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Minor Thermostable Alkaline Protease Produced by *Thermoactinomyces* sp. E79

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Abstract *Thermoactinomyces* sp. E79 produced two types of thermostable alkaline proteases extracellularly. A minor protease was separated from a major protease by using DEAE-column chromatography. This enzyme was purified to homogeneity by ammonium sulfate and DEAE-Sepharose ion-exchange chromatography. The purified minor protease showed different biochemical properties compared to the major protease. The molecular mass of the purified enzyme was estimated by SDS-PAGE to be 36 kDa. Its optimum temperature and pH for proteolytic activity against Hammarsten casein were 70°C and 9.0, respectively. The enzyme was stable up to 75°C and in an alkaline pH range of 9.0–11.0. The enzyme was inhibited by phenylmethylsulfonyl fluoride (PMSF) and Hg^{2+}, indicating that the enzyme may be a cysteine-dependent serine protease. In addition, the enzyme cleaved the endoproteinase substrate, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, and the K_m value for the substrate was 1.2 mM.

Key words: *Thermoactinomyces*, thermophile, alkaline protease, thermostable enzyme, purification

Many microbial proteases are important in the worldwide enzyme market because they are widely used as detergents, and in pharmaceutical and food processing industries [2]. For detergents, enzymes with high activity, stability at alkaline pHs, and resistance to oxidizing and chelating agents are the known pre requisites. In particular, alkaline proteases have attracted many researchers due to their potential use as laundry additives, and there have been many studies on alkaline proteases derived from various microorganisms. Furthermore, much attention has been given to thermostable proteases from thermophilic bacteria, because of their stability at high temperatures and their resistance to detergents, organic solvents, and chaotropic agents. However, even though proteases from thermophiles are generally shown to be stable at high temperatures, they are unstable at high pHs [5, 9, 17]. On the other hand, alkaline serine proteases from alkalophiles are unstable at high temperatures even though they are mostly stable in highly alkaline conditions [6, 16, 25]. Because of these reasons, proteases with high activity and stability in high alkali range and temperature have attracted a great deal of interest for bioengineering and biotechnological applications as well as for protein chemistry. Unfortunately, only a few studies have been conducted regarding thermostable alkaline proteases [3, 19, 23, 24]. In our laboratory, several thermostable and alkaline protease-producing organisms were isolated from soil [7, 8]. Previously, we characterized a major thermostable alkaline protease from *Thermoactinomyces* sp. E79 and reported the nucleotide sequence of the encoding gene [12, 13]. Many bacteria produce several different types of proteases at the same time [4, 10] and some *Actinomyces* produce and secrete several kinds of proteases in a culture broth, such as pronase from *Streptomyces griseus* [18]. In the case of *Thermoactinomyces* E79, it produces both major and minor proteases at the same time in the culture broth. In the present study, we purified the minor thermostable alkaline protease from *Thermoactinomyces* sp. E79, and compared its properties with those of the major protease.

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Materials and Methods

Production of *Thermoactinomyces* sp. E79 Protease

Thermoactinomyces sp. E79 was cultured in a 500-ml baffled flask with 100 ml enzyme production medium (soytone, 1.0%; soluble starch, 2.0%; K_2HPO_4, 0.3%; $MgSO_4$, 0.05%; pH 7.2) at 50°C for 16 h on a rotary shaker (250 rpm).

Enzyme Assay

Proteolytic activity was measured using the Takami method [23]. Protease activity was assayed at 55°C with

Hammarsten casein (Merck, Germany) as a substrate. Three milliliters of 0.6% (w/v) Hammarsten casein in 50 mM Tris-HCl (pH 9.0) was preincubated for 10 min at 55°C and the reaction was initiated by adding 0.5 ml of the enzyme solution. After 15 min, the reaction was stopped by adding 3.0 ml of TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). Then, the reaction mixture was kept at 4°C for 10 min and filtered through a Whatman No. 5 filter paper. Absorbance of the filtrate was measured at 275 nm. One unit of proteolytic activity was defined as the amount of the enzyme that liberated 1 μg tyrosine equivalent per min.

