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Cloning and Expression of an Intracellular Alkaline Protease Gene from Alkalophilic *Thermoactinomyces* sp. HS682†

Katsumi TSUCHIYA,* Ichiro IKEDA, Takashi TSUCHIYA, and Tetsu KIMURA

Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350–02, Japan
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An intracellular alkaline serine protease gene of alkalophilic *Thermoactinomyces* sp. HS682 was cloned and expressed in *Escherichia coli*. Sequence analysis showed a putative promoter region, a putative transcriptional termination signal, and an open reading frame of 963 bases, coding for a polypeptide of 321 amino acids. The protease expressed in *E. coli* was purified by DEAE-Toyopearl 650M and Sephadex G-75 chromatography. The N-terminal sequence (30 amino acids) of the purified protein was coincident with Asp16–Val45 of the deduced amino acid sequence of the ORF. Fifteen amino acids in the N-terminal region were removed during the purification procedures. The deduced amino acid sequence showed high similarity with microbial intracellular serine proteases. The molecular mass of this enzyme was estimated to be 38 kDa by SDS–PAGE. The enzyme was stable at pH 6.0–12.0 and below 60°C in the presence of Ca^{2+}. The temperature and pH optima of the enzyme were 65°C and pH 11.0, respectively. The enzyme was inhibited by DFP and PMSF, but not by MIA and EDTA.

Key words: intracellular alkaline serine protease; alkalophilic *Thermoactinomyces* sp.; DNA sequence; amino acid sequence homology; protein purification

Proteases with high activity and stability in the regions of high alkali and temperature are interesting for bioengineering and biotechnological applications as well as protein chemistry. There have been many studies on alkaline proteases from various microorganisms.[1–3] Alkalophilic microorganisms among them produce alkaline proteases with high activity and stability in highly alkaline pH.[4] The alkaline protease from alkalophilic *Bacillus* sp. No. AH-101 is characteristic in its highly alkaline pH optimum (pH 12–13) and thermostability at highly alkaline pH.[5] Also, alkalophilic *Thermoactinomyces* sp. HS682, growing optimally at pH 10.3 and 52°C, produces and secretes an alkaline serine protease with highly alkaline pH optimum (pH 11–13), high temperature optimum (80°C), and extreme stability in both ranges of high alkali (pH 4–14) and high temperature (≦75°C).[6] Takami *et al.* isolated the gene encoding a minor alkaline protease from alkalophilic *Bacillus* sp. No. AH-101 by the shotgun cloning method.[7] Some actinomycetes produce and secrete several kinds of protease in the culture broth, like Pronase from *Streptomyces griseus*.[3] Although we purified only an extracellular alkaline serine protease from the culture broth of *Thermoactinomyces* sp. HS682,[6] we found that this strain produces minor proteases in the cells. It is not easy, however, to purify these minor proteases from the cells. Therefore, we tried to clone the gene for minor proteases from this strain by using a shotgun cloning method.

We isolated the gene encoding an alkaline protease different from the major extracellular alkaline protease of *Thermoactinomyces* sp. HS682. This protease was expected to be an intracellular protease from its deduced amino acid sequence. In this report, we describe cloning, sequencing, and expression of the gene and characterization of the purified enzyme.

Materials and Methods

Materials. Restriction endonucleases and plasmids were purchased from Takara Shuzo or Boehringer-Manheim. T4 DNA ligase, Kilo-sequencing deletion kit, BcaBest dideoxy sequencing kit, Random primer DNA labeling kit, and primers for sequencing were purchased from Takara Shuzo. [α-^{32}P]dCTP was obtained from Amersham. Calf intestine alkaline phosphatase, isopropyl-β-D-thio-galactopyranoside (IPTG), 5-bromo-4-chloro-3-indoyl-β-D-thio-galactopyranoside (X-Gal) were purchased from Takara Shuzo. Casamino acids (vitamin assay), yeast extract, and tryptone were from Difco Laboratories. Molecular weight marker proteins for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was obtained from Oriental Yeast Co., Ltd. All other chemicals and reagents were of the highest grade and obtained from commercial sources.

Microorganisms, plasmids, and media. Alkalophilic *Thermoactinomyces* sp. HS682 was used as a DNA donor. *Escherichia coli* JM109 and MV1184 were used as host strains for cloning, sequencing, and expression of the gene for HS682 protease. Plasmids pUC18 and pUC118 were used for cloning and sequencing.

Thermoactinomyces sp. HS682 was cultured in the alkaline medium (1% maltose, 0.5% casamino acids, 0.04% yeast extract, 0.5% K_2HPO_4, 0.02% NaCl, and 1.5% Na_2CO_3, pH 10.3) at 50°C for 24 h. Bacteria were aerobically grown in Luria–Bertani broth (LB broth, 1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 8.0) containing ampicillin (100 μg/ml) at 37°C. LB agar plates containing ampicillin (100 μg/ml) and skim milk (1%) were used for screening of protease-positive transformants of *E. coli*.

