RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2006.32.377

ScienceAsia 32 (2006): **377-383**

Purification and Characterization of Alkaline Protease from *Bacillus megaterium* Isolated from Thai Fish Sauce Fermentation Process

Siriporn Yossan[a], Alissara Reungsang[b*] and Masaaki Yasuda[c]

[a] Department of Biotechnology, Graduate School, Khon Kaen University, Khon Kaen, 40002, Thailand.
[b] Fermentation Research Centre for Value Added Agriculture Products, Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, 40002, Thailand.
[c] Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Okinawa, 9030129, Japan.

* Corresponding author, E-mail: alissara@kku.ac.th

Received 6 Feb 2006
Accepted 31 May 2006

ABSTRACT: An alkaline protease produced by *Bacillus megaterium* isolated from a fermented broth of Thai fish sauce was purified by hydrophobic interaction combined with gel filtration techniques. After final purification step, the enzyme was purified 148-fold with an increase in specific activity from 0.09 to 13.33 U/mg protein. The properties of the purified enzyme were then analyzed. The molecular weight of the enzyme under denaturing condition was estimated to be 27 kDa. The optimum pH and temperature for protease activity were 10 and 50 °C, respectively. This protease could retain the activity in the pH and temperature ranging from pH 7.5 to 9.5 and 30 to 45 °C, respectively, resulting in the relative activity of higher than 80%. The enzyme was completely inhibited by diisopropyl fluorophosphate (DFP) suggesting that it was a serine protease. Phenylmethylsulfonyl fluoride (PMSF), p-Chloromercuribenzoic acid (PCMB), ethylenediaminetetraacetic acid (EDTA), 1, 10-phenanthroline and Fe^{3+} strongly inhibited the activity of this purified enzyme activity, decreasing relative activity to lower than 15%. Protease activity was enhanced by Mn^{2+}, $Ca^{2+}$and Mg^{2+}. Cytochrome C, soybean protein isolate and casein were good substrates specific to the enzyme with the relative activity of more than 100%.

KEYWORDS: alkaline protease, *Bacillus megaterium*, characterization, purification, Thai fish sauce fermentation process.

INTRODUCTION

Fish sauce is one of the fermented sauces commonly used in every Thai household and in most parts of Southeast Asia. The fish sauce fermentation process starts by mixing fish with salts and then transferring it to the underground fermentation tank. Normally the fish sauce fermentation process lasts about 8-12 months[1]. The liquid obtained contains fish protein, which is considered as a good source of protein[2].

The most important enzymes associated with fish sauce fermentation are amino acid degradation catalyzing enzyme called proteases[3]. Protease hydrolyzes proteins into smaller peptide units or free amino acids, but can also catalyzes peptide synthesis in organic solvents or in solvents with low water content. Proteases constitute a large and industrially important group of enzymes. They make up about 60% of the total of worldwide sale of enzymes[4].

In our previous study, *Bacillus megaterium* capable of producing protease at high temperature was isolated from the Thai fish sauce fermentation process. Preliminary characterization indicated that this enzyme was an alkaline protease. However, this enzyme has not been purified and characterized yet. An advantage of enzyme purification is an increase in enzyme specific activity, making the enzyme after purification more specific for industrial applications[5,6]. In order to elucidate the specific functions of the enzyme clearly, there is a need to characterize an enzyme after a purification. Therefore, in this research we attempted to purify the alkaline protease enzyme from *B. megaterium* isolated from Thai fish sauce fermentation process using hydrophobic interaction chromatography coupled with gel filtration and then to characterize the purified enzyme.

MATERIALS AND METHODS

Materials

Butyl-Toyopearl 650 M and Phenyl-Toyopearl 650 M were purchased from Tosoh Corp., Tokyo, Japan.

Superdex 75 10/300 GL was purchased from Amersham Bioscience, Uppsala, Sweden. All chemicals were of analytical grade.