Protein Measurement

Protein profiles were followed by measuring absorbance at 280 nm. The protein concentration was measured by using the Bradford method [1] with the dye reagent purchased from Bio-Rad (Richmond, U.S.A.). Bovine serum albumin was used as a standard.

Purification Procedures

All procedures were carried out at 4°C. Solid ammonium sulfate was added to the culture supernatant to 30% saturation and the resulting precipitate was discarded after centrifugation at 10,000 ×g. Ammonium sulfate was again added to the supernatant to 80% saturation and it was left overnight. The precipitate was collected by centrifugation and dissolved in a buffer A (20 mM phosphate buffer, pH 7.2). Then, it was dialyzed against the same buffer. The supernatant obtained by centrifugation was applied to a DEAE-Sepharose CL-6B column pre-equilibrated with buffer A. The column was washed with buffer A followed by eluting with a linear gradient of 0-0.3 M NaCl. Two protein peaks with protease activity were eluted, one in the buffer A wash and the other with NaCl. The adsorbed protease fractions were combined, concentrated by ultrafiltration using an Amicon PM 10 membrane, and then further dialyzed against buffer B (50 mM Tris-HCl buffer, pH 8.0). The solution was reloaded onto a similar DEAE-Sepharose column equilibrated with buffer B. The active enzyme was eluted with a linear gradient of 0-0.2 M NaCl. The enzymatically active fractions were pooled and used as the purified E79 minor protease for subsequent studies.

Polyacrylamide Gel Electrophoresis and Activity Staining

SDS-PAGE was performed in 12% slab gel as described by Laemmli [11]. Before heating at 90°C for 1 min, 10 mM PMSF was added to the enzyme solution with the sample buffer. For the activity staining, SDS was removed by washing the gel twice in 50 mM Tris-HCl (pH 8.0) containing 25% (v/v) isopropanol for 1 h at room temperature, and then twice for 30 min each in the same buffer without isopropanol. Subsequently, the renatured gel was placed on a 5% skim milk agar plate and incubated at 50°C.

N-terminal Amino Acid Sequencing

The purified protein was subjected to SDS-PAGE and electroblotted on a polyvinylidene difluoride membrane (Bio-Rad Lab). After staining with Coomassie Brilliant blue R-250, protein bands were cut out. The N-terminal amino acid sequence was determined by the automated Edman method using a gas-phase protein sequencer (model 476A, Applied Biosystem Inc., Foster, U.S.A.).

Effect of Temperature and pH on Proteolytic Activity

The optimum temperature for the enzyme activity was determined by assaying protease activity at various temperatures. To test the effect of temperature on the stability of the enzyme, 1.0 μg of protease in 90 μl of 50 mM Tris-HCl buffer (pH 8.0) was incubated for 10 min at various temperatures, and then residual activities in the supernatants were assayed.

To find an optimum pH of the enzyme, protease activities were assayed at various pHs. To determine pH stability of the enzyme, 15 μl of the enzyme solution was added to 585 μl of 0.1 M sodium acetate (pH 4.0-6.0), potassium phosphate (pH 6.0-7.5), Tris-HCl (pH 7.5-9.0), and glycine-KCl-KOH (pH 9.0-13.0) buffers. After incubation for 1 h at room temperature, the remaining protease activity was measured with 70 μl of each enzyme solution.

Substrate Specificity

Peptidase activity was routinely assayed by monitoring the release of nitroaniline at 410 nm [22]. The standard assay mixture contained 5 to 100 μl of the enzyme solution, 50 mM Tris-HCl buffer (pH 9.0) up to 1.25 ml, and 25 μl of the substrate from the stock solution of 5 mg/ml in dimethylformamide. After 15 min of incubation at 55°C, the reaction was stopped with 0.25 ml of 2 M sodium citrate buffer (pH 5.0). The amount of released *p*-nitroaniline was then measured by determining absorbance at 410 nm ($\varepsilon = 8480\ M^{-1}cm^{-1}$). One unit of activity is equal to the amount of the enzyme that hydrolyzes 1 μmol of the substrate per min.

