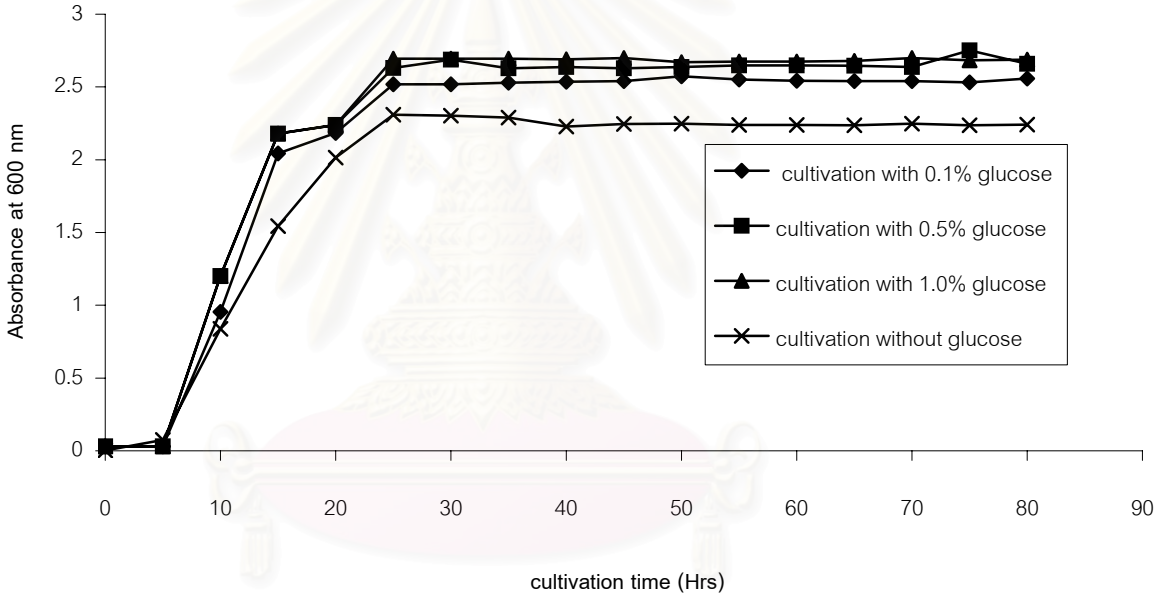

Figure 25. Growth pattern of *Bacillus mycoides* at different glucose concentrations in the culture medium

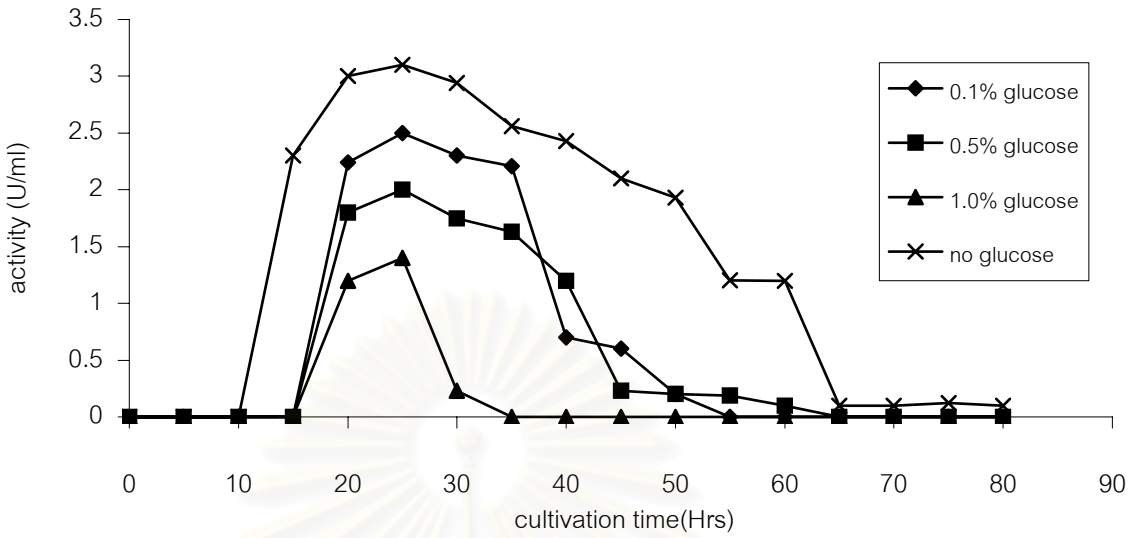

Figure 26. Activity profile of protease from *Bacillus mycoides* at different glucose concentrations.

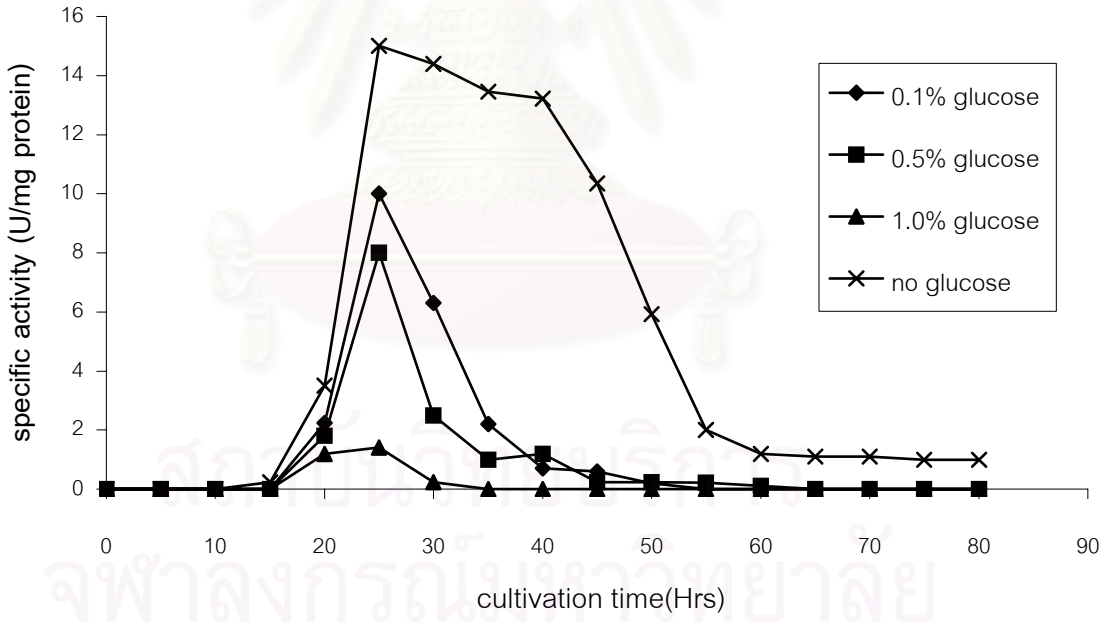

Figure 27. Comparison of protease activity at different glucose concentrations.

3.3.6 Determination on optimum nitrogen source

The optimum nitrogen source was determined by using peptone, tryptone, yeast extract, beef extract, $(NH_4)_2SO_4$ and $NaNO_3$. Unfortunately this bacteria could not grow on medium which contained inorganic nitrogen source such as $(NH_4)_2SO_4$ and $NaNO_3$. The result revealed that tryptone gave the highest protease activity. Growth and activity pattern of this bacteria were shown in figure 28-30.

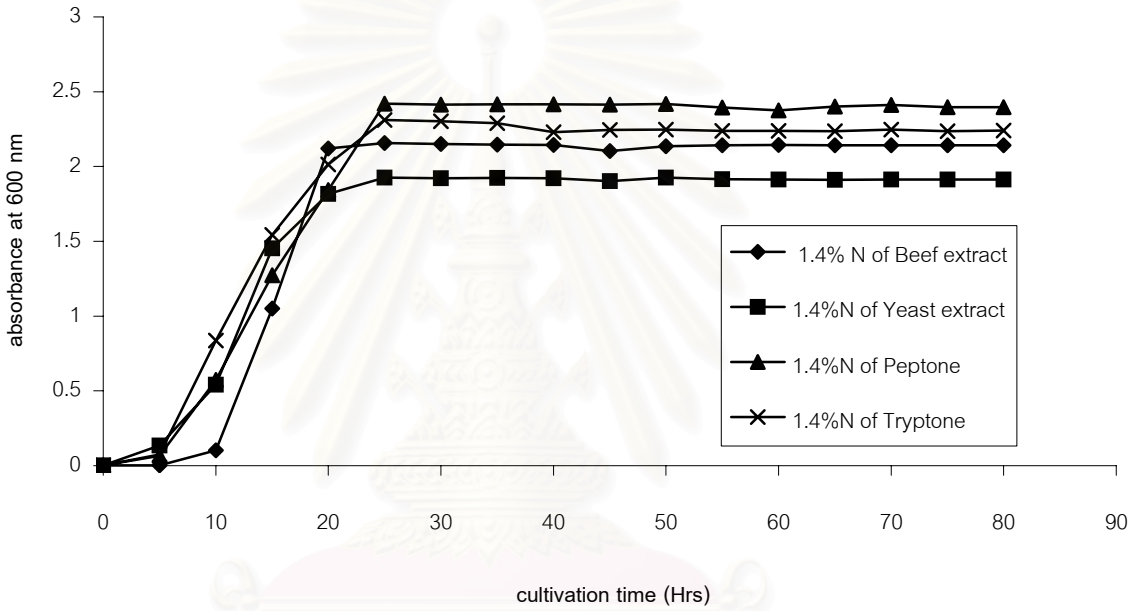

Figure 28. Growth pattern of *Bacillus mycoides* obtained from different nitrogen sources.

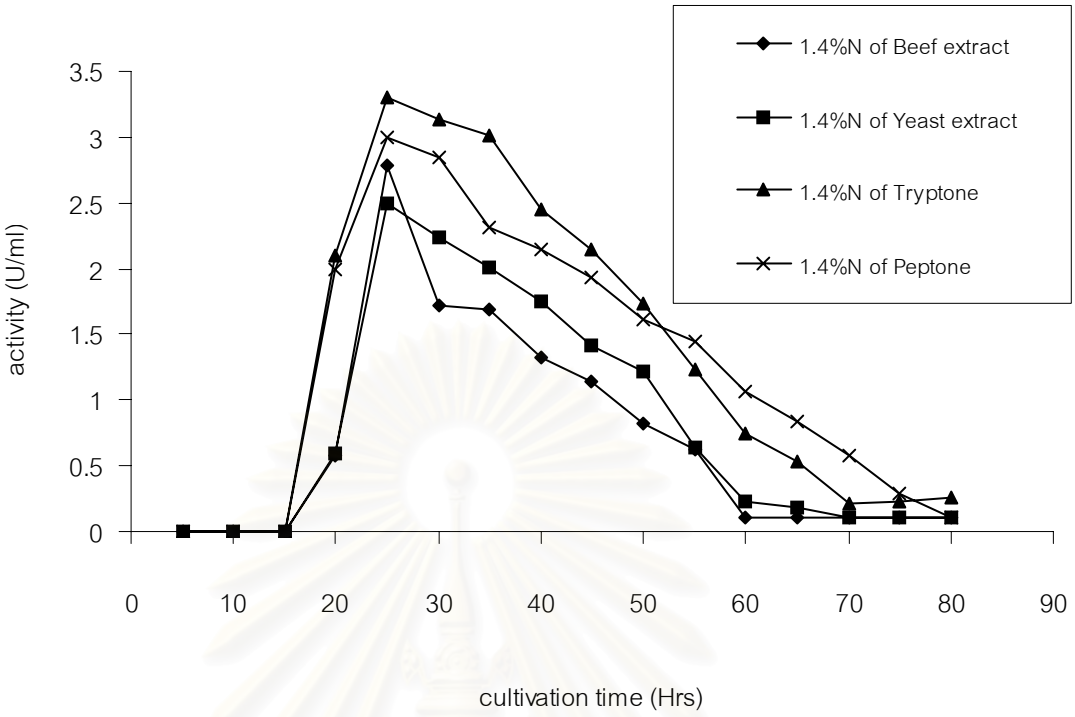

Figure 29. Activity profile of protease from *Bacillus mycoides* grown in different nitrogen sources.