Microorganism and Culture Conditions

Bacillus megaterium isolated from Thai fish sauce fermentation process was used for the protease production. It was cultured in mM73 broth consisting of 0.8% skim milk, 0.1% yeast extract, 1% $MgSO_4.7H_2O$, 0.5% KCl, 0.02% $CaCl.2H_2O$, and 0.5% NaCl. The mM73 was prepared by mixing all compounds except skim milk together in distilled water. pH was adjusted to 9 before sterilization at 110 °C for 20 min. Skim milk solution was separately sterilized at 110 °C for 20 min. These two solutions were mixed aseptically before use. The culture was incubated at 45 °C, 200 rpm for 24 h. The cells were harvested by centrifugation at 10,000 g, 4 °C for 10 min. The supernatant was used as a crude enzyme solution to be purified in the further experiments.

Enzyme Assay and Protein Determination

Alkaline protease activity was determined by measuring the release of trichloroacetic-acid soluble peptides from 1% casein in 10 mM borate buffer (pH 9) at 55 °C for 30 min (modified from Aoyama[7]). One unit enzyme activity was defined as the amount of enzyme that releases 1 µg of tyrosine per ml per min under the above assay conditions. Specific enzyme activity was expressed as units/mg protein.

Protein was measured by the method of Lowry assay using the DC protein assay kit (Bio-Rad, CA, USA) with bovine serum albumin (BSA) as the standard protein.

Purification Procedure

All operations, except for HPLC, were performed at 4 °C

Step 1 Hydrophobic interaction treatment was applied to purify the crude enzyme solution. The resin, butyl-Toyopearl 650 M, was equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 30% saturated ammonium sulfate. The liquid part in the saturated resin was vacuum pumped out from the resin before the crude enzyme solution, 1,000 ml, treated with ammonium sulfate (30% saturation) was applied to the resin at 4 °C. The enzyme solution was then pumped out from the resin and assayed for total protease activity. The bound enzyme on the resin was then eluted again with 150 ml of 10 mM Tris-HCl buffer. The eluted solution was assayed for total protease activity. These steps were repeated three times to obtain the active enzyme solution with the total protease activity more than 1,000 mU. These active fractions were pooled and concentrated by ultrafiltration (UHP-90K, Advantec, Japan). The concentrated enzyme was dialyzed against 10 mM Tris-HCl (pH 8.5).

Step 2 The dialyzed enzyme solution (from step 1) treated with ammonium sulfate to obtain 30% saturation was then purified by hydrophobic interaction column. This solution was applied to a phenyl-Toyopearl 650 M column (1.2 x 18 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 30% saturated ammonium sulfate at 4 °C. After application of enzyme, the bound protein was eluted thoroughly from the column using 10 mM Tris-HCl buffer (pH 8.5) with a linear gradient from 30% to 0% of saturated ammonium sulfate in this buffer. Each fraction of 5 ml at the flow rate of 1.5 ml/min of eluted enzyme solution from the column was collected to be assayed for protease activity and protein content (OD 280) until the optical density of the effluent at 280 nm approached to zero. In this research, the active fractions justified by high protease activity of more than 10 mU/ml coupled with low protein content (OD 280 of less than 0.07) were pooled and concentrated by ultrafiltration (UHP-90K, Advantec, Japan). The concentrated enzyme was dialyzed against 10 mM borate buffer (pH 9.0).

Step 3 Gel filtration chromatography was applied to purify the enzyme solution obtained from Step 2. Each 0.5 ml of enzyme solution was injected into a superdex 75 10/300 GL column equilibrated with 10 mM borate buffer (pH 9). The protein was eluted from the column using the same buffer at the flow rate of 0.3 ml/min. Different types of protein were eluted from the column at the different retention times depending on the molecular weight of the proteins. The fraction of each type of protein was collected and analyzed for protease activity. The active fractions were pooled and concentrated by ultrafiltration (Centriprep YM-30, Millipore) and then stored at -20 °C prior to further study on the enzyme properties.