DNA manipulations. Chromosomal DNA of *Thermoactinomyces* sp. HS682 was prepared by the method of Saito and Miura.[8] Plasmid DNA was isolated by the alkaline extraction method.[9] Competent cells of *E. coli* JM109 and MV1184 were prepared by the method of Hanahan.[10] All the procedures for cloning were done according to the standard

† The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D87557.

* To whom correspondence should be addressed.

Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; DFP, diisopropyl fluorophosphate; MIA, monoiodoacetic acid; ORF, open reading frame; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

procedures of Sambrook *et al.*[11] or the manufacturer's recommendation.

Cloning of protease gene Chromosomal DNA was partially digested with *Hin*dIII and size-fractionated by sucrose density gradient centrifugation. Digested chromosomal DNA (4–8 kb) was ligated with *Hin*dIII digested pUC18 and used to transform *E. coli* JM109. After incubation on the LB agar plate, transformants that formed clear zones around their margin were picked up from the plates. The fragments containing the protease gene were subcloned based on the restriction enzyme map constructed.

Nucleotide sequencing. Plasmid pUC118 inserted the subcloned fragment was deleted by Kilo-sequencing deletion kit in according with the manufacturer's recomendation.[12] Single-strand DNA was prepared by the alkaline-SDS method,[13] and sequenced by the dideoxy nucleotide chain-termination method[14] using the BcaBest dideoxy sequencing kit. Sequence data were analyzed using GENETYX-MAC Software (Software Development).

Protease and protein assays. Caseinolytic activity was assayed by the method described previously.[6] Protein was measured by the method of Bradford[15] using Coomassic brilliant blue G-250 or absorbance at 280 nm with bovine serum albumin as a standard.

Localization of alkaline protease in E. coli JM109 harboring pTIAP. E. coli JM109 harboring pTIAP was cultured in 4 ml of LB broth (pH 8.0) containing 100 μg/ml of ampicillin for 18 h at 37°C and collected by centrifugation at 8000 × *g* for 10 min. The cells were suspended in 2 ml of 25% sucrose 1 mM EDTA–10 mM Tris-HCl buffer (pH 8.0). One milliliter of the cell suspension was incubated with 0.15 mg/ml of lysozyme for 1 h at 37 °C. The periplasmic fraction was obtained from the supernatant after centrifugation of the solution at 15,000 × *g* for 10 min. The precipitate was completely disrupted by sonication (20 kHz, 50 W, 3 min, Ohtake Works) to obtain the cytoplasmic fraction. Another one milliliter of the cell suspension was completely disrupted by sonication under the same conditions to obtain the whole intracellular fraction.

Purification of cloned enzyme. E. coli JM109 harboring pTIAP was aerobically grown in 4 liters of LB broth (pH 8.0) containing ampicillin (100 μg/ml) at 37 °C for 24 h. The cells were collected by centrifugation at 8000 × *g* for 10 min, washed twice with 10 mM Tris-HCl buffer (pH 8.0), and suspended in the same buffer. The suspended cells were disrupted 3 times with a sonicator (20 kHz, 50 W) for 5 min in the ice bath, and centrifuged at 15,000 × *g* for 20 min. Protein in the supernatant was precipitated with 70% saturated ammonium sulfate at 4°C overnight. The precipitate was collected by the centrifugation at 10,000 × *g* for 10 min and dissolved in a minimum volume of 10 mM NH_4Cl–NH_4OH buffer (pH 10.0) containing 5 mM $CaCl_2$. The enzyme solution was dialyzed against the same buffer, and then chromatographed on a DEAE-Toyopearl 650M (Tosoh) column (1.6 × 18 cm) eluting with a linear gradient of 0 to 0.4 M NaCl in the same buffer. An active fraction of the protease was collected, concentrated by ultrafiltration with a Diaflo membrane YM-05 (Amicon, exclusion limit 5 kDa), and chromatographed on a Sephadex G-75 (superfine, Pharmacia Biotech Co.) column (1.6 × 85 cm) eluting with the same buffer. A major active fraction was collected, dialyzed against 10 mM NH_4OH solution, lyophilized, and stored at −20 °C until use.

For molecular mass estimation by the gel filtration on Sephadex G-75, bovine serum albumin (67 kDa), ovalbumin (43.3 kDa), trypsinogen (24 kDa), and cytochrome *c* (13 kDa) were used as the references. All procedures were done at 4 °C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. SDS-PAGE was done by the method of Laemmli[15] using 12.5% (w/v) acrylamide. The enzyme solution inhibited with 5 mM DFP was heated for SDS-treatment at 100 °C for 3 min. Proteins were stained with Coomassie brilliant blue R-250. For molecular mass measurement, oligomers of cytochrome *c* of molecular mass of 12.4–74.4 kDa were used as the references.

