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# Purification and Characterization of α-Neoagarooligosaccharide Hydrolase from *Cellvibrio* sp. OA-2007

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α-Neoagarooligosaccharide (α-NAOS) hydrolase was purified from *Cellvibrio* sp. OA-2007 by using chromatographic techniques after hydroxyapatite adsorption. The molecular masses of α-NAOS hydrolase estimated using SDS-PAGE and gel filtration chromatography were 40 and 93 kDa, respectively, and the optimal temperature and pH for the enzyme activity were 32°C and 7.0–7.2. α-NAOS hydrolase lost 43% of its original activity when incubated at 35°C for 30 min. The enzyme hydrolyzed neoagarobiose, neoagarotetraose, and neoagarohexaose to galactose, agarotriose, and agaropentaose, respectively, and produced 3,6-anhydro-L-galactose concomitantly; however, it did not degrade agarose.

**Keywords:** *Cellvibrio* sp., α-NAOS hydrolase, neoagarooligosaccharide

The bioactivities of agarose hydrolyzates have attracted the attention of researchers in diverse fields developing products ranging from food additives to pharmaceuticals [2, 3, 10]. Although numerous studies have been conducted on neoagarooligosaccharides, the bioactivity of agarooligosaccharides produced by α-agarase remains poorly investigated. Enoki *et al.* [4, 5] reported inhibition of pro-inflammatory mediator release by agarooligosaccharides, and Hatada *et al.* [8] reported that α-agarase-digested porphyran enhances the antioxidant activity. Thus, α-agarase may have promising applications. Conversely, marine polysaccharides have attracted attention as future biomass resources, and an alginate from brown algae has been studied especially well [6, 11, 17]. However, only a few reports have described bioethanol production from agar [9, 14]. For completely bioconverting agarose into its constituent sugars, α-agarase is the most critical enzyme, and although substantial effort has been devoted to studying agarolytic enzymes, α-agarase has so far been purified and investigated genetically only from the genera *Alteromonas* [7, 13], *Bacillus* [16], *Thalassomonas* [12] and *Vibrio* [15]. While purifying agarases from *Cellvibrio* sp. OA-2007, we discovered an enzymatic activity that hydrolyzed neoagarobiose to produce a galactose. In this report, we describe the purification and characterization of α-neoagarooligosaccharide hydrolase from *Cellvibrio* sp. OA-2007. This is the first report on α-agarase from *Cellvibrio*.

Protein purification details are described in a previous paper [1]. After cultivating *Cellvibrio* sp. OA-2007 as previously described [1], cells were collected by centrifugation, suspended in 50 mM Tris-HCl buffer (pH 7.0), and sonicated. After centrifuging to remove cell debris, 2% (w/v) hydroxyapatite (Sigma) was added to the supernatant and the suspension was placed on ice for 1 h and intermittently agitated gently. The hydroxyapatite particles were collected by centrifugation and washed using a cold 10 mM Tris-HCl buffer (pH 7.0) to remove nonspecifically bound proteins, and the proteins bound tightly to the particles were recovered by soaking the particles in a cold 50 mM phosphate buffer (pH 7.0). The supernatant collected after centrifuging the sample was used as the initial fraction for purification of α-NAOS hydrolase by employing chromatographic techniques according to methods described previously [1]. The homogeneity of proteins in the active fractions was confirmed using SDS-PAGE and the molecular mass of the target protein was estimated.

To assay for hydrolysis activity against α-linkages, neoagarobiose was purified from a neoagarooligosaccharide mixture, which was prepared by hydrolyzing agarose with cell homogenates of recombinant *Escherichia coli* as described previously [1]; neoagarobiose was purified using charcoal adsorption and a flash chromatography apparatus, YFLC AI-580 (Yamazen Science Inc., Japan). To measure enzymatic activity under standard assay conditions, a reaction mixture containing the enzyme and 4 g/l neoagarobiose in 20 mM phosphate buffer (pH 7.0) was incubated at specified temperatures for 1 h and then boiled to terminate the reaction. The galactose produced in the reaction was measured using HPLC as described previously [1]. One unit of enzyme activity was defined as the amount of the enzyme that produces 1 μmol galactose/min.

To determine the optimal temperature and pH for the enzyme activity, hydrolysis reactions were conducted under standard assay conditions at various temperatures and at various pH settings at 25°C. Thermal stability of the enzyme was assessed by measuring (under standard conditions at 25°C) the activity remaining after incubating the enzyme solution for 30 min at various temperatures

To examine the mode of action of the purified enzyme on neoagarooligosaccharides, the enzyme was incubated with neoagarooligosaccharides and the resulting hydrolysis products were identified using thin layer chromatography as described by Suzuki *et al.* [16]. Protein concentrations were determined using the BCA assay kit (Bio-Rad, CA, USA).