Molecular Weight Determination

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% acrylamide gel by the method of Laemmili[8]. Proteins were stained with Coomassie brilliant blue R-250 (Sigma). The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins consisting of myosin, 200 kDa; ²-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

Characterization of the Enzyme

Optimal pH for enzyme activity

The purified protease was determined for an enzyme activity by using 1% casein as a substrate dissolved in different pH of the buffer as follows: 10 mM McIlvaine (pH 3.0-7.0), borate buffer (pH 7.0-10.0), Tris-HCl buffer (7.0-10.0) and sodium borate buffer (pH 10.0-12.0).

pH stability of the enzyme

To determine the pH stability of an enzyme, a purified protease was pre-incubated at 37 °C for 60 min at different pH of the buffer as follows: 10 mM McIlvaine (pH 3.0-7.0), borate buffer (pH 7.0-10.0), Tris-HCl buffer (7.0-10.0) and sodium borate buffer (pH 10.0-12.0). The enzyme activity was then determined by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 10.0).

Optimal temperature for enzyme activity

The purified protease was determined for an enzyme activity by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 10.0) and incubated at varied temperatures of 30, 40, 45, 50, 55, 60, 70, 80 and 90 °C.

Thermal stability of the enzyme

To determine the thermal stability of an enzyme, a purified protease was pre-incubated in 10 mM borate buffer (pH 8.0) for 30 min followed by incubation at varied temperatures of 0, 30, 40, 45, 50, 55, 60, 70, 80 and 90 °C for 15 min. The enzyme activity was further determined by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 10.0) at 50 °C.

Substrate inhibitor of the enzyme

The purified protease was determined for an enzyme activity. The substrate inhibitor was added to a purified protease at a final concentration of 1 mM before analyzing the enzyme activity. The substrate inhibitors tested were chymostatin, antipain, pepstatin A, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), E-64, trypsin inhibitor, monoiodacetic acid (MIA), 1, 10-phenanthroline, p-Chloromercuribenzoic acid (PCMB), sodium dodecyl sulfate (SDS), N±-tosyl-L-lysyl chloromethyl ketone (TLCK), L-1-tosyl-phenyllalanyl-chloromethyl ketone (TPCK), N-ethylmaleimide (NEM) and diisopropyl fluorophosphate (DFP). The enzyme activity was then determined by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 10.0) at 50 °C.

Effect of metal salts on enzyme activity

The metal salt at a final concentration of 1mM was added to the purified protease before analyzing the enzyme activity. The metal salts tested were $AlCl_3$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_3$, $CuSO_4$, $FeCl_3$, $HgCl_2$, $MgCl_2$, $MgSO_4$, $MnCl_2$, $NiCl_2$, $ZnCl_2$. The enzyme activity was then determined by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 10.0) at 50 °C.

Substrate specificity of the enzyme

The purified protease was assayed at 50°C for substrate specificity by using different substrates dissolved in 10 mM borate buffer (pH 10.0) at the concentration of 1 mg/ml. The substrates tested were casein, gelatin, hemoglobin, albumin (egg), soybean protein isolate, gluten, albumin (bovine) and cytochrome C.

Results and Discussion

Purification of Protease

The purification procedure is summarized in Table 1, showing that the enzyme was purified 3-fold with a specific activity of 0.29 U/mg protein after butyl-Toyopearl 650M. The enzyme solution was further purified using phenyl-Toyopearl 650 M column. This purification step showed 107-fold enzyme purification with a specific activity of 9.66 U/mg protein. The dialyzed enzyme was then purified using a Superdex 75 10/300 GL column. The final purification step presented 148-fold enzyme purification with a specific activity of 13.33 U/mg protein. These results indicated the effectiveness of purification method. However, the yield of the enzyme after purification was found to be low (2%). This might be due to the result of autolysis of the enzyme during purification. Chomsri[9] suggested that the low enzyme yield (4%) obtained after enzyme purification was probably owing to partial autolysis by molecular unfolding of the enzyme[10].

