A Novel Protein with Alkaline Phosphatase and Protease Inhibitor Activities in *Streptomyces hiroshimensis*

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A novel alkaline phosphatase (S-ALP) was found in the culture filtrate of *Streptomyces hiroshimensis* IFO 12785. Purification was achieved on Sephadex G-75 column, palmitoylated gauze column, and Superdex 75 HR column chromatographies. The molecular weight of S-ALP was estimated to be 14200 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isoelectric point is 9.2. S-ALP had maximum enzyme activity at pH 9.5. S-ALP efficiently catalyzed both p-nitrophenyl phosphate and p-nitrophenyl phosphorylcholine substrates, particularly the latter. The N-terminal amino acid sequence (25 residues) of S-ALP was 60 to 72% identical to that of *Streptomyces subtilisin* inhibitor-like proteins. S-ALP exhibited trypsin inhibition in addition to a strong inhibition of subtilisin.

**Key words** *Streptomyces* hiroshimensis; alkaline phosphatase; protease inhibitor; subtilisin inhibitor; trypsin inhibitor

Alkaline phosphatases (ALPs) (EC 3.1.3.1) in various species of the genera *Streptomyces* and *Streptovercillium* have been known to play an important role in the biosynthesis of aminoglycoside antibiotics.1—4) Recently, the purification and properties of the extracellular enzyme from *Streptomyces griseus* have been studied in detail.5) The *Streptomyces* subtilisin inhibitor (SSI) is a well-characterized proteinaceous inhibitor of proteases.6) The structure–function relationships of SSI and SSI-like proteins have been extensively studied in combination with a variety of physicochemical methods and the generation of site-specific mutants.7—10) Almost all of these inhibitors exist as dimeric proteins consisting of two identical subunits.8,10) A novel monomeric subtilisin inhibitor has been isolated from the culture medium of a *Streptomyces* species 1-72.11

*Streptomyces hiroshimensis* is known to produce phospholipase and hemolysin.12,13) We have recently found that this strain produces a novel ALP with protease inhibitor activity in culture broth. This paper describes the purification and some properties of the enzyme.

**MATERIALS AND METHODS**

**Organism and Growth Conditions** *S. hiroshimensis* IFO 12785 was obtained from the Institute for Fermentation, Osaka (Juso Nishinocho, Osaka, Japan). The strain was precultured as described in a previous paper,14) in medium A (200 ml) composed of 1.0% soluble starch, 1.0% glucose, 0.75% meat extract, 0.5% peptone, 0.3% NaCl, and 0.1% MgSO₄, and stored at 30 °C in an orbital shaker at 150 rev/min for 3 d. The culture (10 ml) was transferred and recultured in the new medium A. After further cultivation for 5 d, the mycelium was removed by centrifugation at 4 °C, and the supernatant was freeze-dried. The freeze-dried materials were used for the purification of the enzyme.

**Enzyme Assay** ALP activity was colorimetrically assayed using p-nitrophenyl phosphate (pNPP, Nacalai Tesque) and p-nitrophenolphosphoryl choline (NPPC, Sigma) as substrates.15) Fifty microliters of enzyme solution was added to 20 mM Tris–HCl buffer 50 μl (pH 8.0) containing 10 mM substrate, and incubated at 37 °C for specified times. The reaction was stopped by adding 0.1 M NaOH 100 μl, and the absorbance of the p-nitrophenol formed was measured at 405 nm. The units of activity were calculated as micromoles of p-nitrophenol released per minute. To determine the $K_m$ and $V_{max}$ constants, the enzyme was incubated with increasing amounts of substrate and the measured activities from duplicate experiments were plotted in a Hanes–Woolf diagram.

**Purification of S-ALP** The freeze-dried culture supernatants (8 g) were dissolved in 1 ml Tris–HCl buffer 40 ml (pH 8.0) and applied to a Sephadex G-75 Superfine (Amersham Pharmacia Biotech) column (97×215 mm) equilibrated with the same buffer and then eluted with the buffer. The pieces of prepared palmitoylated gauze (Pal-G) were washed and packed onto the column with 50% ethanol.16) After washing with water, the column was equilibrated with 1 mM Tris–HCl buffer (pH 8.0). The active fractions from the Sephadex column were applied to the Pal-G column (15×340 mm). The enzyme adsorbed on the column was eluted with the buffer containing 0.5% (w/v) Triton X-100. The active fractions were further applied to a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech) using a Superdex 75 HR (Amersham Pharmacia Biotech) gel filtration column (10×300 mm) equilibrated with 1 mM Tris–HCl buffer (pH 8.0) and then eluted with the same buffer.