N-Terminal amino acid sequencing of purified protein Further purification was done by HPLC on Vydac 214TP column (ODS, 5 μ, 4.6 × 250 mm) eluting with a linear gradient of 30% to 60% CH_3CN in 0.1% TFA. N-Terminal amino acid sequence of the purified enzyme was assayed using a Protein Sequencer (Shimadzu, model PSQ-1).

Results and Discussion

Cloning of the gene of alkaline protease from alkalophilic Thermoactinomyces sp. HS682

After about 10,000 transformants were screened, three colonies were found to express the protease activity. All plasmids in the clones had a 6.4-kb insert in the *Hin*dIII site of pUC18. All three inserts were cleaved at the same positions by *Sal*I, *Sac*I, *Kpn*I, *Eco*RI, and *Bgl*II. One of them was inserted in the reverse direction as compared with others, and showed only slight protease activity in both the presence and absence of isopropyl-1-thio-β-D-galactopyranoside (IPTG). On the other hand, the two clones inserted in the same direction, which was the inverse direction toward the *lacZ* promoter, produced high protease activity in the absence of IPTG (data not shown). This results suggest that the cloned gene should contain a promoter and be expressed by its promoter. The 6.4-kb *Hin*dIII fragment was labeled with ^{32}P by the random primer labeling method[17] and used as a probe for Southern hybridization.[18] This probe showed hybridization to the chromosomal DNA of *Thermoactinomyces* sp. HS682 (data not shown). A restriction map of the insert in one of them is shown in Fig. 1. Based on this map, various subclones were constructed and compared in their potential for protease production. The fragment of *Hin*dIII–*Bgl*II (2.6 kb) with a potential of protease production was ligated with *Hin*dIII–*Bam*HI digested pUC18. The inserted plasmid was partially digested with *Eco*RI and completely with *Sma*I, which cleaved at the 5′-end position of the fused site, *Bgl*II–*Bam*HI, in the inserted plasmid. The obtained fragment of *Eco*RI–*Sma*I (1.5 kb) was ligated with *Eco*RI–*Sma*I digested pUC118, and this plasmid was designated as pTIAP.

Nucleotide sequence of the gene for alkaline protease

The nucleotide sequence of the cloned gene and its flanking regions (1486 bp) was analyzed (Fig. 2). We found an open reading frame (ORF) with an ATG initiation codon at nucleotide 1 and a TAA termination codon at nucelotide 964. This ORF encoded a polypeptide of 321 amino acids. The putative Shine–Dalgarno (SD) sequence,[19] GGAGG, was located 8 bases upstream from the initiation codon. The putative promoter region (−35 and −10 regions) could

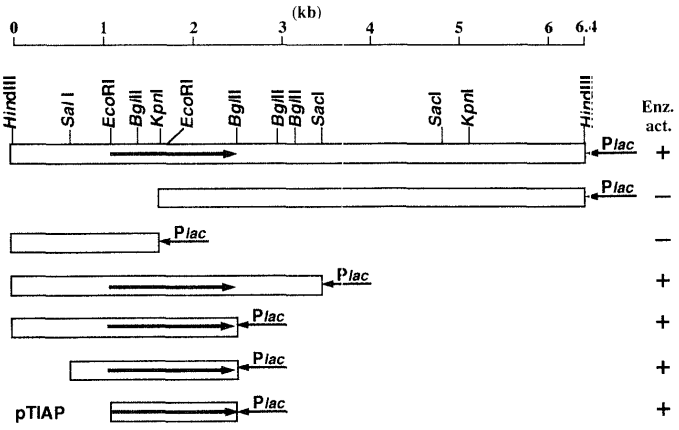

Fig. 1. Restriction Endonuclease Map and Subcloning of pTIAP.

Thin lines represent the plasmid vector pUC18, and P*lac* represents the lactose promoter in the plasmid pUC18. Rectangles represent chromosomal DNA digested by restriction enzymes. The arrows indicate the orientation of transcription of protease gene.