RESULTS AND DISCUSSION

Purification of Minor Protease from *Thermoactinomyces* sp. E79

Thermoactinomyces sp. E79 produced two different proteases simultaneously in the culture broth. The protease activity was separated into two distinct peaks on DEAE-Sepharose ion-exchange column chromatography, corresponding to unbound and bound fractions (Fig. 1A). The protease in the unbound fraction was dominant, comprising up to 88% of the total activity [12]. Therefore, the protease in the bound fraction was referred to as the minor protease. The

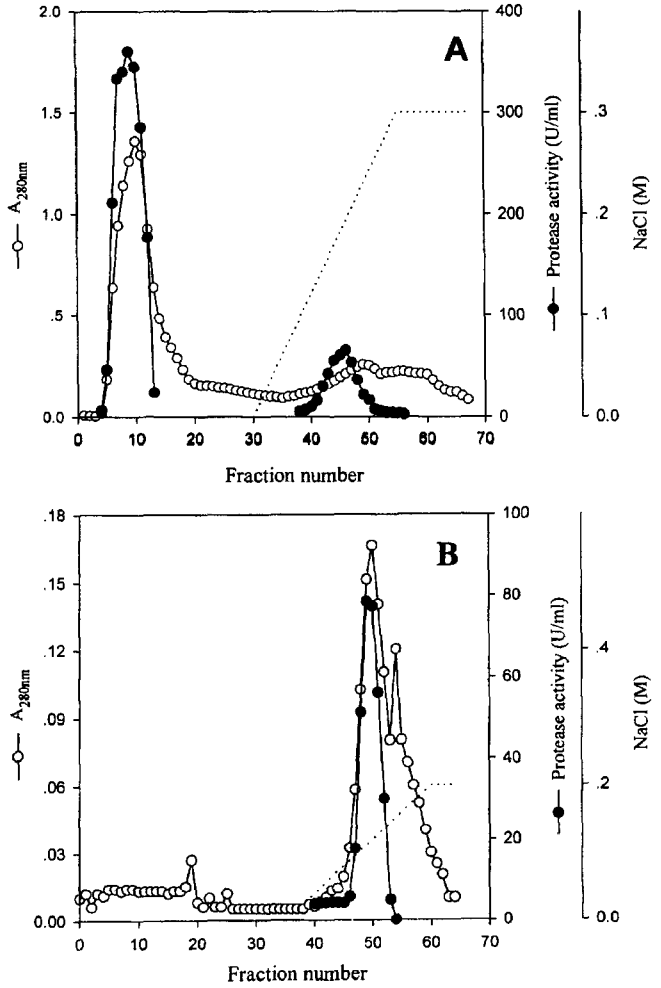

Fig. 1. Chromatography of *Thermoactinomyces* sp. E79 protease.
(A) Chromatography of crude enzyme on the first DEAE-Sepharose CL-6B column. The two enzyme peaks were eluted, one with a washing fraction, and the other with a linear gradient of NaCl in 20 mM phosphate buffer (pH 7.2), at a flow rate of 55 ml/h. Each fraction was 4.5 ml. **(B)** Re-chromatography of the minor protease on DEAE-Sepharose CL-6B column. The enzyme was eluted with a linear gradient of NaCl in 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 45 ml/h, and the volumn of each fraction was 4 ml.