**Protein Quantification** The amount of protein was spectrophotometrically determined using a protein assay (BCA Protein Assay, Pierce) with bovine serum albumin as the standard.17

**Electrophoresis** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an 8—25% gradient gel in the PhastSystem under reducing conditions. An appropriate amount of protein was incubated at 90 °C for 5 min with 2% (w/v) glycerol and 50 mM dithiothreitol in 58 mM Tris–HCl buffer, pH 6.8, and then subjected to electrophoresis. The gel was stained using a Silver Staining Kit (Amersham Pharmacia Biotech). Nondenaturing SDS-PAGE was carried out in the same system but without dithiothreitol. An LMW Electrophoresis Calibration Kit (Amersham Pharmacia Biotech) was used as the molecular
calibration proteins.

Phast Gel IEF 3-9 (Amersham Pharmacia Biotech) on the PhastSystem was used for isoelectric focusing. The gel was stained with Coomassie brilliant blue (Amersham Pharmacia Biotech). A pi Calibration Kit 3-10 (Amersham Pharmacia Biotech) was used as the pi marker.

**Molecular Mass Estimation** The relative molecular mass of the purified enzyme was estimated both by FPLC gel filtration on a Superdex 75 HR column and by SDS-PAGE as described above. For the gel filtration method, dextran blue and protein molecular mass standards (Sigma) were applied to the column to obtain a calibration curve.

**Determination of N-Terminal Amino Acid Sequence** The phosphatase protein (S-ALP) obtained by Superdex 75 HR column chromatography was further purified by reverse-phase HPLC using a Cosmosil 5C18 AR-II (Nacalai Tesque) column (2 x 150 mm). The N-terminal amino acid sequence was determined using the Protein Sequencing System (Model HP G1005A, Hewlett-Packard, Palo Alto, CA, U.S.A.).

**Measurement of Protease Inhibitory Activity** Inhibitory activities toward trypsin (TPCK-treated, Sigma), subtilisin (Sigma), and α-chymotrypsin (Sigma) were measured as described by Kojima et al.7 except that 100 mM Tris–HCl buffer (pH 7.5) was used for subtilisin.

**Measurement of ALP Activity in the Presence of Proteases** The proteases used were trypsin, subtilisin, and α-chymotrypsin. A mixture of S-ALP (0.1 mU) and protease (from 15 mg to 1.5 mg) in 75 μl of 1 mM Tris–HCl buffer (pH 8.0) was incubated at 37 °C for 10 min. Twenty-five microliters of substrate solution (20 mM NPPC or 20 mM pNPP) was added to the preincubation mixtures and incubated at 37 °C for 60 min. The reaction was stopped by adding 0.1 M NaOH 100 μl and the absorbance was measured at 405 nm.

**RESULTS AND DISCUSSION**

**Purification of ALP** As mentioned in MATERIALS AND METHODS the freeze-dried culture supernatant of *S. hiroshimensis* was initially fractionated on Sephadex G-75 Superfine column chromatography, and the ALP fractions were obtained. With Pal-G column chromatography (Fig. 1a), most of the enzyme activity was eluted from the column with the buffer containing 0.5% (w/v) Triton X-100. The enzyme was purified approximately 10 times in this step. Figure 1b shows the elution profile on the FPLC system using Superdex 75 HR column chromatography. The enzyme activity was separated into two distinct peaks, fraction I and fraction II. Fraction II was designated as S-ALP. A summary of the purification of the ALPs from the culture supernatant of *S. hiroshimensis* is shown in Table 1. S-ALP was purified 6.7-fold to a final specific activity of 10 mU/mg of protein with 3.4% recovery.

The purity and homogeneity of fraction I and S-ALP were determined by SDS-PAGE. The peak I preparation gave a broad band on SDS-PAGE under reducing conditions (Fig. 2a, lane 2). S-ALP gave only a single band on the same SDS-PAGE (Fig. 2a, lane 3). All subsequent experiments for the biochemical and biological characterizations were performed using only S-ALP.

Purification of ALP using the Pal-G column (Fig. 1a) was sufficient to recover the homogeneous enzyme protein. This result seems to show that Pal-G is useful tool for the purification of phosphatase in addition to lipase and phospholipase.16,18

**Enzymatic Properties of S-ALP** The relative molecular mass of S-ALP was estimated to be 14500 by FPLC gel fil-

![Fig. 1. Elution Profiles of Column Chromatography](image-url)

(a) Elution of alkaline phosphatase from a Pal-G column. After washing the column with 1 mM Tris–HCl buffer (pH 8.0), elution was carried out with 0.5 mM Tris–HCl buffer (pH 8.0) containing 0.5% (w/v) Triton X-100 (arrow). (b) Separation of active Pal-G fractions on a Superdex 75 HR column.