Characterization of the Purified Enzyme

The enzyme purity was confirmed by SDS-PAGE which demonstrated a single band (Fig. 1), indicating a homogeneous preparation[6]. The molecular weight of

Table 1. Summary of purification procedure.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude enzyme	19.8	227.36	0.09	1	100
Butyl-Toyopearl 650 M	13.0	44.91	0.29	3	66
Phenyl-Toyopearl 650 M	2.8	0.29	9.66	107	14
Superdex 75 10/300 GL	0.4	0.03	13.33	148	2

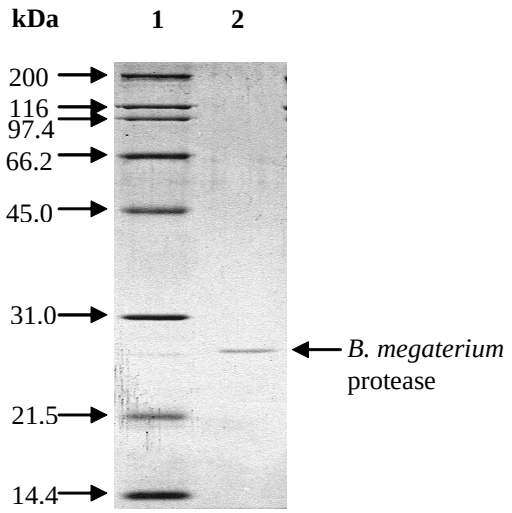

Fig 1. SDS-PAGE of the purified protease from *B. megaterium*. Lane 1 molecular markers (kDa) were as follows: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. Lane 2 purified enzyme.

the enzyme under denaturing condition was estimated to be 27 kDa, similar to the molecular masses of alkaline proteases, which were in the ranges of 15 to 30 kDa[11].

Optimal pH for Enzyme Activity

The optimum pH was found to be 10 (data not shown), indicating that this enzyme might be the alkaline protease[12,13]. An optimum pH of 10 of alkaline protease was observed in the protease produced by *Bacillus* sp.[14], *B. subtilis* CN2[15] and *B. subtilis* PE-11[6].

pH Stability of the Protease

The protease activity had relative activity of more than 80% in borate buffer pH ranging from 7.5 to 9.5 (data not shown). However, within this pH range, the relative activities were observed to be less than 20% in Tris-HCl buffer. Thus, protease activity depended on buffer type even at the same pH ranges. This was in agreement with a research by Chomsri[9] who reported a protease activity which was stable in the pH range of 7 to 10 with the relative protease activity of 100% in phosphate and Tris-HCl buffer. Nascimento[16] also found an alkaline protease which was active in a broad pH range of 6 to 10, with a relative activity of 60% retained at pH above 9. The pH stability of protease produced from *B. subtilis* PE-11[6] and *B. subtilis* CN2[15] were found to be in the ranges of 8 to 11 and 7 to 11 with the relative activity of more than 90% and 70%, respectively.

Optimal Temperature for Protease Activity

The optimum temperature for protease activity was assayed at different temperature from 30 to 90 °C using casein as a substrate dissolved in borate buffer pH 10. The protease activity was active at 30 to 50 °C, but inactive in the ranges of 55 to 90 °C. The optimum temperature of protease activity was 50 °C (Fig. 2), similar to the report[12] that the optimum temperature of alkaline proteases ranged from 50 to 70 °C. Optimum temperature for production of protease by *B. subtilis* CN2 and *B. pumilus* was at 50 °C [7,15]. However, a higher optimum temperature of 60 °C was observed in *Bacillus* sp. SMIA-2[16], similar to the result reports of Banerjee[17] and Horikoshi[18], in which the optimum temperature for *Bacillus* protease was 60 °C.

