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

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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–Val48 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.* 8) Although we purified only an extracellular alkaline serine protease from the culture broth of *Thermoactinomyces* sp. HS682,9) 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, BaxBest dideoxy sequencing kit, Random primer DNA labeling kit, and primers for sequencing were purchased from Takara Shuzo. [α-32P]dCTP was obtained from Amersham. Calf intestine alkaline phosphatase, isopropyl-β-D-thio-galactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-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 MV184 were used as host strains for cloning, sequencing, and expression of the gene for HS682 protease. Plasmids pUC18 and pUC19 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% K₂HPO₄, 0.02% NaCl, and 1.5% Na₂CO₃, 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 Mitura.10) Plasmid DNA was isolated by the alkaline extraction method.9) Competent cells of *E. coli* JM109 and MV184 were prepared by the method of Hanahan.9) All the procedures for cloning were done according to the standard

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* The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D87557.
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**Abbreviations:** IPTG, isopropyl-β-D-thio-galactopyranoside; DFP, diisopropyl fluorophosphate; MIA, monoiodoacetic acid; ORF, open reading frame; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonil fluoride.

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Results and Discussion
Cloning of the gene of alkaline protease from alkalaiophilic Thermaactinomyces 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 HindIII site of pUC18. All three inserts were cleaved at the same positions by SalI, SacI, KpnI, EcoRI, and BglII. 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 reverse direction toward the lacZ promoter, produced high protease activity in the absence of IPTG (data not shown). These results suggest that the cloned gene should contain a promoter and be expressed by its promoter. The 6.4-kb HindIII fragment was labeled with 32P by the random primer labeling method and used as a probe for Southern hybridization. This probe showed hybridization to the chromosomal DNA of Thermaactinomyces 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 HindIII–BglII (2.6 kb) with a potential of protease production was ligated with HindIII–BanHI digested pUC18. The inserted plasmid was partially digested with EcoRI and completely with SmaI, which cleaved at the 5'-end position of the fused site, BglII–BanHI, in the inserted plasmid. The obtained fragment of EcoRI–SmaI (1.5 kb) was ligated with EcoRI–SmaI 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 nucleotide 964. This ORF encoded a polypeptide of 321 amino acids. The putative Shine–Dalgarno (SD) sequence, GGAGG, was located 8 bases upstream from the initiation codon. The putative promoter region (−35 and −10 regions) could...
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 S' end of initiation codon of the gene, and that of amino acids at the N-terminal of pro-enzyme. The dashed underline indicates N-terminal sequence of alkaline serine protease purified from *Escherichia coli* IM109 harboring pTIAP.

be identified at nucleotide -60 (TTGTC) 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. 46 bases downstream of the termination codon (TAAT). Free energy of this sequence for a stem-loop structure was calculated to be -110.8 kJ/mol, 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 (QTVPGWGI5FN10QAHNRGIF) 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 preceded 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 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
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 proteases from Bacilli, which were removed from the primary translation products. The deduced amino acid sequence showed higher homology, 47-56%, with those of intracellular proteases than the 37-40% homology of extracellular proteases. 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 ISPI from Bacillus subtilis and ISPP from Bacillus polymyxa, and 2 residues of IAP221 from alkalophilic Bacillus sp. No. 221, while extracellular serine proteases except for thermitase, 1 residue, from Thermactinomyces vulgaris 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' 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 instability 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.* 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.](Image)

The protein solution was put on the column (1.6 x 18 cm) equilibrated with 10 mM NH₄Cl-NH₄OH buffer (pH 10.0) containing 5 mM CaCl₂. 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

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>2440</td>
<td>1199</td>
<td>0.49</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>70% (NH₄)₂SO₄</td>
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<td>1134</td>
<td>0.70</td>
<td>95</td>
<td>1.4</td>
</tr>
<tr>
<td>DEAE Toyopearl 650M</td>
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<td>784</td>
<td>7.06</td>
<td>65</td>
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</tr>
<tr>
<td>Sephadex G-75</td>
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<td>342</td>
<td>22.80</td>
<td>29</td>
<td>46.5</td>
</tr>
</tbody>
</table>

![Fig. 5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of the Purified Alkaline Protease.](Image)

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.](Image)

(A) The proteolytic activities were measured at each temperature for 30 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 CaCl₂; closed symbols, with 5 mM CaCl₂.
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