Table 1. Purification of Alkaline Phosphatase from *S. hiroshimensis*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (mU)</th>
<th>Specific activity (mU/mg)</th>
<th>Recovery of activity (%)</th>
<th>Purification</th>
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<tr>
<td>Culture supernatant</td>
<td>2723</td>
<td>4180</td>
<td>1.5</td>
<td>100</td>
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<td>Lyophilization</td>
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<td>2243</td>
<td>0.085</td>
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<td>0.57</td>
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<td>1269</td>
<td>3.6</td>
<td>30.4</td>
<td>2.40</td>
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<tr>
<td>Pal-G</td>
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<td>640</td>
<td>42</td>
<td>15.3</td>
<td>28.0</td>
</tr>
<tr>
<td>Superdex 75</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fraction I</td>
<td>1.8</td>
<td>90</td>
<td>50</td>
<td>2.15</td>
<td>33.3</td>
</tr>
<tr>
<td>Fraction II (S-ALP)</td>
<td>13.5</td>
<td>139</td>
<td>10</td>
<td>3.33</td>
<td>6.67</td>
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</table>
tration. SDS-PAGE under both reducing (Fig. 2a) and nonreducing (data not shown) conditions gave a molecular mass of 14200. The SSI-like proteins were composed about 110 amino acids that existed as stable dimer proteins, but S-ALP could be regarded as a monomer protein and a subtilisin inhibitor as reported by Kourteva and Boteva. The isoelectric point was determined to be 9.2 (Fig. 2b).

The optimum pH for S-ALP was 9.5 under with both substrates NPPC and pNPP (Fig. 3). S-ALP had the optimum temperature of 50—60 °C, and was stable in the temperature range from 40 to 50 °C. At a concentration of 1 mM, ZnCl₂ and EDTA for the enzyme on pNPP caused 55% and 40% inhibition, respectively. SDS (1%) exerted a 65% inhibition. S-ALP exhibited simple Michaelis–Menten kinetics. The biochemical constants Kₘ and Vₘₐₓ were calculated using NPPC as the substrate, resulting in a Kₘ value of 9.7×10⁻⁵ M and a Vₘₐₓ value of 9.9 nmol/min/mg. The Kₘ and Vₘₐₓ values for pNPP as the substrate were found to be 3.8×10⁻⁶ M and 4.8 nmol/min/mg, respectively.

S-ALP was distinctly different from the other Streptomyces ALPs in its molecular mass, isoelectric point, and Ca⁺⁺ requirement.

**N-Terminal Sequence of S-ALP** Automated Edman degradation determined the partial N-terminal amino acid sequence (25 residues) of S-ALP to be S-L-Y-A-S-S-A-L-V-L-T-V-G-H-G-A-N-S-T-E-A-X-V-D-R. We did not completely detect other N-terminal sequences different from this sequence in S-ALP. This result also shows that S-ALP is a purified protein. From the DNASIS analysis, the N-terminal sequence of S-ALP showed a low homology (only 24, 4 and 28%) to the N-terminal sequences of each phospholipase C from *Streptomyces albus* and *Streptomyces exfoliatus*, and of the alkaline phosphatase from *Streptomyces griseus*. On the other hand, the N-terminal sequence of S-ALP showed a high identity (60—72%) with various protease inhibitors produced by *Streptomyces* species through similarity searches using BLAST algorithms (Table 2).

**Protease Inhibition Activity of S-ALP** Figure 4 shows the inhibitory effect of S-ALP on the activity of three proteases. S-ALP showed dose-dependent specific inhibitory activity against trypsin and strong inhibition against subtilisin,
and had no effect on \( \alpha \)-chymotrypsin.

The inhibitory specificity is similar to that of the SSI-like proteins because of the strong inhibition against subtilisin compared with trypsin.\(^8,10\) On the basis of the N-terminal amino acid sequence, the structural characteristics, and the inhibitory properties, S-ALP could be defined as a novel extracellular protease inhibitor from \textit{Streptomyces}. Thus S-ALP is a novel protein with ALP and protease inhibitor activities. It appears that S-ALP acts as a multifunctional protein both for dephosphorylation of biologically active intermediates by ALP activity,\(^1—5\) and for the inhibitory regulation of proteases by protease inhibitory activity, similar to the SSI-like proteins.\(^6—11\)

**ALP Activity of S-ALP in the Presence of Proteases**

The ALP activity of S-ALP in the presence of various concentrations of proteases was not inactivated (data not shown). This shows that the ALP activity of S-ALP is not influenced by the proteases. This may demonstrate that the ALP part of S-ALP is different from the protease inhibitor part. A detailed characterization of the structure and functions of S-ALP is now in progress.

**REFERENCES**