Note

Purification and Some Properties of a Keratinolytic Enzyme from an Alkaliphilic Nocardioopsis sp. TOA-1

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A novel alkaliphilic Nocardioopsis sp., strain TOA-1, was isolated from a tile-joint of a bathroom. Strain TOA-1 produced a variety of alkaline hydrolytic enzymes. An alkaline protease, designated NAPase, was purified and characterized. NAPase had a very high keratinolytic activity and high stability under acidic conditions.

Key words: alkaliphilic actinomycetes; Nocardioopsis sp.; alkaline protease; keratinolytic enzyme

Although the tile-joints of bathrooms are highly alkaline, it is well known that many microorganisms, especially certain fungi, can grow preferentially in such environments.13 Attempts to prevent microbial growth in bathrooms have been continuously conducted. In a series of these studies, we have isolated a number of alkaliphilic bacterial strains from the tile-joints.

The isolation of alkaliphilic microorganisms was done out using an alkaline medium containing 10 g of glucose, 5 g of peptone, 1 g of K2HPO4, 0.5 g of MgSO4·7H2O, 15 g of agar, and 10 g of Na2CO3 (per liter). Among a number of alkaliphilic strains isolated in this study, an alkaliphilic actinomycetes strain, TOA-1, was chosen as a protease producer. The substrate mycelium of this strain was colorless. The spores were in straight chains between 10 and 50 in number, and the surface was smooth and white. Strain TOA-1 grew at pH 7.5-13.0 between 15-40°C. The optimal pH and temperature for growth were 10.0 and 30°C, respectively. Diaminopimelic acid was the meso type and diagnostic sugars were not detected.

The genomic DNA of TOA-1 was extracted by the method of Murray and Thompson.23 The 16S rDNA was amplified by PCR using the following oligonucleotide primers: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTACGAC-
Keratinolytic Enzyme from Alkaliphilic *Nocardiopsis* sp. TOA-1

**Strain TOA-1**
- *Nocardiopsis alba*
- *Prausnia hordei*
- *Nocardiopsis listeri*
- *Nocardiopsis lucentensis*
- *Nocardiopsis dussonnvillei*
- *Nocardiopsis santarcitica*
- *Nocardiopsis alborubida*
- *Streptomonospora salina*
- *Thermonospora alba*
- *Streptosporangium pseudovulgaris*
- *Actinomadura oligospora*
- *Kitasatospora setae*
- *Streptomyces griseus*

**Bacillus subtilis**

**Fig. 1.** Phylogenetic Tree Depicting the Relationship of TOA-1 to Other Related Organisms Based on 16S rDNA Sequences. *Bacillus subtilis* was incorporated as an outgroup.

Medium (0.5% skim milk, 0.1% yeast extract, 1.0% Na₂CO₃) at 30°C for 4 days with shaking (120 rpm). The keratinolytic activity was assayed by the method of Takami. One unit of keratinolytic activity was defined as the amount of enzyme that released 1 µg of tyrosine per h. During purification of keratinolytic enzyme, caseinolytic activity was measured instead of keratinolytic activity. Caseinolytic activity was assayed by the method of Takami. One unit of caseinolytic activity was defined as the amount of enzyme that released 1 µg tyrosine per min. The protein content was measured by Lowry’s method. The culture broth was precipitated by adding (NH₄)₂SO₄ to 80% saturation and the precipitate was dialyzed against 20 mM 3-[n-morpholino]propanesulfonic acid (MOPS)-NaOH buffer, pH 7.5, and used as a cell-free crude enzyme. The crude enzyme was put onto a column of CM-Toyopearl 650M (φ2.5 cm × 10 cm) that had been equilibrated with 10 mM MOPS-NaOH buffer (pH 7.5). The column was washed and protein was eluted with the same buffer containing 0.2 M NaCl. Fractions containing the caseinolytic activity were collected and dialyzed against 10 mM Tris-HCl buffer (pH 9.0). The dialyze was put onto a column of DEAE-Toyopearl (φ1.0 cm × 5.0 cm) that had been equilibrated with 10 mM Tris-HCl buffer (pH 9.0). The column was washed with the same buffer and the fractions with caseinolytic activity were pooled as purified enzyme. The results of the purification are summarized in Table 1.