Thermal Stability of the Enzyme

The purified protease solution was incubated at various temperatures, 0 to 90 °C, for 30 min to determine the thermal stability. The results indicated that at 30 min, the protease activity was more than 95%

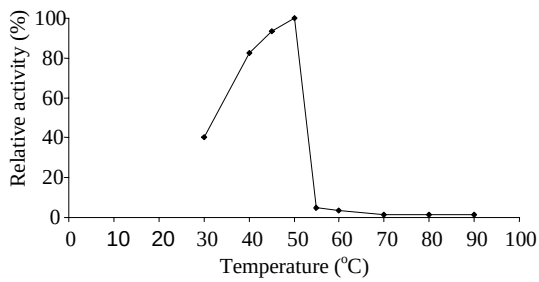

Fig 2. Protease activity at various temperatures.

at 30 to 45 °C (Fig. 3), similar to protease enzyme produced by *B. clausii*, which retained 100% after incubation at temperatures ranging from 30 to 65 °C for 60 min[19]. Protease produced by *Bacillus* sp. F603.1 was stable at 60 °C for 90 min[9] with a relative activity of 75%, while *Bacillus* sp. SMIA-2 produced protease, which was stable at 30 °C for 2 h with a 100% relative activity[16]. The proteases obtained from cultivation of *B. pumilus* were stable at 45 and 50 °C for 30 min with the relative activity of 100% and 47%, respectively[20,21].

Inhibitors of the Protease

The effect of various inhibitors on protease activity is shown in Table 2. The enzyme was completely inhibited by 1 mM DFP, a well-known inhibitor of serine protease[4,21] suggesting that this enzyme was a serine protease. Similar results of serine proteases completely inhibited by DFP were observed in serine protease produced by *B. licheniformis*[22], *B. pumilus*[7,21] and *B. intermedius* 3-19[23].

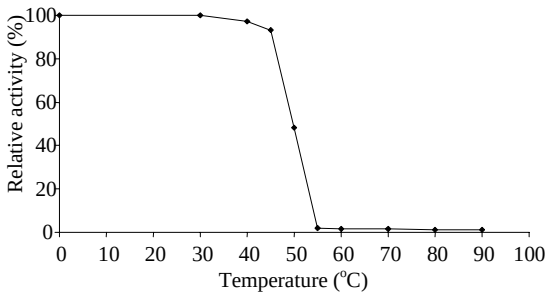

Fig 3. Thermal stability of protease activity.

Other strong inhibitors were found to be PCMB, EDTA, PMSF and 1, 10 Phenanthroline, with resulted relative activity of 2, 7, 7 and 12%, respectively. Gold[24] described an alkaline protease which was completely inhibited by PMSF and DFP in which PMSF sulfonated the essential serine in the active site and resulted in the complete loss of activity. Our result was supported by that of Adinarayana[6], who found that protease produced by *B. subtilis* PE-11 was completely inhibited by PMSF and 94% inhibited by DFP.

In contrast, the activity of this enzyme was not inhibited by pepstatin A, Chymostatin, E-64, Trypsin inhibitor, TLCK, TPCK, Antipain, MIA and NEM as indicated by relative activity of greater than 100%.

Effect of Metal Salts on Enzyme Activity

The protease activity was enhanced with an addition of Mn^{2+}, $Ca^{2+}$and Mg^{2+} resulting in the relative activity of 151, 130 and 121%, respectively, (Table 3) suggesting that metal ions had a capability to protect enzyme against denaturation. Nascimento[16] reported that protease produced by *Bacillus* sp. SMIA-2 was enhanced by Mn^{2+} and Ca^{2+}. These metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature. In addition, alkaline protease required a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations for its maximum activity[12]. These cations enhanced the stability of a *Bacillus* alkaline protease[25]. A strong inhibitory effect on protease activity of our *B. megaterium* was observed in the presence of Al^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+}, with relative activities of 19, 12, 28 and 28%, respectively (Table 3). Zn^{2+}, $Cu^{2+}$and Hg^{2+} were found to inhibit the catalytic activity of alkaline protease secreted by *B. brevis* [17].