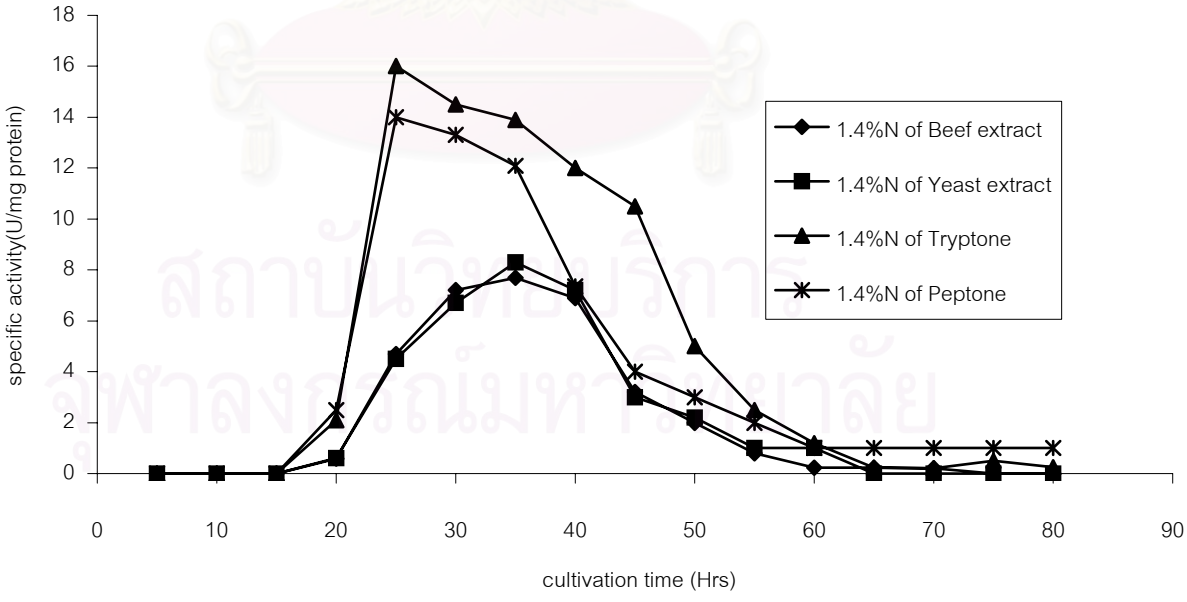

figure 30. Comparison of protease activity obtained from different nitrogen sources

3.3.7 Determination of optimum nitrogen content

Nitrogen content of tryptone were analyzed by using Kjeldahl method and was added in the medium to adjust nitrogen content 0.7, 1.4 and 2.8%. Growth and activity patterns of *Bacillus mycoides* were shown in figures 31-33. This result revealed that 1.4 % nitrogen content gave the highest protease activity.

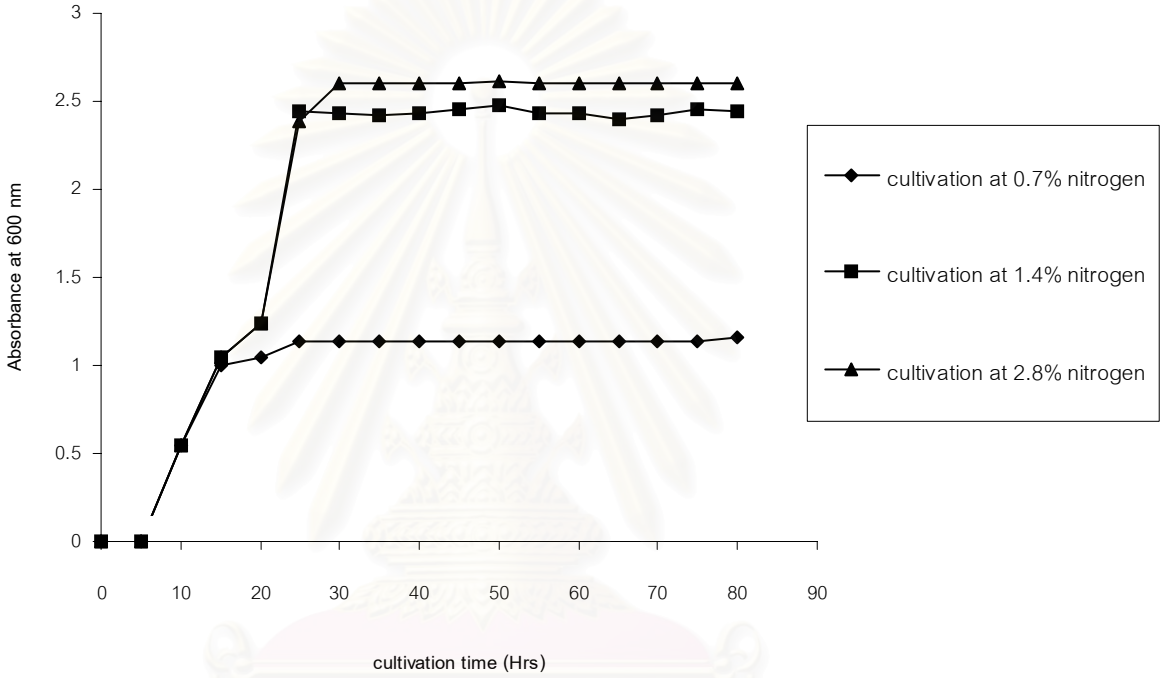

Figure 31. Growth pattern of *Bacillus mycoides* at various percentages of nitrogen content in culture medium.

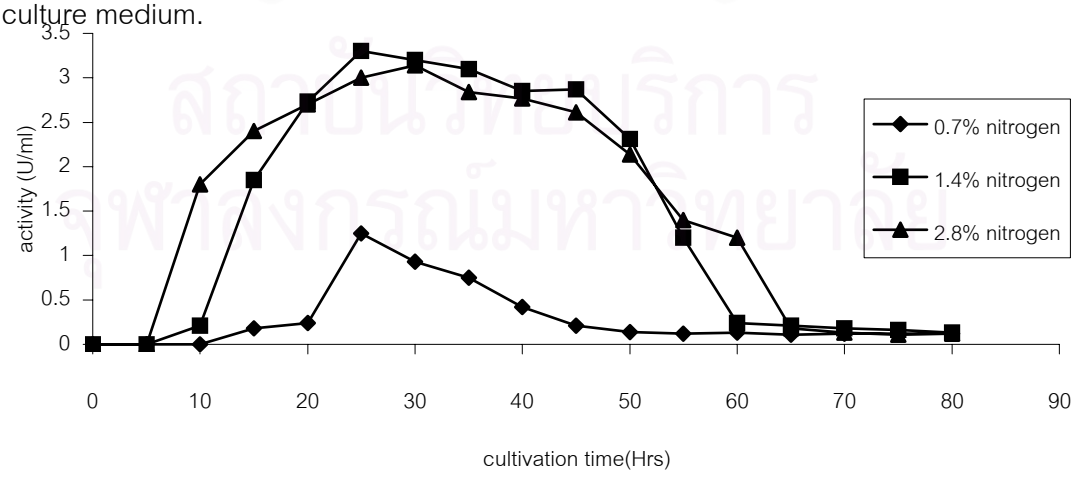

Figure 32. Activity profile of protease from *Bacillus mycoides* at various percentages of nitrogen content.

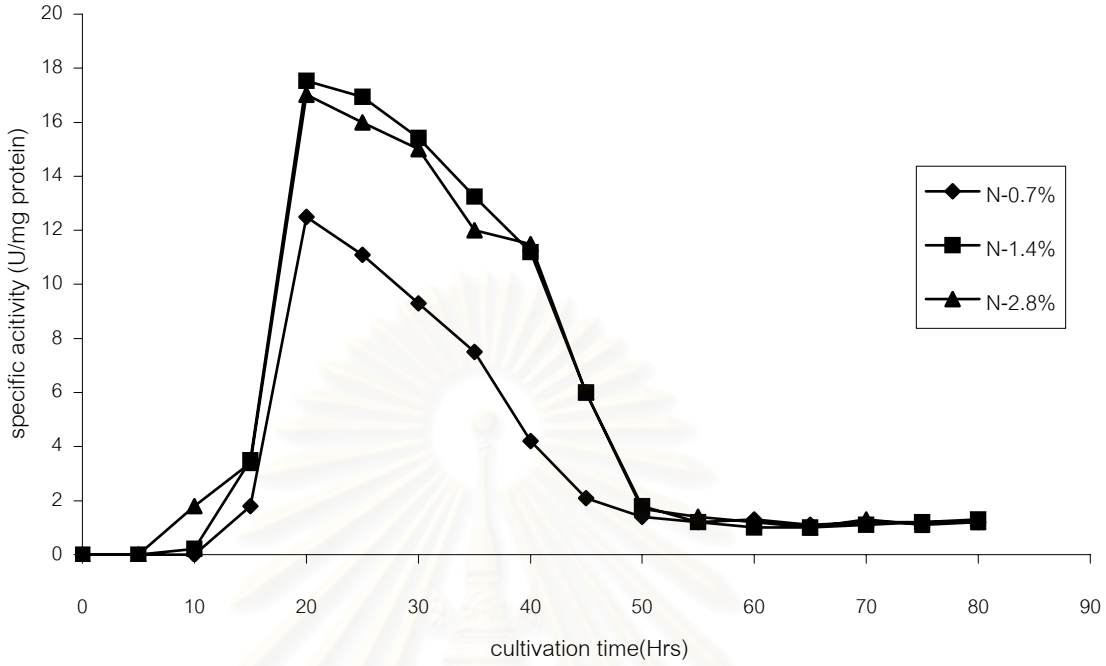

Figure 33. Comparison of protease specific activity obtained from different percentages of nitrogen content.

3.3.7. Determination on optimum agitation rate

The rate of shaking affects oxygen dissolving in liquid medium. The dissolving oxygen has major effect on the growth rate of aerobic bacteria. The growth and activity patterns from various rates of shaking are shown in figures 34-36. These results indicated that 250 rpm was optimum condition to cultivate *Bacillus mycoides*.