minor protease was purified effectively from the culture supernatant of the *Thermoactinomyces* sp. E79 by re-chromatography on a DEAE-Sepharose CL-6B column (Fig. 1B). The homogeneity of the final enzyme preparation was determined by SDS-PAGE (Fig. 2A) and the proteolytic activity was further examined by using the substrate gel electrophoresis containing 5% skim milk (Fig. 2B). A summary of the purification procedures is shown in Table 1. The yield of the purified minor protease was only 2%. This represents a low recovery, since the minor protease was only 12% of the total protease in the culture supernatant. The purified enzyme had a specific activity of 9×10^4 U/mg. By SDS-PAGE, the molecular mass of the minor protease was estimated to be 36 kDa,

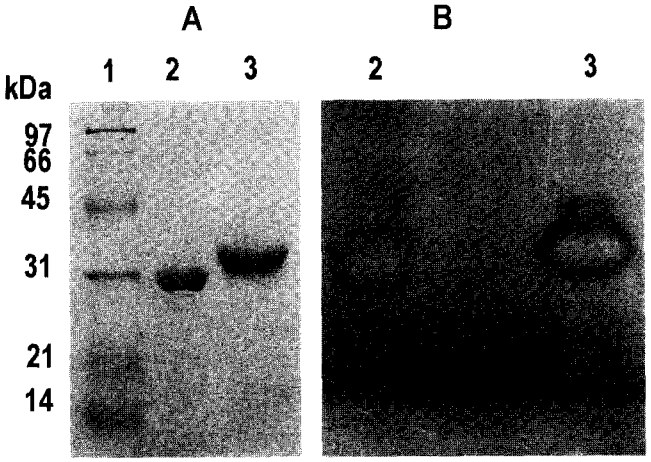

Fig. 2. SDS-PAGE of E79 proteases **(A)**, and activity staining of SDS-PAGE gel **(B)**.
Lane 1, molecular weight marker; lane 2, major protease from DEAE-unbound fraction; lane 3, minor protease from DEAE-bound fraction.

Table 1. Purification of the minor protease from *Thermoactinomyces* sp. E79.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Culture supernatant	31.4	2.9×10^6	9.2×10^4	100
30-80% $(NH_4)_2SO_4$	14.1	2.1×10^6	1.5×10^5	70
DEAE-Sepharose (I)	1.8	1.6×10^5	9×10^4	6
DEAE-Sepharose (II)	0.6	5.4×10^4	9×10^4	2

which was larger than the major protease with a molecular mass of 31 kDa [12].

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence of the purified enzyme was determined by an automated Edman method after SDS-PAGE and electroblotting, and found to be Phe-Thr-Pro-Asn-Asp-Pro-Ala-Tyr-Asp-Glu-Gln-Tyr-Ala-Pro-Glu. This sequence was significantly different from major and other alkaline proteases, showing only 28% identity compared to the major protease (Fig. 3). In addition, the sequence of the minor protease did not match with any deduced amino acid sequence of the major protease, whose gene was sequenced previously [12]. Therefore, this result indicated that the minor protease was not a pre-pro- or a pro-form of the major protease which was characterized earlier [12].

Effects of Temperature and pH

The effect of temperature on the protease activity of the minor protease was examined at various temperatures ranging from 30°C to 80°C. As shown in Fig. 4, the enzyme exhibited a maximum activity at 70°C. This result was similar to that of the E79 major protease, which showed an optimum temperature of 75°C [12], and thermostable proteases

	+1														+14
E79 minor protease	F-	T-	P-	N-	D-	P-	A-	Y-	D-	E-	Q-	Y-	A-	P-	E
E79 major protease	W-	T-	P-	N-	D-	L-	T-	S-	R-	Q-	W-	G-	P-	Q-	K
Thermitase	Y-	T-	P-	N-	D-	P-	Y-	F-	S-	S-	R-	Q-	Y-	G-	P
AK1	W-	T-	P-	N-	D-	T-	Y-	Y-	Q-	G-	Y-	Q-	Y-	G-	P

Fig. 3. Comparison of N-terminal amino acid sequence of the E79 minor protease with other microbial proteases.
E79, thermitase, and AK1 represent extracellular proteases produced from *Thermoactinomyces* sp. E79, *Thermoactinomyces vulgaris*, and *Bacillus* sp. AK1, respectively.

