A novel alkaliphilic *Nocardiopsis* 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; *Nocardiopsis* 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. Thus, 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 K$_2$HPO$_4$, 0.5 g of MgSO$_4$$\cdot$7H$_2$O, 15 g of agar, and 10 g of Na$_2$CO$_3$ (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. The 16S rDNA (accession no. AY027776) obtained by PCR was 1,515 bp in size. The BLAST search of this sequence was done to a non-redundant DNA database. The phylogenetic tree obtained with a mammal-likelihood algorism is presented in Fig. 1. The highest similarity of 97.6% was shown between strain TOA-1 and *Nocardiopsis alba*. The result suggested that strain TOA-1 would be a member of the genus *Nocardiopsis*.

It is well-known that actinomycetes secrete a variety of hydrolytic enzymes. However, knowledge of alkaline enzymes from alkaliphilic actinomycetes is very scarce. Only two types of alkaline serine protease from *Nocardiopsis dassonvillei* and alkaline cellulase from *Streptomyces lividans* have been reported to date. We investigated the production of the extracellular alkaline enzymes from strain TOA-1.

Plate assays were first attempted using alkaline media including various substrates to detect alkaline enzyme activities. Strain TOA-1 formed large clear zones on plates containing the following substrates: soluble starch, carboxymethyl cellulose and Avicel, chitin, tween 80, pectin, casein and keratin powder, and xylan. This shows that strain TOA-1 produces at least seven alkaline hydrolytic enzymes such as amylase, cellulase, chitinase, lipase, pectinase, protease, and xylanase.

One of the interesting features of strain TOA-1 was that it formed extremely large clear zones on keratin powder plates. For purification of the keratinolytic enzyme, strain TOA-1 was cultivated in 500-ml Sakaguchi flasks containing 100 ml of skim milk.
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.⁷) 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.⁸) 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.⁹) 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 x 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 dialyzate was put onto a column of DEAE-Toyopearl (φ1.0 cm x 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

![Phylogenetic Tree Depicting the Relationship of TOA-1 to Other Related Organisms Based on 16S rDNA Sequences. Bacillus subtilis was incorporated as an outgroup.](image)

![SDS-PAGE of Purified NAPase.](image)

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.10) Protein was stained with Commassie 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 Nocardiopsis dassonvillier.5) 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
was 3,300 U/mg protein. Phenylmethanesulfonyl 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-101.1,13) However, NAPase showed higher stability than the keratinase from Bacillus halodurans AH-101 under acidic conditions.8) To further investigate the unique properties at molecular level, the cloning of NAPase is now in progress.

References