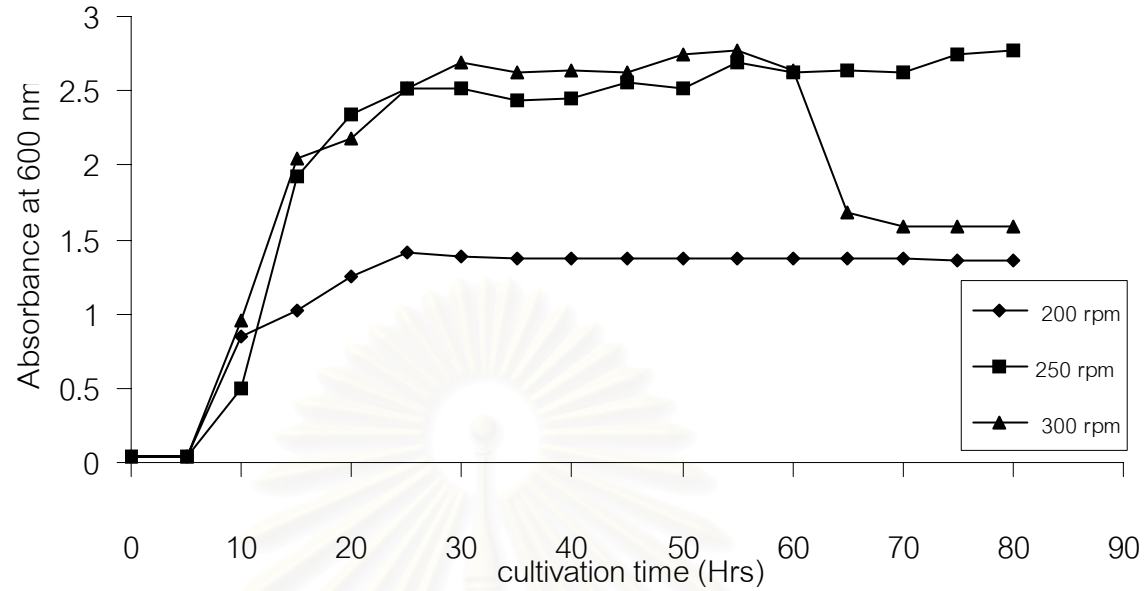

Figure 34.Growth pattern of *Bacillus mycoides* at various rates of shaking.

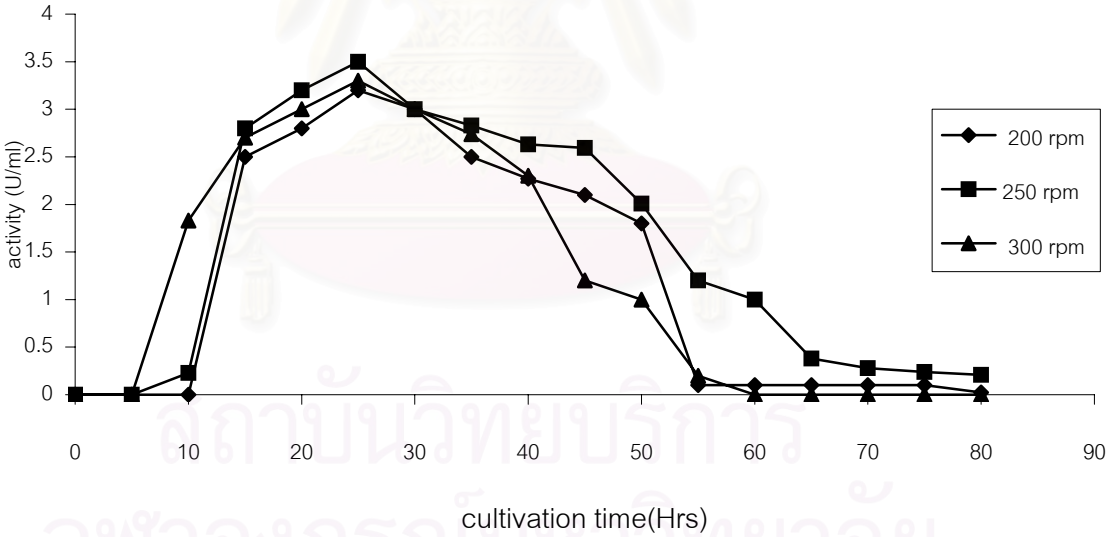

Figure 35. Activity profile of protease from *Bacillus mycoides* at various rates of shaking.

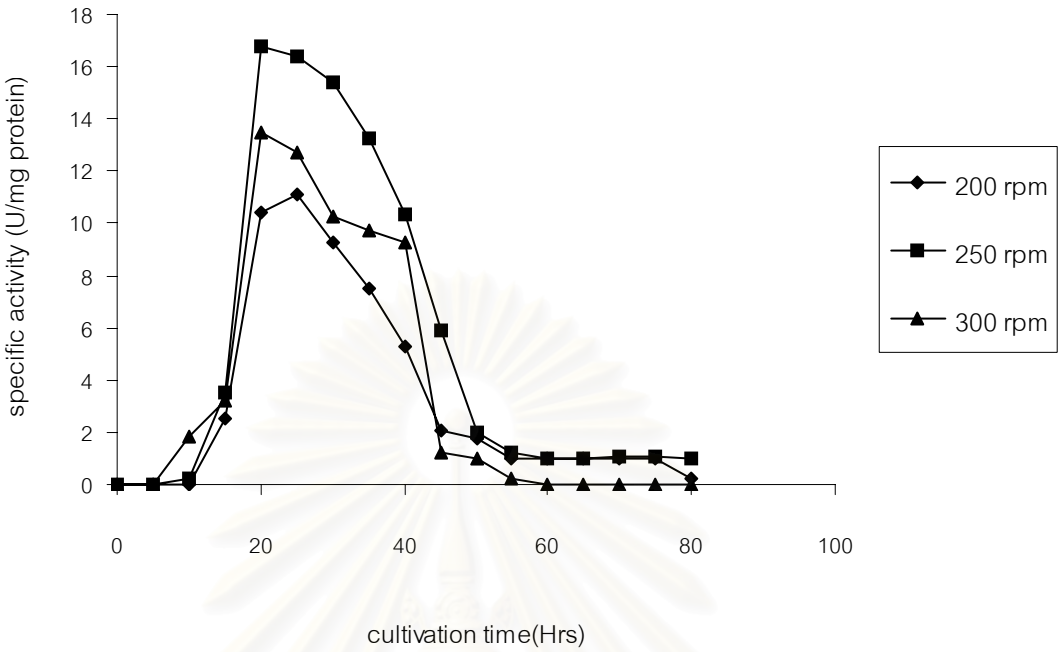

Figure 36. Comparison of protease specific activity at various rates of shaking.

3.4 Purification of protease

3,000 ml of crude enzyme protease was prepared for purification. The 3 liters of supernatants were concentrated by ultrafiltration to decrease volume into 300 ml. Then cold acetone was slowly added to the enzyme solution to make final concentration of 50%. The precipitated protein was dissolved with 10 mM Tris –HCl, pH 9. The protein was loaded on to Sephadex G-75 column. The enzyme was eluted by 0.1mM Tris-HCl, pH 9. It was found that the fraction number 37-55 had protease activity (Figure 37.) Fractions with high enzyme activity (fractions 40-50) were pooled. The pooled fraction were analyzed for purity by native PAGE. It was found that only one band was observed in the pooled fraction from Sephadex G-75 column chromatography. The activity stain was performed to compare with protein stain. The result was shown in figure 38. It was found that protein stain in lane 5-6 showed a single band, which corresponded to the activity stain, indication that the enzyme from Sephadex G-75 was pure enzyme.

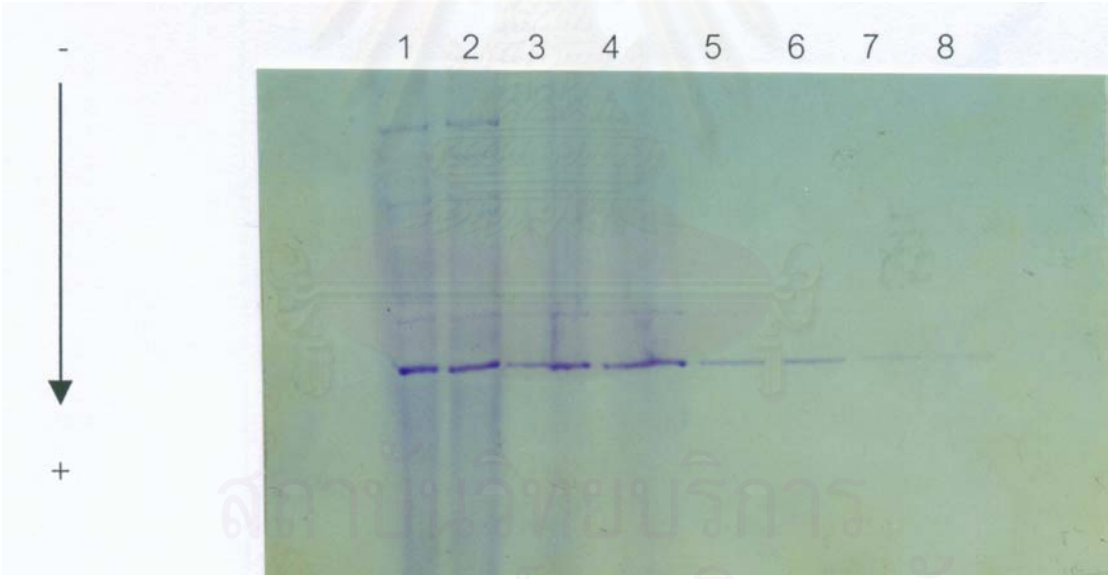

Figure 37. Non – denaturing polyacrylamide gel electrophoresis of protease from each step of purification

A: protein stain

Lane 1-2 crude enzyme	63.7 μg
Lane 3-4 Ultrafiltration	70.0 μg
Lane 5-6 50% acetone precipitation	35.4 μg
Lane 7-8 Sepadex G-75 column chromatography	7 μg

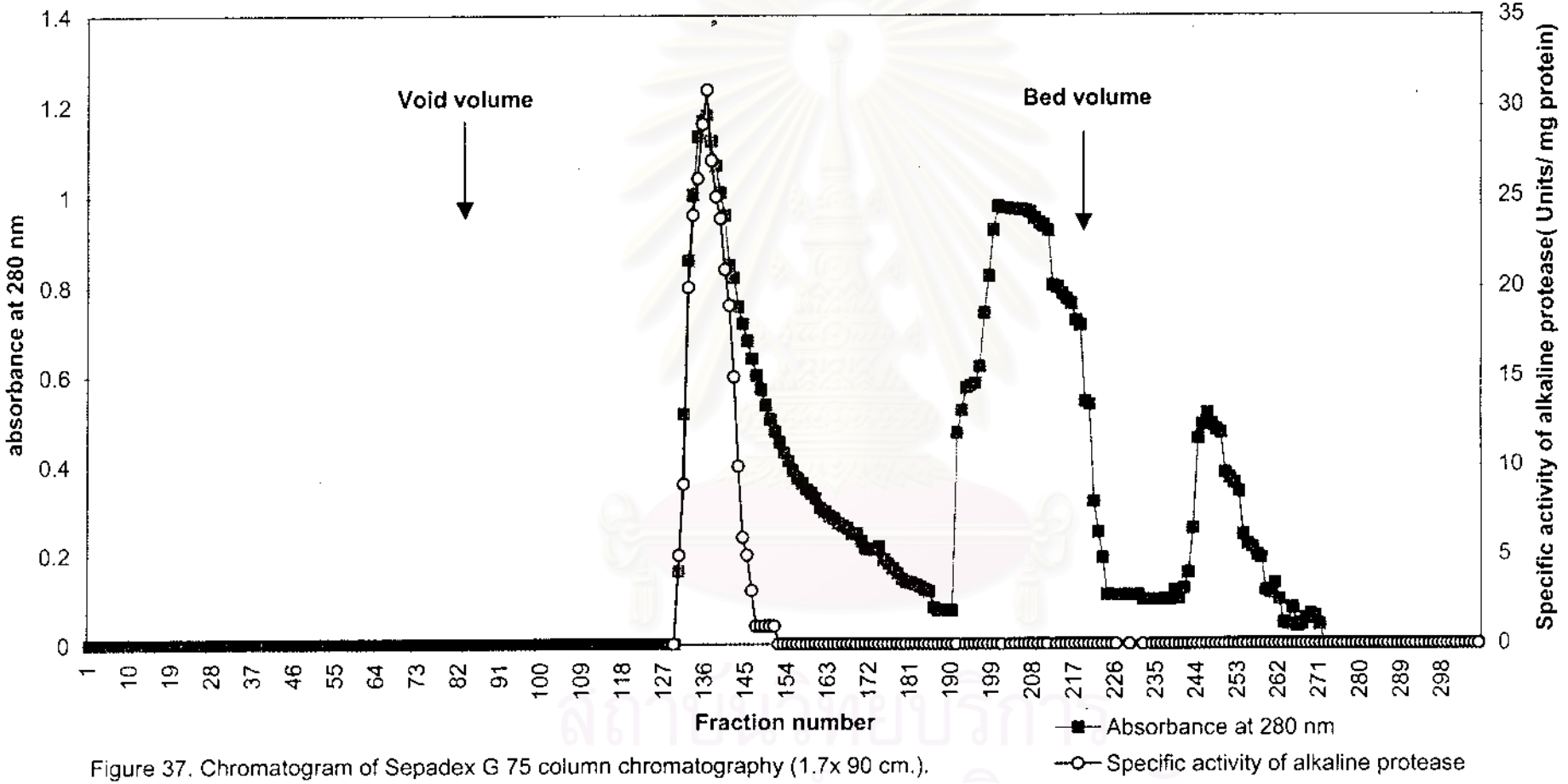

Figure 37. Chromatogram of Sepadex G 75 column chromatography (1.7x 90 cm.).