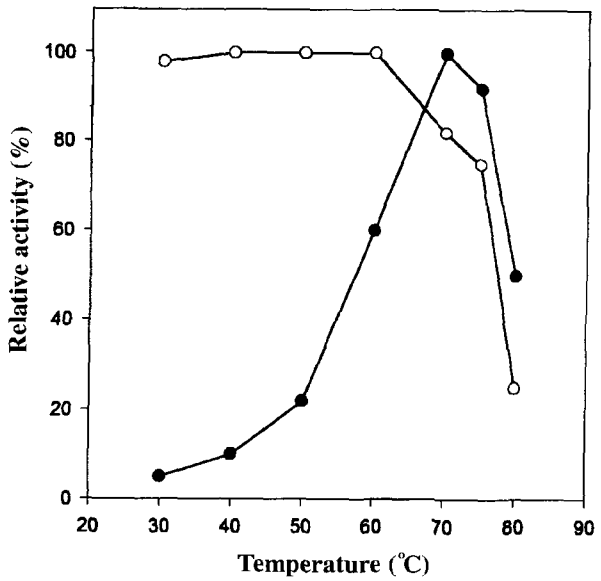

Fig. 4. Effect of temperature on the enzyme activity and stability of the E79 minor protease.
The enzyme was assayed at various temperatures (●). For the temperature stability, the enzyme was preincubated at various temperatures for 10 min and the remaining activity was measured at 55°C for 15 min (○).

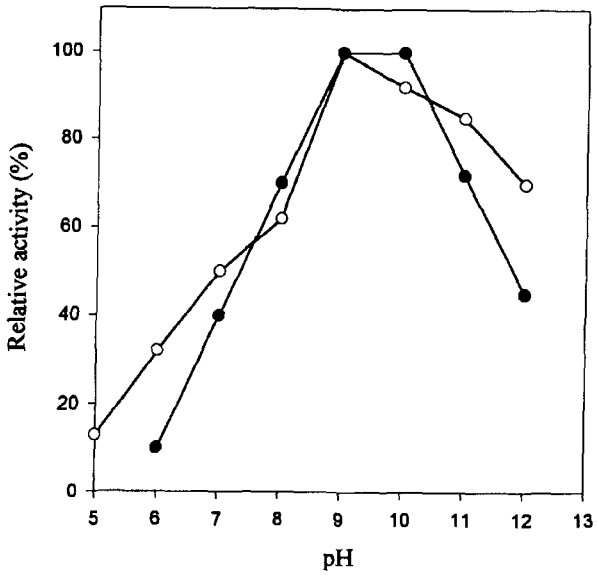

Fig. 5. Effect of pH on the enzyme activity and stability of E79 minor protease.
The enzyme was assayed at various pHs (●). For the pH stability, the enzyme was preincubated in buffers with different pHs for 1 h and the remaining activity was measured at pH 9.0 (○).

produced from *Bacillus stearothermophilus* F1 [20] and *Bacillus* sp. B18' [3]. To test thermostability, activity of the minor enzyme was measured after incubation at various temperatures for 10 min. The enzyme was stable up to 75°C, but rapidly inactivated at 80°C, and calcium ions had a small effect on the thermal stability of the minor protease (data not shown). In the case of the major protease, thermal stability increased considerably when calcium ions were added, showing that the metal ion stabilized the protein structure by preventing unfolding at high temperatures [13]. In addition, the enzyme was very stable at 90°C in the presence of Ca^{2+}.