In Fig. 1, the left vertical axes show the relative UV (280 nm) absorbance of an eluate and the right vertical axes show the enzyme activity relative to maximal activity. Active fractions of the enzyme were detected at 0.12–0.15 M NaCl in a linear gradient elution of the anion-exchange chromatography (Fig. 1A); these fractions were pooled and further purified using a gel-filtration column (Fig. 1B). The active fractions were collected and subjected to SDS-PAGE (Fig. 2). The final enzyme preparation was purified 100-fold to homogeneity (data not shown). A single protein band was observed at 40 kDa, a molecular mass that is comparable to 42 kDa for α-NAOS hydrolases from *Bacillus* sp. MK03 [16] and *Vibrio* sp. strain JT0107 [15]. The molecular weights of α-agarase from *Thalassomonas* sp. and *Alteromonas agarlyticus* strain GJ1B are 85 kDa [12] and 180 kDa [13], respectively. In this study, the molecular mass estimated for the enzyme by gel filtration chromatography was 93 kDa, which suggests that the native enzyme is a dimer: native α-NAOS hydrolase from *Vibrio* sp. strain JT0107 is 84 kDa [15], whereas α-NAOS hydrolase from *Bacillus* sp. MK03 is 320 kDa [16].

The optimal temperature for enzyme activity was 32°C (Fig. 3A, closed circle), which is similar to the optimal temperatures for α-NAOS hydrolases from *Bacillus* sp. MK03 [16] and *Vibrio* sp. strain JT0107 [15]. The enzyme lost 43% of its original activity after incubation at 35°C (Fig. 3A, open circle). The α-NAOS hydrolases from *Bacillus*

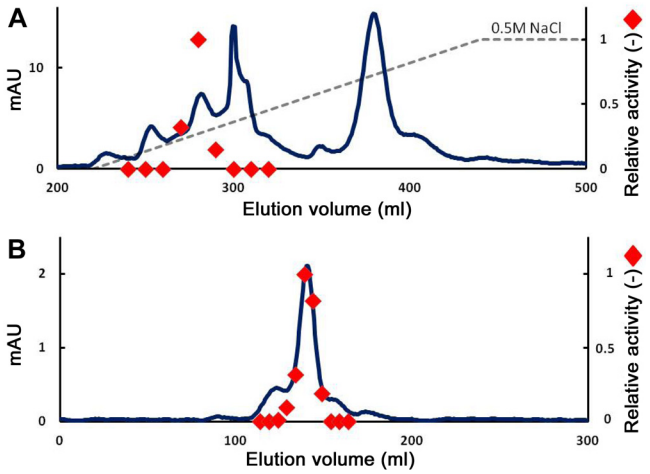


**Fig. 1.** Chromatographic purification of cell homogenate.
(**A**) Anion-exchange chromatography on DEAE-Toyopearl PAK-650M. The phosphate buffer containing gradient NaCl rising from 0 to 0.5 M (broken line) was used to wash out the active fraction. (**B**) Gel filtration chromatography on HiPrep 26/60 Sephacryl S-200 HR. Fractions were monitored at 280 nm for protein content (curved line). Symbol: diamond, the relative activity of the active fraction.

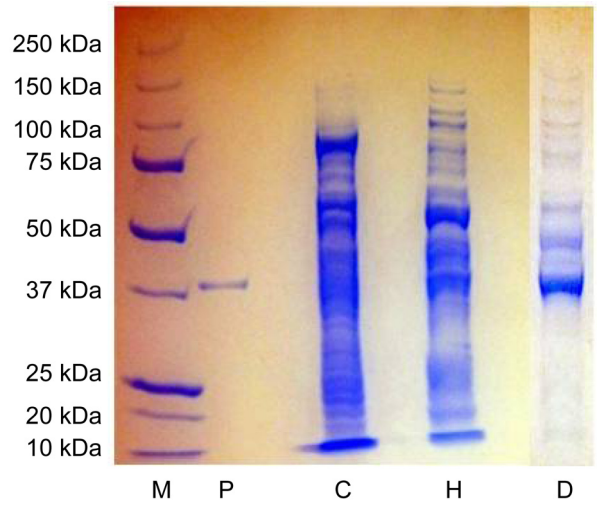


**Fig. 2.** SDS-PAGE images.
Lane: M, size marker; P, purified protein; C, cell homogenate; H, after hydroxyapatite treatment; D, after anion-exchange chromatography.