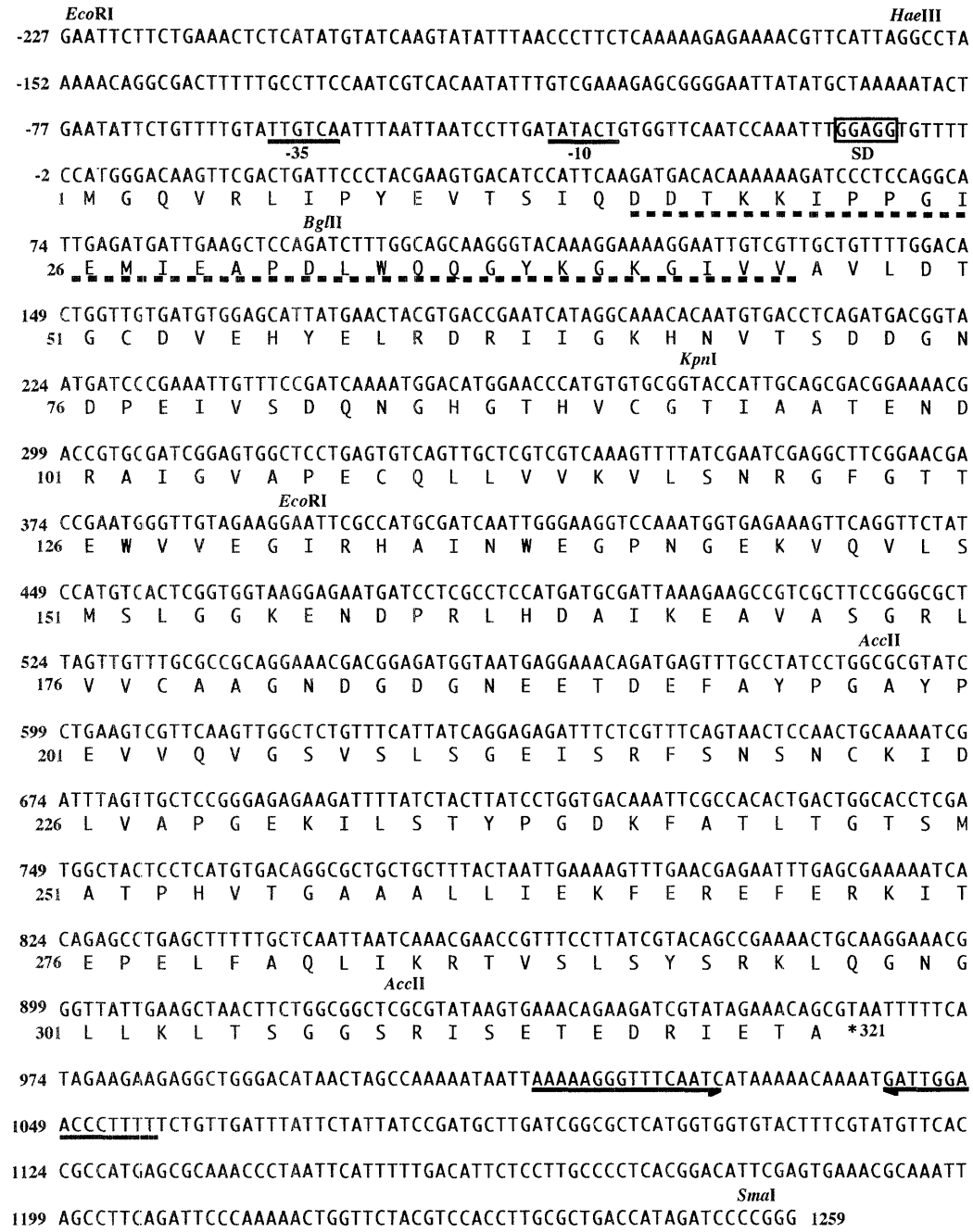

Fig. 2. Nucleotide Sequence of the Gene Encoding Alkaline Protease from *Thermoactinomyces* sp. HS682 and Its Deduced Amino Acid Sequence.

The putative expression signals, −35 and −10 regions, a Shine–Dalgarno Sequence (SD), the stop codon (*), and a potential transcriptional termination signal (inverted arrows) are indicated. The restriction sites are shown over the nucleotide sequence. The numbering of nucleotides starts at the 5′ end of initiation codon of the gene, and that of amino acids at the N-terminus of pro-enzyme. The dashed underline indicates N-terminal sequence of alkaline serine protease purified from *Escherichia coli* JM109 harboring pTIAP.

be identified at nucleotide −60 (TTGTCA) and −37 (TATACT), with a spacing of 17 bases. The ORF was followed by a putative palindromic sequence, which may act as the rho-independent transcriptional terminator of bacterial genes,[20] 46 bases downstream of the termination codon (TAA). Free energy of this sequence for a stem-loop structure was calculated to be −110.8 kJ/mol,[21] which would be sufficient for the termination of transcription.

Deduced amino acid sequence of alkaline protease

We could find no identical sequence to the N-terminal amino acid sequence (QTVPWGISFINTQQAHNRGIF)[6] of extracellular alkaline protease from alkalophilic *Thermoactinomyces* HS682 in this ORF. An internal amino acid sequence (Asp16–Val45) deduced from the ORF was coincident with the N-terminal amino acid sequence (Asp1–Val30) of alkaline protease purified from *E. coli* harboring pTIAP (Fig. 2). The N-terminal amino acid of the purified alkaline protease was proceeded by a peptide of 15 amino acids in the deduced sequence. We could not find any core of hydrophobic residues and signal cleavage sequence characteristic for a signal peptide[3] in this sequence. This suggests that the sequence from the primary translation product could be removed as a consequence of some artifact of the purification as proven for the intracellular serine protease of *Bacillus subtilis*.[22]