The optimum pH of the major protease was observed to be 11.0 [12], while that of the minor protease was in the range of 9.0-10.0. The enzyme was stable after incubation at room temperature for 1 h at pHs 8.0 to 12.0, but it showed a slow decrease of the activity above pH 9.0 (Fig. 5). On the other hand, the major protease retained about 95% of its original activity in the pH range of 9.0-12.0 [12]. In summary, the temperature and pH stability of the purified minor protease were slightly lower compared with those of the major protease from *Thermoactinomyces* sp. E79.

Effect of Metal Ions and Inhibitors on Protease Activity

To study the effect of various metal ions and inhibitors, the enzyme was incubated with different inhibitors and metal ions at 30°C for 10 min in 50 mM Tris-HCl buffer (pH 8.0). After incubation, the remaining protease activity of the enzyme was assayed. As shown in Table 2, the enzyme was markedly inhibited by 1 mM phenylmethylsulfonyl fluoride (PMSF), but was not affected by EDTA and *N*-ethylmaleimide, indicating that the minor protease was a serine protease. It was also observed that the enzyme activity was completely inhibited by Hg^{2+}, suggesting that the minor protease contained one or more cysteine residues which might be involved in enzyme activity by the sulfhydryl group. It has been reported that there are proteases classified

Table 2. Effects of inhibitors, metal ions, and detergents on proteolytic activity.

Reagents	Concentration	Relative activity (%)
	(mM)	
PMSF	1	1
EDTA	1	90
N-Ethylmaleimide	1	96
KCN	1	95
Iodoacetamide	1	100
$ZnCl_2$	1	99
$CaCl_2$	1	113
$CuCl_2$	1	47
$HgCl_2$	1	2
$MnCl_2$	1	94
$MgCl_2$	1	93
KCl	1	88
	(%)	
Triton X-100	1	86
Tween 20	1	93
Tween 80	1	110
SDS	1	30

under a subfamily of subtilisins, which show no distinct similarity in the N-terminal chains with the sequence of subtilisins represented by subtilisin BPN'. In addition, they have mercuric ion-sensitive free cysteines in the equivalent position of the primary structure [12, 14, 15, 21]. In the case of the major protease, a single cysteine (Cys-180) was present and shown to be inhibited by Hg^{2+} [12]. From the above observations, we predict that the minor protease may also be a cysteine-dependent serine protease, similar to the major protease. When the minor protease was incubated for 10 min with different detergents at 30°C, 1% SDS showed 70% loss of the enzymatic activity. On the other hand, other detergents showed little effect.

Substrate Specificity and Enzyme Kinetics

Specificity of the minor protease towards various synthetic substrates was examined. The minor protease failed to hydrolyze a simplest nitroanilide such as Bz-Arg-pNA, Bz-Leu-pNA, and Bz-Tyr-pNA. Suc-Ala-Ala-Pro-Phe-pNA was most susceptible to hydrolysis, showing a specific activity of 0.74 U/mg, and Tos-Gly-Pro-Lys-pNA was hydrolyzed with less activity (0.14 U/mg). Employing Suc-Ala-Ala-Pro-Phe-pNA as a substrate, Michaelis-Menten kinetics was followed. The values obtained from the Lineweaver-Burk double-reciprocal plots for V_{max} and K_m were 0.79 μmole of *p*-nitroaniline released per min per mg of enzyme and 1.2 mM, respectively. In the case of the major protease, the values were 6.1 μmol and 1.49 mM. The above results indicated that the minor protease exhibited similar affinity to this substrate as the major (Fig. 6).

The purified minor protease was distinctly different from the major protease in terms of its molecular mass, N-terminal amino acid sequence, and enzymatic properties.