Table 3. Purification and recovery of the protease

Purification step	Volume (ml)	Total Protein (mg)	Total activity (units)	Specific activity (unit/mg protein	Yield (percent)	Purification fold
Crude enzyme	3000	408.39	600.00	1.46	100.00	1.00
Ultrafiltration	300	58.42	87.00	1.49	14.75	1.01
50% Acetone precipitation	50	10.40	57.50	5.53	9.58	3.78
Sephadex G-75 Column chromatography	10	0.09	2.75	30.97	0.46	21.21

3.5 Characterization of protease

3.5.1 Relative molecular mass determination

The denatured enzyme, which migrated as a single band of stained protein by SDS-PAGE, had molecular mass of 31,000 (Figure.39). However, the molecular mass on Sephadex G-75 column chromatography gave a value of 27,600 Da. The molecular mass was under estimated by gel filtration and has been observed for a number of extracellular microbial protease (Peek et al. 1992; Gusek and Kinsella 1987). The molecular mass obtained from SDS – PAGE was closed to those reports for most bacterial proteases (15,000 to 30,000) (Morihara, 1974). Manachini et al. (1988) had reported a higher molecular mass of 39,000 Da for thermostable protease produced by *B. Thermoruber*. From this result, we could get thermostable protease with molecular mass 27,600 Da.

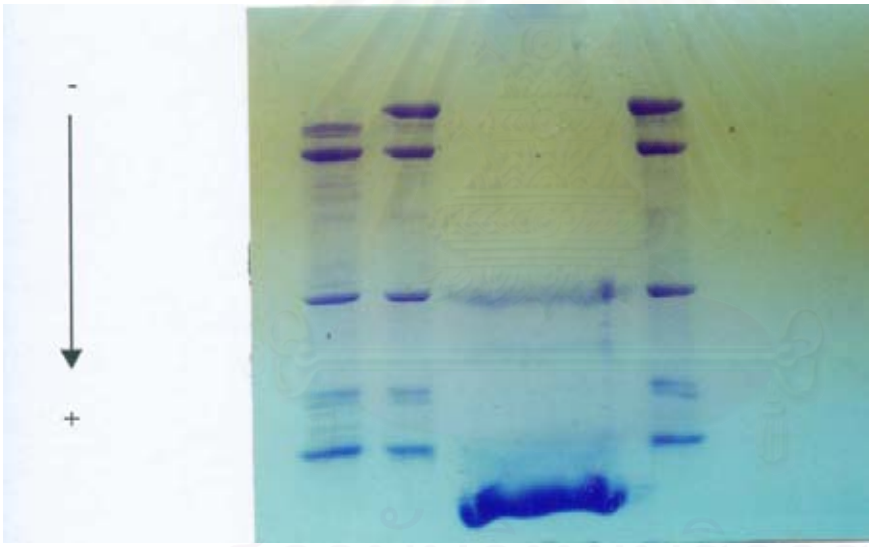

Figure 39. Denaturing(SDS) polyacrylamide gel electrophoresis of protease

Lane 1,2,4	Standard protein marker	63.7 μg
Lane 3	protease from *Bacillus mycoides*	35.4 μg

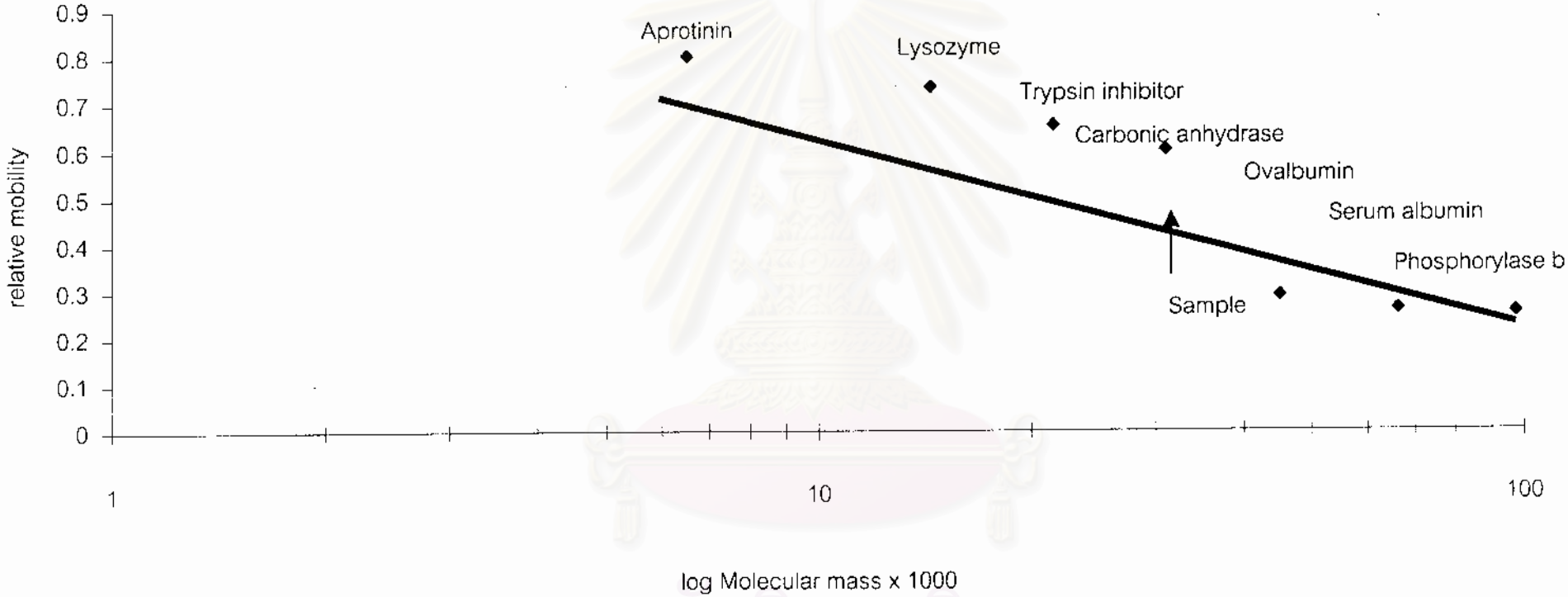

Figure 40. Relative mobility of standard proteins and sample from SDS- Polyacrylamide gel electrophoresis

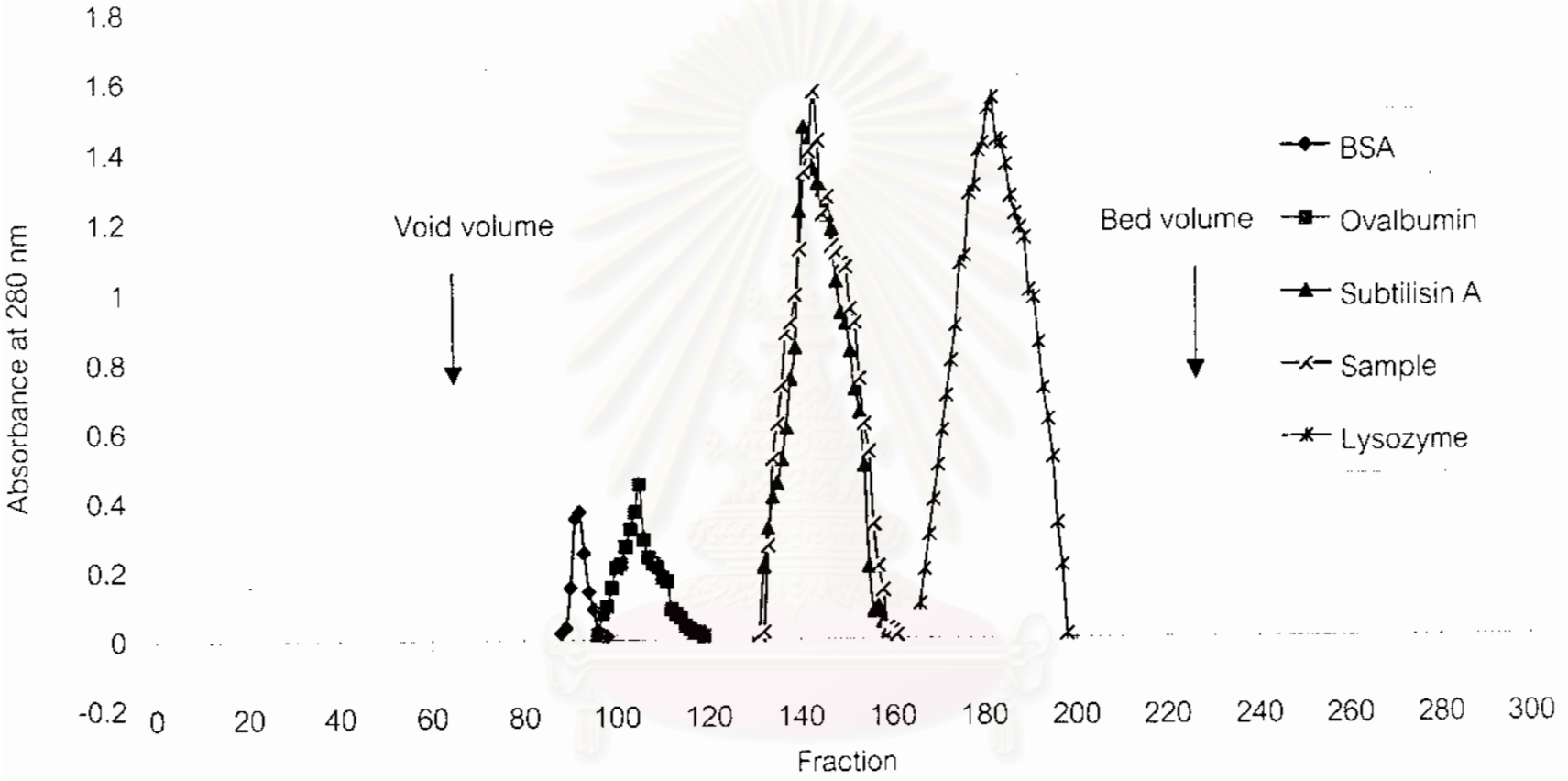

Figure41. Chromatogram of standard protein and sample on Sephadex G-75 column chromatography (1.7x90 cm.)