The deduced amino acid sequence of cloned protease was compared with those of various microbial serine alkaline

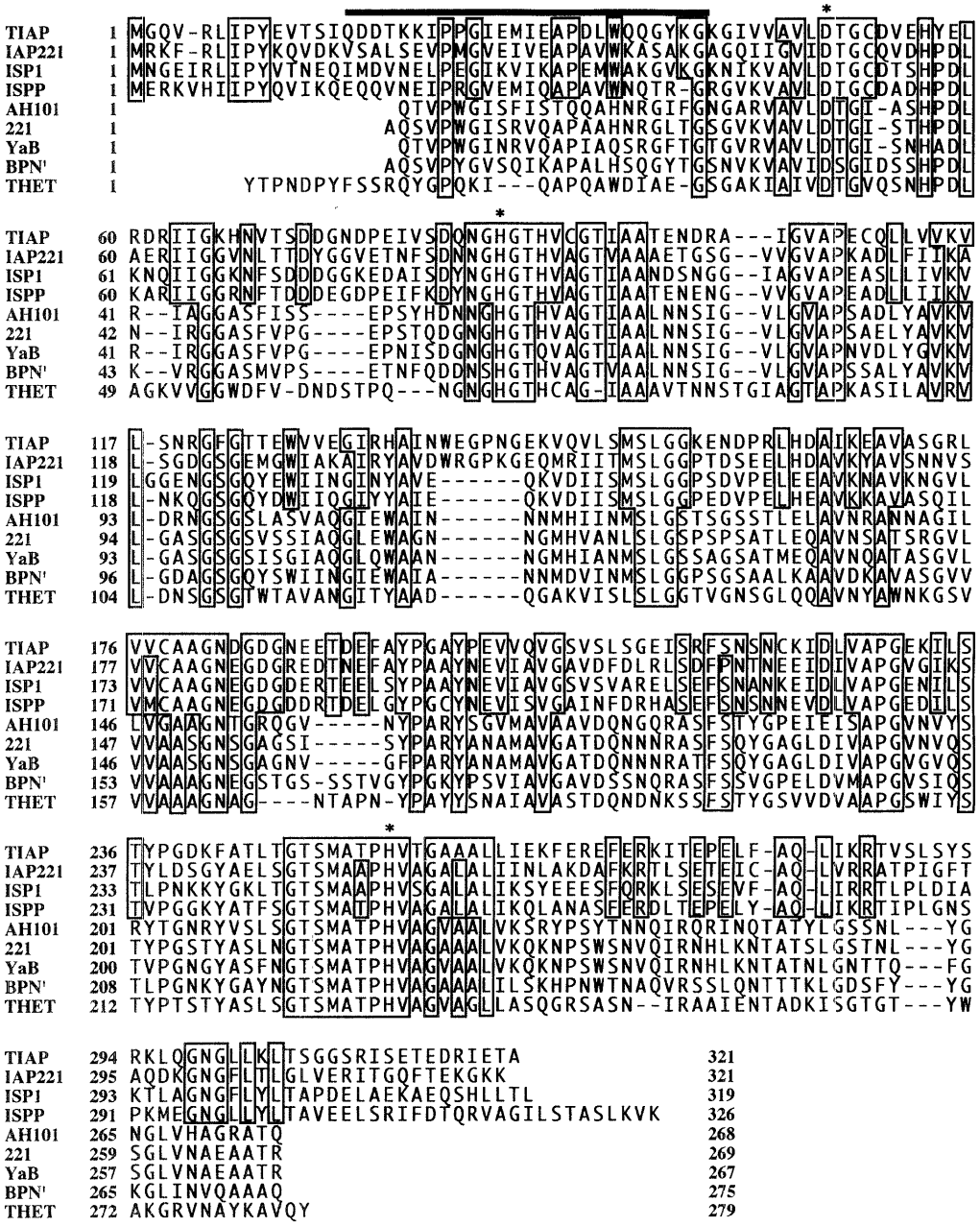

Fig. 3. Comparison of the Deduced Amino Acid Sequence with Those of Various Serine Proteases.

The boxes represent identical amino acid residues in all, intracellular, or extracellular serine proteases. Identical cysteine residues in intracellular serine proteases are shadowed. TIAP, cloned alkaline serine protease; IAP221, intracellular alkaline serine protease from alkalophilic *Bacillus* sp. No. 221; ISP1, major intracellular serine protease from *B. subtilis*[23]; ISPP, intracellular serine protease from *B. polymyxa*[24]; AH101, extracellular alkaline serine protease from alkalophilic *B.* sp. AH101[25]; 221, extracellular alkaline serine protease from alkalophilic *B.* sp. No. 221[26]; YaB, extracellular alkaline elastase from alkalophilic *B.* sp. Ya-B[27]; BPN′, extracellular alkaline serine protease from *B. amyloliquefacience*[28]; THET, thermitase from *Thermoactinomyces vulgaris.*[29] "–" Gaps introduced for maximum homology. * Proton relay system. Solid line, N-terminal sequence of alkaline serine protease purified from *Escherichia coli* JM109 harboring pTIAP.