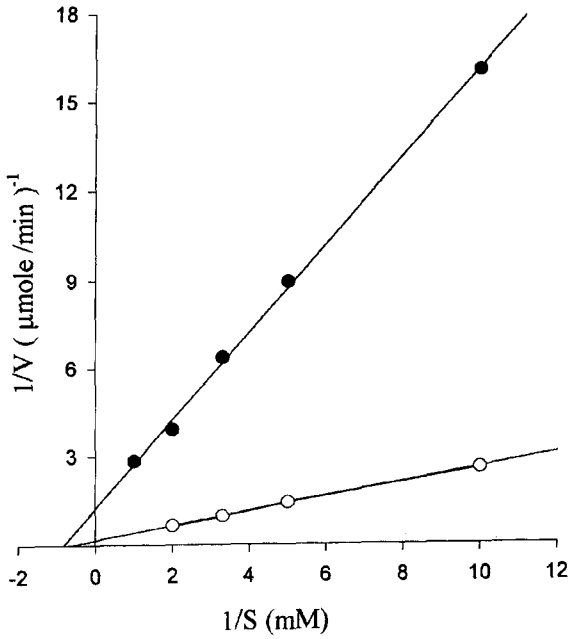

Fig. 6. Lineweaver-Burk plot of E79 major (○) and minor (●) proteases on Suc-Ala-Ala-Pro-Phe-pNA.

When information is readily available, dealing with some different properties of these two proteases, it may be possible to deduce one or the other of its functions. We are now in a process of cloning the gene encoding this minor protease. We strongly expect that this investigation will provide more information on the relationship between the protease structure and function. In addition, it will be possible to elucidate the relation and the difference in optimum pH and thermostability between the minor and major proteases of *Thermoactinomyces* sp. E79.

REFERENCES

1. Bradford, M. M. 1976. A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
2. Frost, G. M. and D. A. Moss. 1987. In *Biotechnology*, vol. 7a. Enzyme Technology, J. F. Kennedy (ed.), pp. 157-168. VCH, Weinheim, Germany.
3. Fujiwara, N., A. Mausi, and T. Imanaka. 1993. Purification and properties of the highly thermostable alkaline protease from an alkalophilic and thermophilic *Bacillus* sp. *J. Biotechnol.* **30:** 245-256.
4. Hellen C., D. A. Cowan, and R. J. Sharp. 1991. Heterogenecity of proteinase from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *J. Gen. Microbiol.* **137:** 1193-1199.
5. Horikoshi, K. and T. Akiba. 1982. *Alkalophilic Microorganism: A New Microbial World*, pp. 93-101, Japan Scientific Societies Press, Tokyo.