3.5.2 Effect of temperature on enzyme activity

The effect of temperature on the protease activity toward azocasein was shown in figure 43. The optimum temperature for the protease acitivity was 60°C. The enzyme was stable at temperature between 40°C and 60°C (Appendix D).

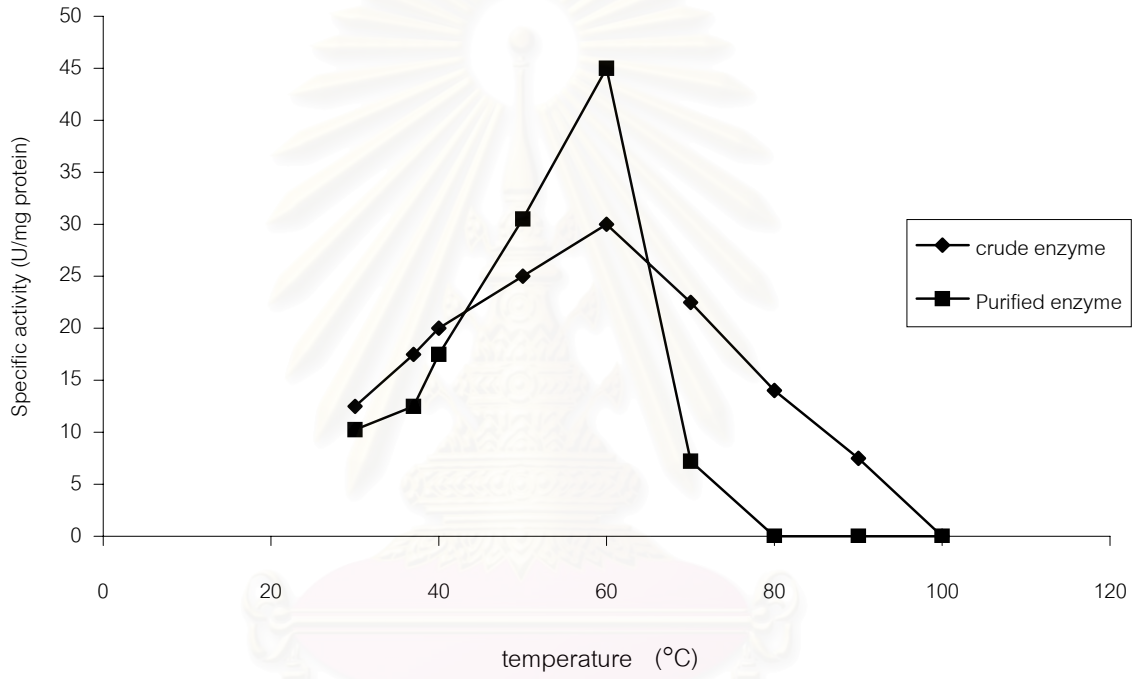

Figure 43. Optimum temperature for activity assay of protease activity on azocasein hydrolysis

3.5.3 Effect of pH on enzyme activity

Effect of pH on enzyme activity was examined in the pH range 4 – 12 (figure 44). Maximal proteolytic activity toward azocasein was observed at pH 8.0. The enzyme was stable between pH 7 and 10 during incubation for 30 min (Appendix D).

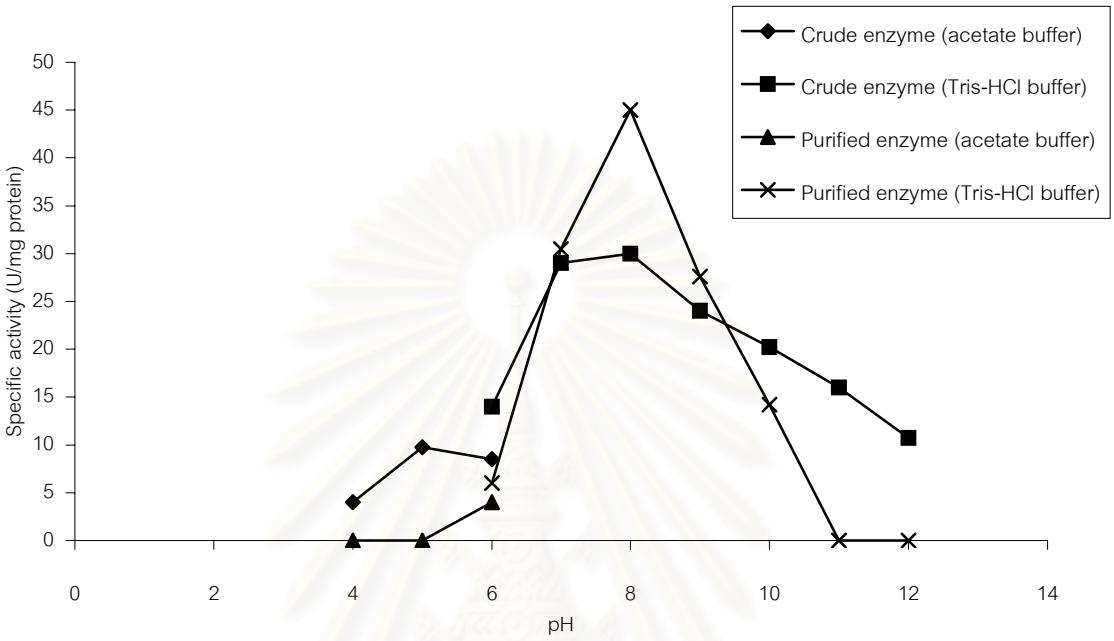

Figure 44. Optimum pH for assay protease activity on azocasein hydrolysis

3.5.4 Effect of metal ions and inhibitors on enzyme activity

The effect of metal ions on enzyme activity were tested . Ion were used as the Following $FeCl_3.6H_2O$,$NaNO_3$,$MnCl_2$,$FeSO_4$,$AgNO_3$,$ZnSO_4$,NH_4Cl,$MgSO_4$,KBr and $LiCl_2$. The presence of $HgCl_2$,$ZnCl_2$,$CuSO_4.5H_2O$,$CoCl_2.6H_2O$, $K_2CO_3.6H_2O$ and $RbCl_2$ at 5 mM inhibited protease activity. However, $MgCl_2.6H_2O$,$MnCl_2$ and $CaCl_2$ was shown to activate the activity of a thermostable protease. Similarly, Shimogaki et al.(1991) showed that Mn^{2+} had little effect on the *Bacillus sp.Y* protease.

Protease could be classified by their sensitivity to various inhibitors (North,1982). The effect of various inhibitors on purified protease was shown in table 3. The enzyme was partially inhibited by PMSF, a serine protease inhibitor. However, the protease was sharply inhibited by hydroxyamine HCl, EDTA, and guanidium hydrochloride.

Table 3. Effect of inhibitors(10mM) and metal ions(10mM) on protease activity

Metal ion or inhibitor	Residual activity after 20 minutes (units/ mg protein)	Residual relative protease activity (%)
none	18.00	100.00
$FeCl_3.6H_2O$	8.94	49.60
$HgCl_2$	0.00	0.00
$NaNO_3$	17.00	94.44
NaN_3	0.00	0.00
$MnCl_2$	18.20	101.10
$FeSO_4$	0.00	0.00
$ZnCl_2$	0.00	0.00
$AgNO_3$	10.00	55.55
$ZnSO_4$	0.00	0.00
NH_4Cl	17.90	99.44
$MgSO_4$	16.42	91.22
$CuSO_4.5H_2O$	8.42	46.77
$CoCl_2.6H_2O$	0.00	0.00
$MgCl_2.6H_2O$	18.70	103.80
$K_2CO_3.6H_2O$	16.30	90.50
KBr	2.50	13.88
$CaCl_2.2H_2O$	19.00	105.50
$RbCl_2$	9.38	51.70
$LiCl_2$	3.80	21.10
NaCl	16.80	93.30
DTT	9.42	52.30
Hydroxyamine HCl	11.50	63.80
EDTA	0.00	0.00
Guanidium hydrochloride	5.70	31.60
PMSF	7.45	41.38

3.5.5 Substrate specificity

The protease was active on modified protein (azocasein) and natural protein (casein). The K_m on casein and azocasein were 125.00 mg/ml and 26.31 mg/ml consequently. Likewise protease exhibited V_{max} on casein and azocasein were 3.80 Units/mg protein and 17.00 Units/mg protein. The results were shown in figures 45- 48.

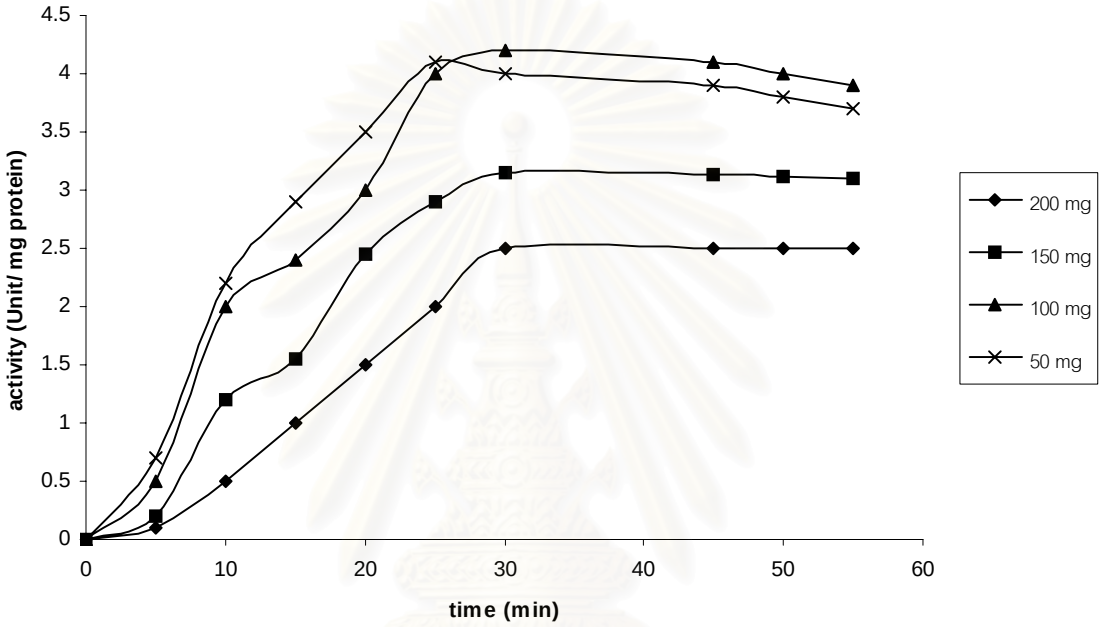

Figure 45. Activity as a function of time at different concentrations of casein substrate.

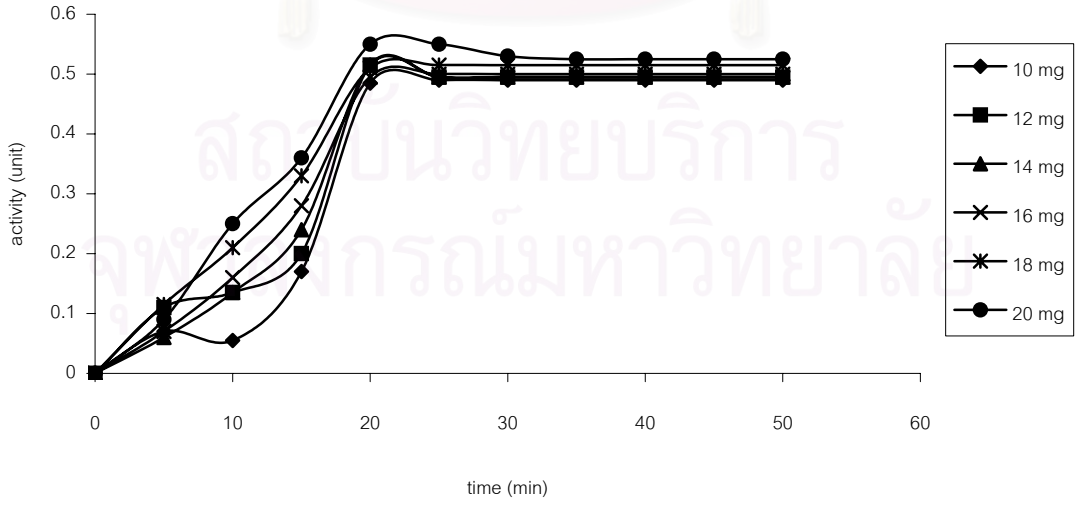

Figure 46. Activity as a function time at different concentrations of azocasein substrate.