proteases in the SWISS-PROT of GENETYX-Biodatabase (Fig. 3). The sequence removed from primary translation product showed distinct homology with the N-terminal sequences of intracellular serine proteases from *Bacilli*,[23,24] which were removed from the primary translation products. The deduced amino acid sequence showed higher homology, 47–56%, with those of intracellular serine proteases than the 37–40% homology of extracellular serine proteases.[23–29] The cloned enzyme contains five cysteine residues in the deduced sequence. Intracellular serine proteases compared in Fig. 3, also, contain several cysteine residues, 3 residues of ISP1 from *Bacillus subtilis*[23] and ISPP from *Bacillus polymyxa*,[24] and 2 residues of IAP221 from alkalophilic *Bacillus* sp. No. 221, while extracellular serine proteases except for thermitase, 1 residue, from *Thermoactinomyces vulgaris*[29] contain no cysteine residue. The two cysteine residues in the cloned enzyme were identical with those of intracellular serine proteases. The amino acid sequences around the proton relay system of the catalytic triad, corresponding Asp32, His64, and Ser221 of subtilisin BPN′,[28] were found to be conserved in the cloned protein, like other intracellular or extracellular serine proteases. These characteristics of the deduced sequence suggests that the cloned enzyme should be an intracellular serine protease.

Localization of alkaline protease in E. coli JM109 harboring pTIAP

E. coli JM109 harboring the plasmid pTIAP were treated

with lysozyme in a hypertonic solution and sonicated, and the cellular fractions were assayed for alkaline protease. When expressed in *E. coli* JM109, about 80% of the alkaline protease activity was found in the cytoplasmic fraction. This suggests also that the cloned enzyme should be an intracellular serine protease.

Purification of cloned alkaline protease

The alkaline protease was purified from the cell extract of *E. coli* JM109 harboring pTIAP. The alkaline protease activity was separated into two active fractions by an ion-exchange chromatography on DEAE-Toyopearl 650M (Fig. 4). Major and minor fractions were eluted at the concentrations of 0.12 M and 0.18 M NaCl, respectively. Each fraction was collected, concentrated with a Diaflo membrane YM-05, and put on the column of Sephadex G-75. The major fraction was eluted with only one active fraction at the position of 38 kDa. On the other hand, the minor fraction was eluted mainly at the position of 68 kDa with a small active fraction at the position of 38 kDa (data not shown). This 68-kDa protease could not be purified because of its unstability during the purification procedures. Typical purification procedures for the major alkaline protease are summarized in Table. The alkaline protease was purified 46.5-fold over the cell extract and recovered in 29% yield. The purified enzyme gave a single band on SDS–PAGE and its apparent molecular mass was estimated to be 38 kDa (Fig. 5). This molecular mass of the purified enzyme was larger than that of the extracellular alkaline protease produced by alkalophilic *Thermoactinomyces* HS682, 25 kDa.[6] The crude 68-kDa protease showed the highest activity at pH 11.0–12.0 like the purified 38-kDa protease. It might be estimated from these results that the cloned alkaline protease may be expressed as a dimer of 38-kDa subunit and degraded partially into a monomer at the same time of artificial removal of 15 amino acids from the N-terminus of the primary translation product during the purification procedures. It is necessary, for this estimation, to purify this enzyme and analyze its N-terminal sequence. The purification of the 68-kDa protease is in

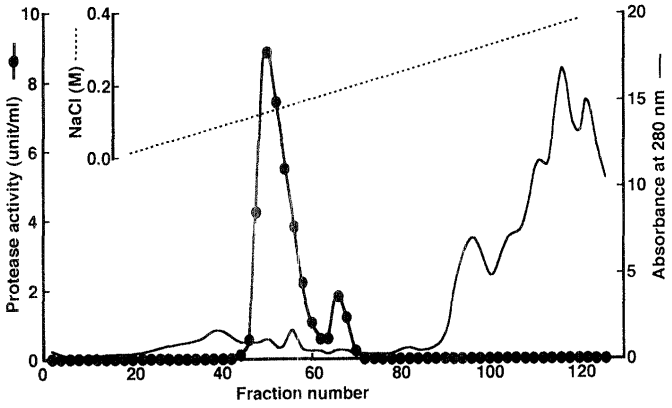

Fig. 4. DEAE-Toyopearl 650M Chromatography of the Cloned Alkaline Protease.

The protein solution was put on the column (1.6 × 18 cm) equilibrated with 10 mM NH_4Cl NH_4OH buffer (pH 10.0) containing 5 mM $CaCl_2$. The protease was eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer at a flow rate of 25 ml/h and collected in a volume of 4 ml.