Alkaline protease from strain TOA-1, designated

**Fig. 2.** SDS-PAGE of Purified NAPase.
Lane 1, molecular mass marker proteins (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; lysozyme, 14.3 kDa); lane 2, purified NAPase.
NAPase, was purified to homogeneity by the criterion of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). SDS-PAGE was done with 12% polyacrylamide gel as described by Laemmli.\(^9\) Protein was stained with Comassie Brilliant Blue.

Some enzymatic properties of the purified NAPase were investigated. Optimal temperature of NAPase was between 70–75°C and optimal pH was 11.0–11.5 (Fig. 3). NAPase was found to be stable below 60°C after 10 min of incubation at various temperatures at pH 8.0 (Fig. 3). NAPase was stable in wide pH range from 1.5 to 12.0 after incubation at 30°C for 24 h (Fig. 3). The molecular mass of NAPase, estimated as 20 kDa (Fig. 2), was close to other proteases of actinomycetes origin such as Protease A (18 kDa) and B (19 kDa) from Streptomyces griseus,\(^{11}\) SFase-2 (19 kDa) from Streptomyces fradiae,\(^{12}\) and protease I (21 kDa) from Nocardiosis dassonvillei.\(^9\) The isoelectric points was above pH 10.0. The specific activity of NAPase toward casein was 1,100 U/mg protein. The specific activity of NAPase toward keratin

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Table 1. Summary of Purification of Kerationalytic Enzyme from Alkaliphilic Nocardiosis sp. TOA-1

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Activity (units/ml)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg-protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture fluid</td>
<td>3790</td>
<td>29</td>
<td>769</td>
<td>143</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>157</td>
<td>440</td>
<td>137</td>
<td>504</td>
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</tr>
<tr>
<td>CM-Toyopearl</td>
<td>17</td>
<td>2470</td>
<td>40</td>
<td>1050</td>
<td>38</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
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<td>1940</td>
<td>30</td>
<td>1100</td>
<td>30</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of pH and Temperature on Activity and Stability of NAPase.
A: Effects of pH on enzyme activity. The enzymatic activity toward casein was assayed at pHs ranging from 7.0 to 13.0. B: Effects of pHs on enzyme stability. The reaction mixtures were incubated at pH ranging from 1.0 to 13.0, at 30°C for 24 h and caseinolytic activity was assayed at 30°C, pH 10.5. Symbols: ○; KCl-HCl buffer, △; glycine-NaCl-HCl buffer, △; acetate buffer, ■; phosphate buffer, ○; Tris-HCl buffer, ●; glycine-NaCl-NaOH buffer, ◆; KCl-NaOH buffer. C: Effect of temperature on enzyme activity. The enzymatic activity toward casein was assayed at temperatures from 40°C to 90°C, at pH 10.5. D: Effects of temperatures on enzyme stability. The enzyme in 20 mM Tris-HCl buffer (pH 8.0) was incubated at temperatures from 40°C to 80°C. After 10 min of incubation, the caseinolytic activity was assayed at 30°C, pH 10.5.
was 3,300 U/mg protein. Phenylmethylsulfonyl fluoride (PMSF) and diisopropylphosphorofluoridate (DFP) completely inhibited the activity of NAPase. EDTA and p-chloromercuribenzoate (PCMB) slightly inhibited it. These results suggested that NAPase could be a serine protease.

NAPase had a very high specific activity toward keratin (3,300 U/mg protein), but the specific activity was slightly lower than that of keratinase (3,970 U/mg protein) from Bacillus halodurans AH-101. However, NAPase showed higher stability than the keratinase from Bacillus halodurans AH-101 under acidic conditions. To further investigate the unique properties at molecular level, the cloning of NAPase is now in progress.

References