Table 2. Effect of various inhibitors on protease activity.

Inhibitors	Relative activity (%)
None	100
EDTA	7
PMSF	7
1,10 Phenanthroline	12
PCMB	2
pepstatin A	156
SDS	93
Chymostatin	176
E-64	151
Trypsin inhibitor	154
TLCK	180
TPCK	149
Antipain	120
MIA	151
NEM	139
DFP	0

Note: The activity in absence of inhibitor (None), referred to 100% relative activity, was 178.15 mU.

Substrate Specificity of the Protease

The enzyme had a capability to effectively catalyze the hydrolysis of various proteins, including cytochrome C, soybean protein isolate, casein, human hemoglobin and wheat gluten with relative activity of more than 70% (Table 4). The best substrate for the enzyme was cytochrome C with the highest relative activity of 114% followed by soybean protein isolate (109% relative activity) and casein (100% relative activity). In contrast, gelatin, egg albumin and bovine albumin were less good substrates for the protease with lower than 50% relative activity (Table 4). Aoyama[7] reported that cytochrome C, casein and soybean protein isolate were good substrates for serine protease enzyme from *B. pumilus* with the relative protease activity of 145, 130 and 100% respectively.

Table 3. Effect of various metal salts on protease activity.

Metal salts	Relative activity (%)
None	100
$AlCl_3$	19
$CaCl_2$	130
$CdCl_2$	98
$CoCl_2$	102
$CuCl_3$	88
$CuSO_4$	91
$FeCl_3$	12
$HgCl_2$	28
$MgCl_2$	121
$MnCl_2$	151
$NiCl_2$	105
$ZnCl_2$	28

Note: The activity in absence of metal salt (None), referred to 100% relative activity, was 156.12 mU.

Table 4. Substrate specificity of the protease enzyme.

Substrate	Relative activity (%)
Casein	100
Cytochrome C	114
Soybean protein isolate	109
Hemoglobin, human	78
Gluten (wheat)	70
Gelatin, fine powder	48
Albumin (egg)	41
Albumin (bovine)	25

Note: The activity of casein, referred to 100% relative activity, was 143.67 mU.

Conclusion

This research successfully purified alkaline protease from *Bacillus megaterium* by hydrophobic interaction combined with gel filtration techniques. After final purification step, the enzyme was purified 148-fold with an increase in specific activity from 0.09 to 13.33 U/mg protein. The properties of the purified enzyme indicated that this purified protease enzyme functioned at alkaline pH. This enzyme was suggested to be a serine protease since it was completely inhibited by DFP. However, the study of amino acid sequences of this enzyme should be conducted to provide further information on the enzyme.

Acknowledgements

The authors wish to express their gratitude to the Fermentation Research Centre for Value Added Agricultural Products, Faculty of Technology, Khon Kaen University for providing the financial support for this research. Association of International Education, Japan (AIEJ) is gratefully acknowledged. We greatly appreciate Yoichi Toyokawa and Naohito Naka for their valuable technical assistance. This research is conducted under the JSPS-NRCT Core University Program (Microbial Resources).