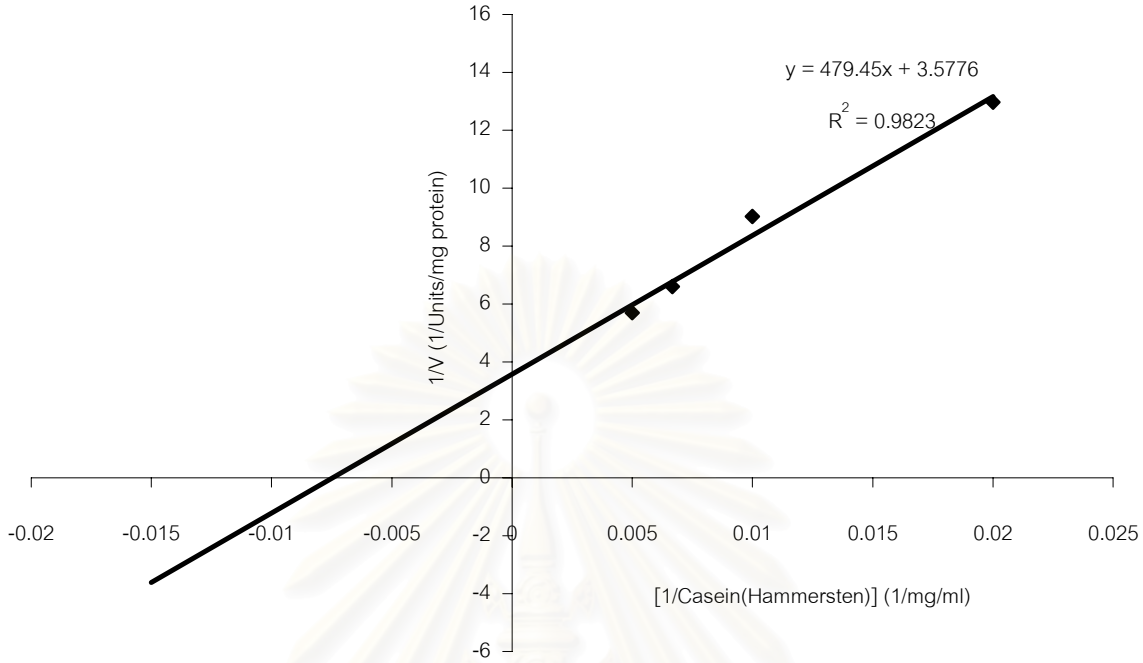

Figure 47. Line weaver Burk plot of protease on casein

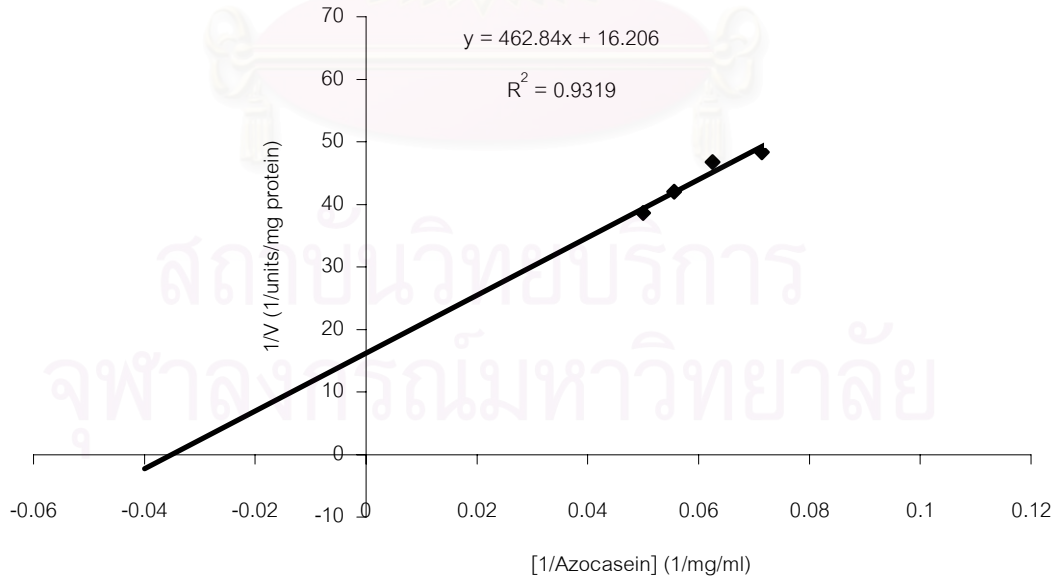

Figure 48. Line weaver Burk plot of protease on azocasein

3.5.6 Determination on suitable storage condition.

Ten percent glycerol were added to the liquid enzyme and storage at –20°C,0°C,5°C, and room temperature. Then the residual activity were monitored and compared with the enzyme solution as a control and the lyophilized enzyme.

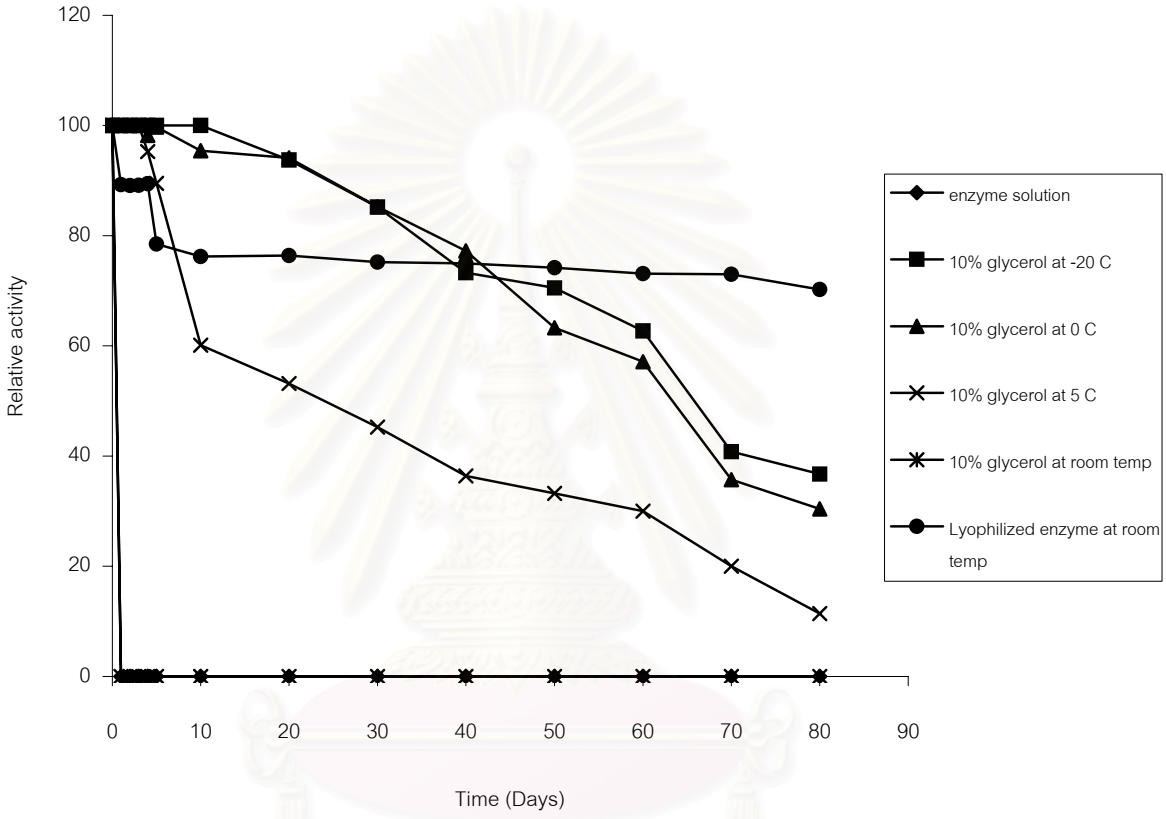

Figure 49. Stability of thermostable protease from *Bacillus mycoides* at various storage conditions.

3.5.7 Hydrolysis of protein wastes from tuna fish canned industry

There were three kinds of the wastes, red meat (solid), drained water, viscera and blood. The mixture was blended by using a blender at high speed for 1 min. For 1 g of red meat, the volume was adjusted to 10 ml by 10mM Tris-HCl pH 9.0 before blending. Drained water was two phases of solid and liquid. The solid wastes

1 g was adjusted to 10 ml by liquid wastes before blending. Likewise 1 g of viscera was adjusted to 10 ml by blood and blended.

Each sample was digested by enzyme and then centrifuged to collect the supernatants. Each supernatant was analyzed by Kjeldahl method.

Table 5. Comparison of percentage of nitrogen content from supernatant of protein wastes with protease digestion

Sample	% nitrogen content		
	Before protease digestion	After protease digestion for 20 min	% increasing
Red meat	4.7	5.9	1.2
Drained water	6.6	7.3	0.7
Viscera and blood	15.2	18.9	3.9

CHAPTER IV
DISCUSSION

4.1 Isolation and identification of protease producing strain

A mesophilic *Bacillus mycoides* that produced a thermostable protease was isolated from soil of hot spring at Ampher Hui Sai Kwao, Chiang Rai province. Firstly the isolated bacteria were screened for skim milk digestion on medium. Two methods (pick and drop in well) were used to select around the colonies on the basis of clear zone formation. Eighty strains were isolated from the screened samples. The proteolytic activity of this strain was selected as the most potential producer of proteolytic enzyme.

The isolated thermostable proteolytic strain, strain No. 2, was a spore forming, gram positive, and identified as *Bacillus mycoides* by microscopic appearance and biochemical test. The principle of using biochemical test to characterize microorganism had gained wide acceptance. The biochemical test was used on basic of chemical composition of cell wall and reaction of product metabolism. This study was performed by using *Bacillus subtilis* TISTR 25 and *Bacillus mycoides* from Bergey's manual characteristic comparison with unknown strains of *Bacillus* sp. The results were compared with the characterized strains in Bergey's manual. Then the analysis of nucleic acid sequences was used to support identification of bacterial strain of this study. Amplification of DNA fragment which encoded for 16S rRNA gene that interspersed among highly conserved regions and have proven useful as primer target sites for establishing partial sequence derived from direct RNA sequencing (Lane, 1985). The DNA sequence of amplified fragment of 16S rRNA gene similar to *Bacillus mycoides.* The identifications by both biochemical tests and DNA sequence of amplified fragment of 16S rRNA gene supported that this bacterium was *Bacillus mycoides.*

4.2 Optimization of protease production

Among various nitrogen sources tested, tryptone produced the maximum protease activity. A similar study by Phadatare et al., 1988 reported the enhancement of protease production of *C .coronatus* in organic nitrogen sources such as tryptone, peptone,yeast extract, skim milk and soybean meal were similar.