Table Purification of Alkaline Protease from *Escherichia coli* Harboring pTIAP

Procedure	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)	Recovery (%)	Purification (fold)
Cell extract	2440	1199	0.49	100	1.0
70% $(NH_4)_2SO_4$	1630	1134	0.70	95	1.4
DEAE Toyopearl 650M	111	784	7.06	65	14.4
Sephadex G-75	15	342	22.80	29	46.5

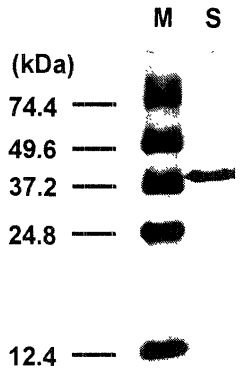

Fig. 5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of the Purified Alkaline Protease.

The purified enzyme was inhibited with 5 mM DFP before incubation with SDS and 2-mercaptoethanol at 100 C. The electrophoresis was done on 12.5% polyacrylamide gel. Lane S, purified protease; lane M, standard proteins including oligomers of cytochrome *c* of molecular mass of 12.4 to 74.4 kDa.

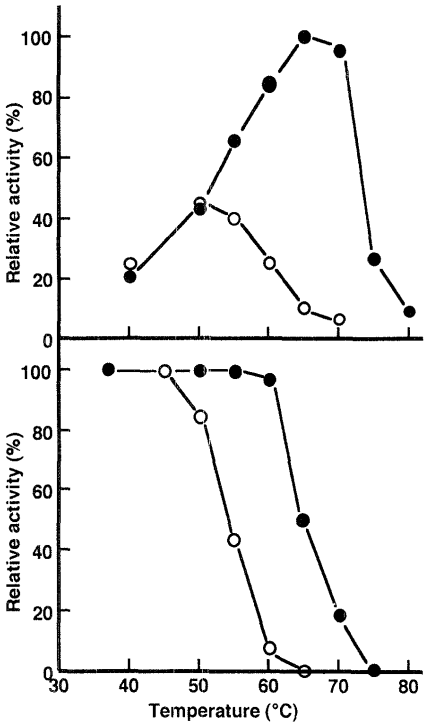

Fig. 6. Effect of Temperature on (A) the Proteolytic Activity and (B) the Stability of Purified Alkaline Protease.

(A) The proteolytic activities were measured at each temperature for 20 min at pH 11.0. (B) The remaining activities were measured at 37 C and pH 11.0 after incubating the protease in the buffer solution of pH 11.0 at each temperature for 10 min. Open symbols, without $CaCl_2$; closed symbols, with 5 mM $CaCl_2$.

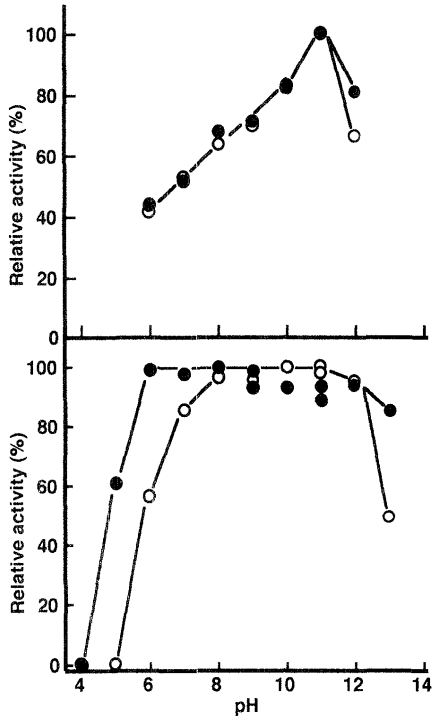

Fig. 7. Effects of pH on (A) the Proteolytic Activity and (B) the Stability of Purified Alkaline Protease.

(A) The proteolytic activities were measured at 37°C for 20 min in the buffer solution of each pH. (B) The remaining activities were measured at 37°C and pH 11.0 after incubating the protease in the buffer solution of each pH at 37°C for 10 min. Open symbols, without $CaCl_2$; closed symbols, with 5 mM $CaCl_2$.

progress in our laboratory.

Characterization of cloned alkaline protease

The effects of temperature on the proteolytic activity and the stability of the purified protease were examined in the absence and presence of 5 mM $CaCl_2$ at pH 11.0 for 10 min (Fig. 6). The maximum activity was obtained at 65–70°C in the presence of 5 mM $CaCl_2$. In the absence of $CaCl_2$, the activity was maximum at 50°C and almost inactivated at 65°C. Below 50°C, the activity without $CaCl_2$ was the same as that with 5 mM $CaCl_2$. The protease was stable below 45°C and showed the remaining activity of 85% at 50°C in the absence of $CaCl_2$. By addition of 5 mM $CaCl_2$, the heat stability was increased to 60°C.