6. Jacobs, M., M. Elirron, M. Uhlen, and J. I. Flock. 1985. Cloning, sequencing and expression of subtilisin carlsberg from *Bacillus licheniformis*. *Nucl. Acids Res.* **13:** 8913-8926.
7. Kim, H.-K., K.-H. Kim, J.-K. Lee, K.-S. Bae, C. Sung, and T.-K. Oh. 1994. Purification and characterization of thermostable protease from *Pseudomonas aeruginosa* NS-83. *J. Microbiol. Biotechnol.* **4:** 113-118.
8. Kim, Y.-O., J.-K. Lee, H.-K. Kim, Y.-S. Park, and T.-K. Oh. 1996. Purification and characterization of an alkaline protease from *Bacillus licheniformis* NS 70. *J. Microbiol. Biotechnol.* **6:** 1-6.
9. Kubo, M., K. Murayama, K. Seto, and T. Imanaka. 1988. Highly thermostable neutral protease from *Bacillus stearothermophilus*. *J. Ferment. Technol.* **66:** 13-17.
10. Kwon, Y. T., J. O. Kim, S. Y. Moon, H. H. Lee, and H. M. Rho. 1994. Extracellular alkaline proteases from alkalophilic *Vibrio metschnikovii* strain RH 530. *Biotechnol. Lett.* **16:** 413-418.
11. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
12. Lee, J.-K., Y.-O. Kim, H.-K. Kim, Y.-S. Park, and T.-K. Oh. 1996. Purification and characterization of a thermostable alkaline protease from *Thermoactinomyces* sp. E79 and the DNA sequence of the encoding gene. *Biosci. Biotech. Biochem.* **60:** 840-846.
13. Lee, J.-K., Y.-O. Kim, K. Sunitha, and T.-K. Oh. 1998. Expression of thermostable alkaline protease gene from *Thermoactinomyces* sp. E79 in *E. coli* and heat activation of the gene product. *Biotechnol. Lett.* **20:** 837-840.
14. Meloun, B., M. Baudys, V. Kostka, G. Hausdorf, C. Frommel, and W. E. Hohne. 1985. Complete primary structure of thermitase from *Thermoactinomyces vulgaris* and its structural features related to the subtilisin type proteinases. *FEBS Lett.* **183:** 195-200.
15. Moriyama, R., S. Kazuhiro, Z. Haishuo, I. Toshihiko, and M. Shio. 1998. A cysteine-dependent serine protease associated with the dormant spores of *Bacillus cereus*: Purification of the protein and cloning of the corresponding gene. *Biosci. Biotech. Biochem.* **67:** 268-274.
16. Murao, S., Y. Nomura, K. Nagamatsu, K. Hirayama, M. Iwahara, and T. Sin. 1991. Purification and some properties of a thermostable metal proteinase produced by *Thermomicrobium* sp. KN-22. *Agric. Biol. Chem.* **55:** 1739-1744.
17. Odonohue, M. J., B. P. Roques, and A. Beaumont. 1994. Cloning and expression in *Bacillus subtilis* of the *npr* gene from *Bacillus thermoproteolyticus* Rokko coding for thermostable metalloprotease thermolysin. *Biochem. J.* **300:** 599-603.
18. Peczynska-Czoch, W. and M. Mordarski. 1988. In *Actinomyces in Biotechnology*, M. Goodfellow, S. T. Williams, and M. Moedarshi (eds.), pp. 246-253. Academic press, New York, U.S.A.
19. Peek, K., R. M. Daniel, C. Monk, L. Papker, and T. Coolbear. 1992. Purification and characterisation of thermostable proteinase isolated from *Thermus* sp. Strain Rt41A. *Eur. J. Biochem.* **207:** 1035-1044.
20. Rahman, R. N. Z. A., C. N. Razak, K. Ampon, M. Basri, W. M. Z. W. Yunus, and A. B. Salleh. 1994. Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1. *Appl. Microbiol. Biotechnol.* **40:** 822-827.
21. Stepanov, V. M., G. G. Chestukhina, G. N. Rudenskaya, A. S. Epremyan, A. L. Osterman, O. M. Khodova, and L. P. Belyaniya. 1981. A new subfamily of microbial serine proteinases? Structural similarities of *Bacillus thuringenesis* and *Thermoactinomyces vulgaris* extracellular serine proteinases. *Biochem. Biophys. Res. Commun.* **100:** 1680-1687.
22. Strongin, A. Y. A., L. S. Izotova, Z. T. Abramov, D. I. Gorodetsky, L. M. Ermakava, L. A. Baratova, L. P. Belyanova, and V. M. Stepanov. 1978. Two related structural genes coding two homologous serine proteases in the *Bacillus subtilis* genome. *J. Bacteriol.* **133:** 1401-1411.
23. Takami, H., T. Akiba, and K. Horikoshi. 1990. Characterization of an alkaline protease from *Bacillus* sp. no. AH-101. *Appl. Microbiol. Biotechnol.* **33:** 519-523.
24. Tsuchiya, K., H. Nakamura, H. Sakashita, and T. Kimura. 1992. Purification and characterization of a thermostable alkaline protease from alkalophilic *Thermoactinomyces* sp. HS682. *Biosci. Biotech. Biochem.* **56:** 246-250.
25. Zuidweg, M. H. J., C. J. K. Bos, and H. van Welzen. 1972. Proteolytic components of alkaline proteases of *Bacillus* strains: Zymograms and electrophoretic isolation. *Biotechnol. Bioeng.* **16:** 685-714.