References

1. Chaiyanan S (1992) *Involvement of enzyme in fish sauce production process.* Department of Microbiology. Faculty of Microbiology, King Mongkut's University of Technology. [In Thai]
2. Fukami K, Ichiyama S, Yaguramaki H, Masuzawa T, Nabeta Y, Endo K and Shimoda M (2002) Identification of Distinctive Volatile Compounds in Fish sauce. *J Agric Food Chem*. **50**, 5412-6.
3. Poralla K (1971) The induction of a dehydrogenase activity for branced chain amino acids in *Bacillus subtilis*. *Arch Microbiol*. **77**, 339-47.
4. Rao MB, Tanksale AM, Ghatge MS and Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev.* **62**, 597–635.
5. Kumar CG (2002) Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. *Letters Applied Microbiol*. **34**, 13-7.
6. Adinarayana K, Ellaiah P and Prasad DS (2003) Purification and Partial Characterization of Thermostable Serine Alkaline Protease from a Newly Isolated *Bacillus subtilis* PE-11. *AAPS Pharm Sci Tech*. **4,** 1-9.
7. Aoyama M, Yasuda M, Nakachi K, Kobamoto N, Oku H and Kato F (2000) Soybean-milk-coagulating activity of *Bacillus pumilus* derives from a serine proteinase. *Appl Microbiol Biotechnol*. **53,** 390-5.
8. Laemmili UK (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature*. **277**, 680-5.
9. Chomsri N (2001). *Thermostable Protease Enzymes*. [Master thesis in Biotechnology]. Chiangmai: The Graduate school, Chiangmai University.
10. Ward OP (1985) Proteolytic enzymes. In: M. Moo-Young Editor. *Comprehensive Biotechnol*. **3**, 789-818.
11. Fogarty WM, Griffin PJ and Joyce AM (1974) Enzymes of *Bacillus* species-Part 2. *Proc Biochem*. **9**, 27–35.
12. Kumar CG and Takagi H (1999) Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnol Adv*.**17**, 561-94.
13. Alencar RB, Biondi MM, Paiva PMG, Vieira VLA and Junior LBC (2003) Alkaline Proteasesfrom the Digestive Tract of Four Tropical Fishes. *Braz J Food Technol*. **6**, 279-84.
14. Gupta A, Roy I, Patel RK, Singh SP, Khare SK and Gupta MN (2005) One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. *J Chromatography A*. **1075**, 103-8.
15. Uchida H, Kondo D, Yamashita S, Tanaka T, Tran HL, Nagano H and Uwajima T (2004) Purification and properties of a protease produced by *Bacillus subtilis* CN2 isolated from a Vietnamese fish sauce. *World J Microbiol Biotechnol*. **20**, 579–82.
16. Nascimento WCA do and Martins MLL (2004) Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Braz J Microbiol*. **35**, 91-6.
17. Banerjee UC, Sani RK, Azmi W and Soni R (1999) Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Proc Biochem*. **35,** 213-9.
18. Horikoshi K (1990) Enzymes of alkalophilies. *In: Microbial Enzyme and Biotechnology* 2nd, 275-94.
19. Kumar CG, Joo HS, Koo YM, Paik SR and Chang CS (2004) Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate. *World J Microbiol Biotechnol*. **20**, 351-7.
20. Feng YY, Yang WB, Ong SL, Hu JY and Ng WJ (2001) Fermentation of starch for enhanced alkaline protease production by constructing an alkalophilic *Bacillus pumilus* strain. *Appl Microbiol Biotechnol*. **57**, 153-60.
21. Huang Q, Peng Y, Li X, Wang H and Zhang Y (2003) Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. *Current Microbiol*. **46**, 169-73.
22. Tang XM, Lakay FM, Shen W, Shao WL, Fang HY, Prior BA, Wang ZW and Zhuge J (2004) Purification and characterization of an alkaline protease used in tannery industry from *Bacillus licheniformis*. *Biotechnol Lett*. **26**, 1421-4.
23. Balaban NP, Mardanova AM, Sharipova MR, Gabdrakhmanova LA, Sokolova EA, Rudenskaya GN and

Leshchinskaya IB (2004) Purification and characterization of serine proteinase 2 from *Bacillus intermedium* 3-19. *Biochemistry (Moscow)*. **69,** 519-26.
24. Gold AM and Fahrney D (1964) Sulfonyl fluorides as inhibitors of esterases. II. Formation and reactions of phenylmethanesulfonyl alpha-chymotrypsin. *J Biochem*. **3**, 783–91.
25. Paliwal N, Singh SP and Garg SK (1994) Cation-induced thermal stability of an alkaline protease from a *Bacillus* sp. *Bioresource Technol*. **50**, 209–11.