To improve and maximize the production of enzyme, optimization of the environmental conditions on enzyme production in shaking flask was performed. The results of comparative culture medium between peptone medium (Raja, 1994) and yeast extract medium (Kasem, 1987) indicated that peptone medium gave the highest protease activity. First, this experiment was to find the optimum inoculum size. The purpose of this optimization was to determine the growth rate and the time to produce the highest protease activity. The result revealed that 1% of inoculum size was an optimum condition. One percentage of inoculum size gave the highest protease activity at 25 hours while over 2% of inoculum size showed lower activity.

The optimum temperature was 37°C for the cultivation while bacterium was not grown over 40°C. The activity of protease was necessary for survival of bacteria because protease hydrolyzed peptone in medium to amino acids, which provided growth and energy of bacteria. Consequently, growth rate of bacterium was parallel to the activity of protease.

The effect of pH on enzyme activity was examined in the pH range of 4.0 – 11.0. Maximal proteolytical activity toward azocasein was observed at pH 8.0. The pH of culture medium affected ionization of some functional groups on molecule of enzyme. It was found that the pH of medium after inoculum about 30 hours was risen to pH 8-10.

Raja, (1994) reported that addition of glucose in medium was shown the decrease of protease activity. The result of growth pattern of *Bacillus mycoides* showed that the pH of medium was increased to acid range. This result was indicated that these bacteria utilized nutrient in the medium to produce acid. Then the optimum condition showed the highest enzyme activity at pH 7-10. This result supported that these bacteria had preferred to firstly use glucose and followed by amino acids when glucose was absence. Laishley and Berlohr, (1958) studied alkaline protease from *Bacillus*

formis A-5 and reported that the alkaline protease was controlled by catabolic repression system of glucose. It was concluded that alkaline protease from *Bacillus mycoides* was coincidentally controlled by catabolic repression of glucose.

The determination on optimum percentage of nitrogen content was performed. Too much of protein substrate (tryptone) affected over production of amino acids and revealed that 1.4% nitrogen of tryptone was optimum condition for cultivation of these bacteria. From the data of identification method, this bacterium was a facultative aerobic bacterium. Then the result on the optimum agitation rate was 250 rpm. In this study the agitation rate 200 and 250 rpm were slightly different in protease activity but agitation rate of 300 rpm was sharply different from the others. The agitation rate of 300 rpm should provide more of oxygen dissolved on medium and the mechanical forces from shaker could cause cell lysis. The oxygen dissolved had the effect on cell wall component and metabolism of bacteria.

4.3 Purification of protease

The protease from *Bacillus* sp. had been purified and characterized by many researchers. Mc Conn et al, (1964) reported that protease from *B. subtilis* was purified by ion exchange chromatography on DEAE cellulose and CM-cellulose. Leighton and Doi, (1973) reported that protease from *B.subtilis* WB746 was purified by ion exchange chromatography on DEAE Sephadex A-50, CM-cellulose, and Sephadex G-50.

In this study, the purified protease from *Bacillus mycoides* was obtained after 3 steps of purification: ultrafiltration, 50% acetone precipitation, and Sephadex G-75 column chromatography. When the data of purification was analyzed, the enzyme was purified to 21.21 folds and the specific activity was 30.97 units/mg protein with 0.458% yield. Native polyacrylamide gel electrophoresis was the most satisfactory method available to identify the purity of protein. It was found that protein stain in lane 7-8 (figure. 37) showed of a single band corresponded to the activity stain. This indicated that the enzyme from Sephadex G-75 was pure enzyme.

4.4 Characterization of protease

The protease from *Bacillus mycoides* was characterized by many methods. First, the relative molecular mass was determined by SDS-PAGE and Sephadex G-75 column chromatography. Enzyme was reduced by mercaptoethanol and denatured by heated at 100°C for 10 min. This method was damaged disulfide bond and then damaged the folding of protein. The result of SDS-PAGE showed the relative molecular mass of 31,000 Da. In addition, It was a single band of strained protein. While the relative molecular mass by Sephadex G-75 gave a value of 27,600 Da. The difference on value of molecular mass from SDS-PAGE and gel filtration indicated the significant was different between denatured and native protein. The higher value of denatured protein (31,000 Da) indicated that protein was damaged from the compact form holding by disulfide bond and other weak bonds (H-bond, Van der Waals bond etc.). The weak bonds affected protein folding. The defolding might stretch the protein chain. Then the compact or globular protein should be migrated faster than the stretching protein. Since the denatured protein was stretched and the result from denatured protein gave the higher value than native protein. From this result, it was reasonable to conclude that the molecular mass of protein was 27,600 Da.

However, Sookkheo et.al. (2000) who studied thermostable protease from *Bacillus sterothermophillus* TLS 33 reported that there were 3 isozymes of protease and each isozyme showed molecular mass of 36,000, 53,000 and 71,000 Da, respectively. Han et.al (1998) reported a higher molecular mass of 39,000 Da for thermostable alkaline protease produced by *Bacillus thermoruber*. On the other hand, thermostable protease from *Archaebacterium* was studied recently to have biochemical adaptation for their extreme environments. Wilfried et al. (1996) reported that hyperthermostable protease from *Pyrococcus furiosus* showed molecular mass of 150,000 Da and 130,000 Da on SDS-PAGE. Lin et al. (1989) showed that thermostable protease from *Sulfolobus acidocaldarious* (Thermopsin) had molecular mass of 46,000Da (Sephadex G-75, and 51,000Da from its electrophoretic mobility in SDS-PAGE). Kulakova et.al (1992) reported that cold-active serine protease alkaline protease from *Shewanella* sp. Ac10 had molecular mass of 43 kDa on SDS-PAGE.

4.4.1 Effect of temperature and pH on enzyme activity

The effect of temperature on the protease activity toward azocasein was conducted at various temperatures and pHs. The optimal temperature for the protease activity was 60°C and pH 8.0. Although the experiment was replaced carbonate buffer at pH 9-11, the result was not significantly different on activity profiles. Denaturation of protein at elevated temperature was usually the result of unfolding which was followed by an irreversible process, most often aggregation (Agard, 1993). The unfolding process involved in irreversible denaturation often had a partial (as oppose to global) character has been confirmed experimentally in several cases (Lomas, 1992). Thermal denaturation of alkaline protease also depended on partial unfolding processes. However, they were not followed by aggregation and autolytic degradation at unknown sites in the partially unfolding molecule (Dahlquist, 1976). The observation showed that this bacterium was mesophillic bacterium *Bacillus mycoides*. This result of optimum temperature for enzymatic assay was parallel to general mesophilic bacterium. Raja et al. (1993) studied thermostable protease from *Bacillus stearothermophillus* F1 had reported the optimum temperature at 80°C and pH 9.0 (carbonate buffer). Banerjee et al. studied thermostable protease from *Bacillus brevis* had reported that optimum temperature was at 60°C and pH 10.5 (NaOH/glycine buffer).

pH had an effect on ionization of amino acid residues on enzyme molecule. The ionization of amino acid residues on enzyme involved in enzyme and substrate binding. Then the ionization of amino acids had a significant effect on activity and stability of enzyme.

4.4.2 Effect of metal ions and inhibitors on enzyme activity

Metal ions were tested for the effect on enzyme activity as the followings: $FeCl_3.6H_2O$, $NaNO_3$, $MnCl_2$, $FeSO_4$, $AgNO_3$, $ZnSO_4$, NH_4Cl, $MgSO_4$, KBr and $LiCl_2$. The results had revealed that the presence of $HgCl_2$, $ZnCl_2$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $K_2CO_3.6H_2O$ and $RbCl_2$ at 5mM inhibited protease activity. However $MgCl_2.6H_2O$ and $CaCl_2$ was shown to activate the activity of a thermostable protease. The effect of metal ion on enzyme activity was depended on strong binding between amino acids residues

on enzyme and metal ion. A permanently bound between an essential amino acid and metal ion made enzyme lost activity.

The activation of protease activity by metal ion ($CaCl_2$, and $MnCl_2$) had been reported by Banerjee et al. (1999) (*Bacillus brevis*), Raja et al. 1993 (*Bacillus stearothermophillus* F1) Sookkheo et.al (2000)(*Bacillus stearothermophillus* TLS33) and Han et al. (1998) (*Bacillus pumilus*).

These results correlated the earlier findings of metal ions enhancing the production and stabilizing the activity of protease enzyme. Ferrero et al.(1996) reported the use of trisodium citrate along with $MgSO_4$, $CaCl_2$, $MnSO_4$, for protease production by *Bacillus licheniformis* MIR29.

Furthermore, the purified protease from *Bacillus mycoides* was partially inhibited by PMSF, a serine protease inhibitor. However it was inhibited by the denaturant reagents such as hydroxyamine HCl and guanidium hydrochloride . Then it was significantly inhibited by EDTA, a chealating agent. The completely inhibited enzyme by EDTA had revealed that this protease needed to react with metal ion especially divalent cation such as Ca^{2+},Mn^{2+},Mg^{2+}, respectively.

4.4.3 Substrate specificity

The protease from *Bacillus mycoides* was tested with natural (casein) and modified protein (azocasein). From the result it was found that this protease gave higher affinity on azocasein, a modified protein with Km = 26.32 mg/ml than casein, a natural protein with Km = 125.00 mg/ml. Furthermore, this protease exhibited V_{max} on casein and azocasein of 3.80 Units/mg protein and 17.00 Units/mg protein. These results revealed that the high affinity of protease on modified protein should be involved in the structure of azocasein. The azo-group (-N=N-) were random on protein (casein) by modifying reaction of sulfanilide acid at 40% NaOH. Unfortunately, the certain position of this functional group and structure of azocasein were not revealed.

4.4.4 Hydrolysis of protein wastes from tuna canned industry

There were three kind of the wastes; red meat (solid), drained water, and mixture of viscera and blood. The red meat was taken off by trimming process in order to collect only white meat. The water from washing tuna fish processes was enriched with protein. The washing drain were boiled and stored in reservoirs. This process had separated some of proteins into two phases of liquid protein and solid protein. Before the fish were boiled, the fish were separated from blood and viscera. Thus, there were three kinds of protein wastes from this industry.

The wastes could be reused for feed additive and other industries. To make this protein in the form of protein hydrolysate, it was digested with protease. The three kind of wastes were digested with protease from *Bacillus mycoides*. The results had revealed that the digested product (%nitrogen content) from mixture of viscera and blood was given the highest digestion product (39%). The red meat and drained water gave the digested products of 1.2% and 0.7% nitrogen content. The products from digestion of protease were determined by Kjedahl method.

From the analysis of the results, the highest digestion produced from mixture of viscera and blood might be due to the higher initial soluble protein such as hemoglobin, myoglobin, and enzyme in digestive organ (viscera).

From the results of this study, this protease might be used for application of protease in industry. However the future study might be an upscale production of this protease. From the optimization result, this protease could be produced in low prize because this culture medium was inexpensive. Furthermore, the essential amino acid on active site of enzyme should be confirmed in order to understand the biological adaptation of bacteria. Then the other enzymatic application was necessary to fulfill this study in order to make useful in this knowledge.