The effects of pH on the proteolytic activity and the stability of the purified enzyme were examined in the range of 4.0–13.0 at 37°C in the absence and presence of 5 mM $CaCl_2$ (Fig. 7). The optimum pH for the proteolytic activity of the enzyme was pH 11.0–11.5. The protease was stable in the pH range of 7.0–12.0 without $CaCl_2$. By addition of 5 mM $CaCl_2$, the stability was increased to the pH range of 6.0 to 13.0. The heat and pH stabilities of the purified protease was lower than those of the extracellular alkaline protease from alkalophilic *Thermoactinomyces* sp. HS682.[6]

The effects of several inhibitors on the proteolytic activity of the enzyme were examined with each inhibitor at pH 11.0 and 37°C for 10 min. The protease activity was completely inhibited by 1 mM DFP and 10 mM PMSF but not by 1 mM MIA or 1 mM EDTA, which indicates that the protease is a serine protease.

References

1) G. M. Frost and D. A. Moss, in "Biotechnology, Vol. 7a, Enzyme Technology," ed. by J. F. Kennedy, VCH, Weinheim, 1987, pp. 157–168.
2) O. P. Ward, in "Microbial Enzymes and Biotechnology," ed. by W. H. Forgarty, Applied Science Publishers, London, 1983, pp. 251–317.
3) W. Peczynska-Czoch and M. Mordarski, in "Actinomyces in Biotechnology," ed. by M. Goodfellow, S. T. Williams, and M. Moedarshi, Academic Press, New York, 1988, pp. 246–253.
4) K. Horikoshi, in "Microorganisms in Alkaline Environments," VCH, Weinheim, 1991, pp. 187–193.
5) H. Takami, T. Akiba, and K. Horikoshi, *Appl. Microbiol. Biotechnol.*, **30**, 120–124 (1992).
6) K. Tsuchiya, Y. Nakamura, H. Sakashita, and T. Kimura, *Biosci. Biotech. Biochem.*, **56**, 246–250 (1992).
7) H. Takami, T. Akiba, and K. Horikoshi, *Biosci. Biotech. Biochem.*, **56**, 510–511 (1992).
8) H. Saito and K. Miura, *Biochim. Biophys. Acta*, **72**, 619–629 (1963).
9) H. C. Birnboim and J. Doly, *Nucl. Acids Res.*, **7**, 1513–1523 (1979).
10) D. Hanahan, *J. Mol. Biol.*, **166**, 557–580 (1980).
11) J. Sambrook, E. F. Fritsh, and T. Maniatis, in "Molecular Cloning: A Laboratory Manual," 2nd Ed., ed. by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
12) S. Henikoff, *Gene*, **28**, 351–359 (1984).
13) M. Hattori and Y. Sakaki, *Anal. Biochem.*, **152**, 232–238 (1986).
14) F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5463–5467 (1984).
15) U. K. Laemmli, *Nature*, **227**, 680–685 (1970).
16) M. M. Bradford, *Anal. Biochem.*, **72**, 248–254 (1976).
17) A. P. Feinberg and B. Vogelstein, *Anal. Biochem.*, **132**, 6–13 (1983); *ibid.*, **137**, 266–267 (1984).
18) E. M. Southern, *J. Mol. Biol.*, **98**, 503–517 (1975).
19) J. Shine and L. Dalgarno, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1342–1346 (1974).
20) S. Adhya and M. Gottesman, *Ann. Rev. Biochem.*, **47**, 967–996 (1978).
21) I. Tinoco, Jr., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla, *Nature New Biol.*, **246**, 40–41 (1973).
22) S. M. Sheen and R. L. Switzer, *J. Bacteriol.*, **172**, 473–476 (1990).
23) Y. Koide, A. Nakamura, T. Uozumi, and T. Beppu, *J. Bacteriol.*, **167**, 110–116 (1988).
24) S. Takekawa, N. Uozumi, N. Tsukagoshi, and S. Udaka, *J. Bacteriol.*, **173**, 6820–6825 (1991).
25) H. Takami, T. Kobayashi, R. Aono, and K. Horikoshi, *Appl. Microbiol. Biotech.*, **38**, 101–108 (1992).
26) H. Takami, T. Kobayashi, M. Kobayashi, M. Yamamoto, S. Nakamura, R. Aono, and K. Horikoshi, *Biosci. Biotech. Biochem.*, **56**, 1455–1460 (1992).
27) R. Kaneko, N. Koyama, Y. C. Tsai, R. Y. Juang, K. Yoda, and M. Yamasaki, *J. Biochem.*, **171**, 5232–5236 (1989).
28) N. Vasantha, L. D. Thomson, C. Phodes, and C. Banner, *J. Bacteriol.*, **159**, 811–819 (1984).
29) B. Meloun, M. Baudys, V. Kostka, G. Hausdorf, and C. Frommel, *FEBS Lett.*, **183**, 195–200 (1985).