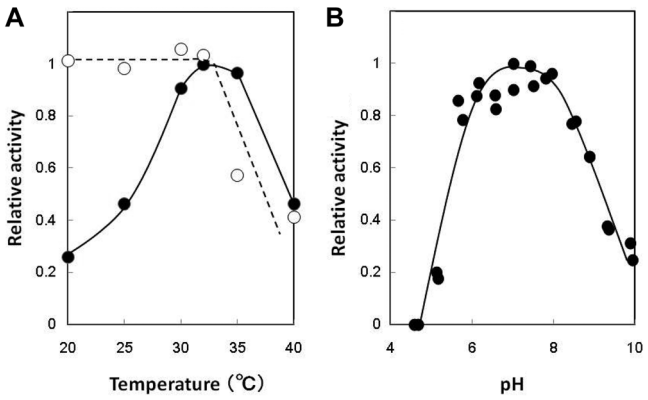


**Fig. 3.** Characteristics of enzymatic activity.
(**A**) Effect of temperature on enzyme activity (closed circle) and on its thermal stability (open circle), (**B**) effect of pH on enzyme activity.

sp. MK03 and *Vibrio* sp. strain JT0107 show slightly higher thermal stability than the enzyme described here, because 50% of the original activities of α-NAOS hydrolases from *Bacillus* sp. MK03 and *Vibrio* sp. strain JT0107 were retained after incubating at 42°C for 30 min and 35°C for 60 min, respectively.

The effect of pH on enzyme activity is shown in Fig. 3B. The optimal pH for hydrolysis of neoagarobiose was at 7.0, and whereas >80% of the activity was measured at pH 5.5–8.0, the relative activity decreased by 20% at pH 5.0. The optimal pH values for α-NAOS hydrolases from *Bacillus* sp. MK03 and *Vibrio* sp. strain JT0107, determined by incubating for 10 min at 30°C, were 6.1 [16] and 7.7 [15], respectively; these enzymes were less active at lower pH settings than the enzyme described here (whose activity was measured by incubating for 60 min at 25°C). The kinetic parameters $K_m$ and $V_{max}$ of purified α-NAOS hydrolase were calculated to be 6.0 mM and 19 U/mg-protein from Lineweaver-Burk plot (data not shown). The $K_m$ of α-NAOS hydrolase from *Vibrio* sp. strain JT0107, which was reported to be 5.37 mM [15], is comparable to that of the enzyme purified in this study, but the $V_{max}$ measured for α-NAOS hydrolase from *Vibrio* sp. strain JT0107 was 92 U/mg-protein, which is considerably higher than that of the enzyme described here.

As shown in Fig. 4, the purified enzyme hydrolyzed neoagarobiose (Lane 2) to produce galactose and 3,6-anhydro-L-galactose, as judged based on the results of Suzuki *et al.* [16]. When 100-fold (Lane 5) or 30-fold (Lane 7) more enzyme was used with neoagarotetraose or neoagarohexaose, the enzyme hydrolyzed these substrates to 3,6-anhydro-L-galactose and agarotriose or agaropentaose,

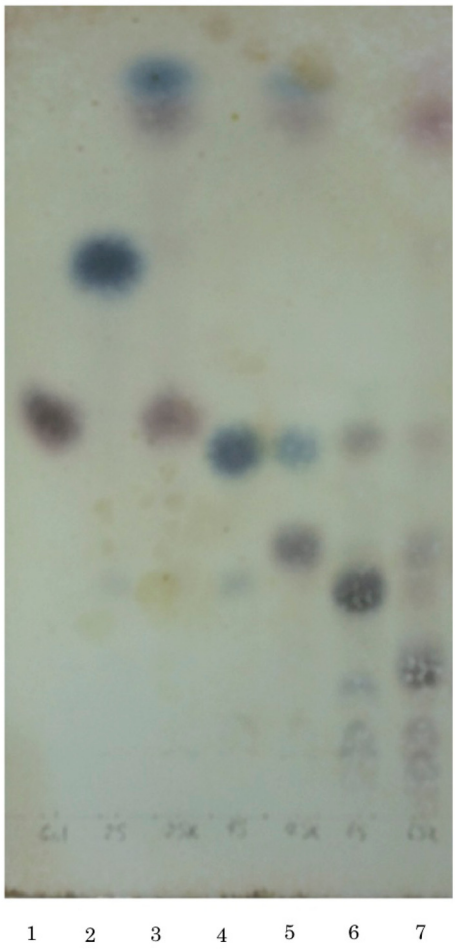


**Fig. 4.** TLC chromatogram of reaction products.
Lanes : 1, galactose; 2, neoagarobiose; 3, neoagarobiose+enzyme; 4, neoagarotetraose; 5, neoagarotetraose+enzyme; 6, neoagarohexaose; 7, neoagarohexaose+enzyme.

respectively. This result suggests that hydrolysis occurred at the nonreducing end of the neoagarooligosaccharides. However, the enzyme did not degrade agarose even at high concentrations. Thus, the enzyme we have purified is similar to the α-NAOS hydrolase from *Vibrio* sp. strain JT0107 in terms of the molecular mass of the native enzyme and its subunit, optimal reaction temperature and pH, thermal stability, and the mode of action on neoagarooligsaccharides.

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