Chapter V
CONCLUSION

1. The screened for thermostable protease was isolated from soil of hot spring water at Amphor Hui Sai Kwao, Chiang Rai province. Biochemical test and 16S rRNA gene sequnce comparison were identified and the results were revealed that the bacterium was *Bacillus mycoides* strain.
2. The optimization conditions of protease production on peptone medium were at 37°C, initial pH 8.0, inoculum size 1.0%, initial nitrogen content 1.4%, and tryptone was used to replace peptone as nitrogen source and carbon source.
3. Thermostable protease from *Bacillus mycoides* was determined the optimum condition for assay activity. The optimum conditions of the enzyme were 60°C, and pH 8.0.
4. Thermostable protease was purified by 50% acetone precipitation and Sephadex G-75 column chromatography with 0.46% yield and specific activity of 30.97 units/mg protein.
5. The results from SDS-PAGE and Sephadex G-75 column chromatography were indicated that the thermostable protease had molecular mass 27,600 Da.
6. The loss of thermostable protease activity in the presence of PMSF and EDTA was indicated the significant of essential amino acid on active site might be serine.
7. Thermostable protease exhibited the K_m on casein and azocasein was 125.00 mg/ml and 26.135 mg/ml and exhibited V_{max} on casein and azocasein was 3.80 Units/mg protein and 17.00 Units/mg protein.
8. The application of this protease toward protein wastes from tuna canned industry exhibited the highest digestion product with the 3.9% nitrogen content on mixture of viscera and blood.

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APPENDICES

Appendix A: Culture medium preparation (1L)

Every culture mediums were adjusted volume to 1 liter with distilled water and sterilized by autoclaving at 121 °C for 20min.

1. Bacteria medium (pH 8.0)

Meat extract	5.0 g
Peptone	10 g
NaCl	5.0 g
Agar	20 g

2. Skim milk Agar medium

Skim milk	10 g
Agar	20 g

3. Peptone medium (Raja, 1993) (pH 7.0)

$CaCl_2.7H_2O$	0.5 g
K_2HPO_4	0.2 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.1 g
Peptone	10 g

4. Yeast extract medium (Kasem,1993) (pH 7.0)

KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2.2H_2O$	0.01g

5. Nutrient Agar (pH 7.0)

Beef extract	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15 g

6. Thioglycolate broth (pH 7.2)

Trypticase	20 g
Glucose	10 g
NaCl	5.0 g
Sodium thioglycolate	2.0 g
Sodium formaldehyde sulfoxylate	1.0 g

7. Voges-Proskauer broth (pH 6.5)

Protease peptone	7.0 g
Glucose	5.0 g
NaCl	5.0 g

8. Basal medium for acid production from carbohydrates (pH 7.4)

Diammonium hydrogen phosphate	1.0 g
KCl	0.2 g
$MgSO_4$	0.2 g
Yeast extract	0.2 g
Agar	15 g

Carbohydrate solutions sterilize 10% (w/v) aqueous solutions of each test substrate by autoclaving at 110°C for 20 min. Then add aseptically sufficient carbohydrate solution to tubes of sterile basal medium to give a final concentration of 0.5%.

9. Citrate utilization medium (pH 6.8)

Trisodium citrate	1.0 g
$MgSO_4.7H_2O$	1.2 g
$(NH_4)_2HPO_4$	0.5 g
KCl	1.0 g
Trace element solution (see below)	40. ml
0.04% (w/v) phenol red	20 ml

10. Trace element solution

EDTA	500 mg
$FeSO_4.7H_2O$	200 mg
$ZnSO_4.7H_2O$	10 mg
$MnCl_2.4H_2O$	3.0 mg
H_3BO_3	30 mg
$CoCl_2.6H_2O$	20 ml
$CuCl_2.2H_2O$	1 mg
$NiCl_2.6H_2O$	2 mg
$Na_2MoO_4.2H_2O$	3 mg

11. Phenylalanine agar (pH 7.3)

Yeast extract	1 g
NaCl	5 g
Agar	12 g
DL-Phenylalanine	2 g
Na_2HPO_4	1 g

12. Egg-yolk reaction medium (pH 7.6)

Tryptone	10 g
Na_2HPO_4	5 g
KH_2PO_4	1 g
NaCl	2 g
$MgSO_4.7H_2O$	0.1 g
Glucose	2 g
Egg-yolk	1.5 ml (aspirated aseptically or a sterile commercial preparation)

13. Nitrate broth (pH 7.0)

Peptone	5 g
Beef extract	3 g
Potassium nitrate	1 g

14. Reagent A

α- Napthylamine	5.0 g
Acetic acid (5N),30%	1000 ml

15. Reagent B

Sulfanilic acid	8 g
Acetic acid (5N), 30%	1000 ml

16. Indole production medium

Tryptone or trypticase	10 g

17. Kovacs indole reagent

Iso-amyl alcohol	150 ml
p- Dimethylaminobenzaldehyde	10 g
HCl (concentrated)	50 ml

18. Starch agar (pH 7.0)

Potato starch	1 g
Beef extract	3 g
Peptone	5 g
NaCl	5 g

19. Hugh and Leifson's Oxidative Fermentation basal medium (pH 7.1)

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	3.0 g
Bromthylmol Blue	0.03-0.08 g

Appendix B. Reagent

1. Tracking dye for SDS – PAGE

1 M Tris-HCl pH 6.8	0.06 ml
50% glycerol	2.5 ml
10% SDS	2.0 ml
β- mercaptoethanol	0.5 ml
1% bromphenol blue	1.0 ml
Distilled water	3.4 ml

2. Electrode buffer (pH = 8.3) for SDS PAGE

Tris (hydroxymethyl) aminomethane	3.0 g
Glycine	1.0 g
SDS	1.0 g

Adjusted volume to 1 liter with distilled water

3. Staining solution

Coomassie brilliant blue R-250	0.1 g
Methanol	45 ml
Acetic acid	10 ml
Distilled water	45 ml

4. Destaining solution

Methanol	45 ml
Acetic acid	10 ml
Distilled water	80 ml

5. Gram 's Crystal violet solution

Reagent A	
Crystal violet	2 g
95% Ethanol	20 ml
Reagent B	
Ammonium oxalate	0.8 g
Distilled water	80 ml

Mix reagent A and B and store in brown bottle.

6. Gram's iodine solution

Iodine (crystal)	0.25 g
KI	10 ml
Distilled water	300 ml

7. Gram's safanin straining solution

Safanin	0.25 g
95 % Ethanol	10 ml
distilled water	100 ml

8. Malachite green solution

Malachite green	5 g
Distilled water	100 ml

9. H_2O_2 solution (3%)

H_2O_2 (30%)	10 ml
Distilled water	90 ml

10. 30% Acrylamide solution

N,N' – methylene-bis-acrylamide	0.8 g
Acrylamide	29.2 g
Distilled water	100 ml

11. Tracking dye for SDS – PAGE

1 M Tris-HCl pH 6.8	0.06 ml
50% glycerol	2.5 ml
10% SDS	2.0 ml
β- mercaptoethanol	0.5 ml
1% bromphenol blue	1.0 ml
Distilled water	3.4 ml

12. Electrode buffer (pH = 8.3) for SDS- PAGE

Tris (hydroxymethyl) aminomethane	3.0 g
Glycine	1.0 g
SDS	1.0 g

Adjusted volume to 1 liter with distilled water

13. Staining solution

Coomassie brilliant blue R-250	0.1 g
Methanol	45 ml
Acetic acid	10 ml
Distilled water	45 ml

14. Destaining solution

Methanol	45 ml
Acetic acid	10 ml
Distilled water	80 ml

15. 1.5 M Tris – HCl (pH 8.8)

Tris(Hydroxymethyl)-aminomethane	18.17 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

16. 2 M Tris – HCl (pH 8.8)

Tris (Hydroxymethyl)-aminomethane	24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

17. 0.5 M Tris –HCl (pH 6.8)

    Tris (Hydroxymethyl)-aminoethane 0.06 g

    Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

18. 1.0 M Tris-HCl (pH 6.8)

    Tris (Hydroxymethyl aminoethane) 12.1 g

    Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

19. Solution B (SDS-PAGE)

    2 M Tris-HCl pH 8.8 75 ml

    10 % SDS 4 ml

    Distilled water 21 ml

20. Solution C (SDS-PAGE)

    1 M Tris –HCl (pH 6.8) 75 ml

    10% SDS 4 ml

    Distilled water 46 ml

21. Tracking dye for non-denaturing PAGE

    1 M Tris –HCl pH 6.8 3.1 ml

    Glycerol 5.0 ml

    1% bromophenol red 0.5 ml

    Distilled water 1.4 ml

22. Electrode buffer (pH8.3) for native PAGE

    Tris (Hydroxymethyl)- aminoethane 3.03 g

    Glycine 14.40 g

    Dissolved in distilled water to 1 liter.

23. 0.2% Azocasein solution

Azocasein	2 g
Ethanol	10 ml

Adjusted volumes with distilled water to 1000 ml then over layer this solution with toluene.

24. Bradford stock solution

95% Ethanol	100 ml
82% Phosphoric acid	200 ml
Serva blue G	350 mg

25. Bradford working solution

Distilled water	425 ml
95% Ethanol	15 ml
85% Phosphoric acid	30 ml
Bradford stock solution	30 ml

Filter thorough Whatman No.1 paper. Store at room temperature in brown glass bottle.

Appendix C: Standard curve of protease activity (Subtilisin A) on azocasein

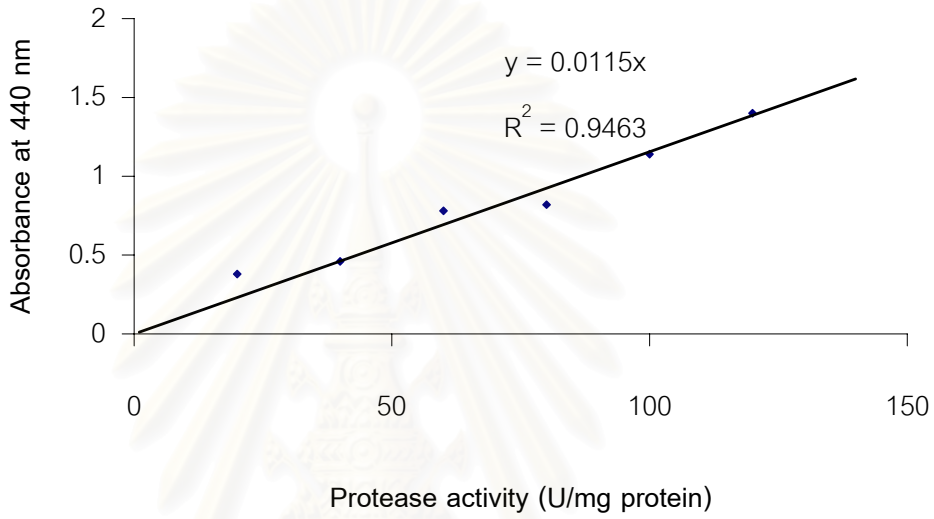

Figure50. Standard curve of protease activity (Subtilisin A)(20 min; pH 9.0)

Appendix D: Stability of thermostable protease from *Bacillus mycoides*

Table 6. Thermostability of thermostable protease from *Bacillus mycoides*

	Temperature(°C)	Remaining activity after incubation for 30 min (Units/mg protein)	Residual activity after incubation for 30 min (%)
Crude enzyme	0	30.14	100
	30	27.21	90.27
	37	26.04	86.39
	40	24.36	80.82
	50	16.78	55.67
	60	16.43	54.51
	70	10.02	33.24
	80	0	0
	90	0	0
	100	0	0
Purified enzyme	0	17.95	100
	30	15.22	84.79
	37	15.04	83.78
	40	14.97	83.39
	50	9.11	50.75
	60	6.22	34.65
	70	2.14	11.92
	80	0	0
	90	0	0
	100	0	0

BIOGRAPHY

Mr. Chaiyasit Sittiwet was born on January 19,1978. He graduated with the Bachelor Degree of Science in Chemistry from Mahasarakham University in 1999 and continued studying for Master in Biochemistry Program, Faculty of Science, Chulalongkorn University.