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Purification and Characterization of a Novel Enzyme, α-Neoagarooligosaccharide Hydrolase (α-NAOS Hydrolase), from a Marine Bacterium, *Vibrio* sp. Strain JT0107

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A novel enzyme, α-neoagarooligosaccharide hydrolase (EC 3.2.1.-), which hydrolyzes the α-1,3 linkage of neoagarooligosaccharides to yield agaropentaose (*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-D-galactose], agarotriose [*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose], agarobiose [*O*-β-D-galactopyranosyl(1→4)-3,6-anhydro-L-galactose], 3,6-anhydro-L-galactose, and D-galactose was isolated from the marine bacterium *Vibrio* sp. strain JT0107 and characterized. This enzyme was purified 383-fold from cultured cells by using a combination of ammonium sulfate precipitation, successive anion-exchange column chromatography, gel filtration, and hydroxyapatite chromatography, gel filtration, and hydroxyapatite chromatography. The purified protein gave a single band (M_r, 42,000) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Estimation of the M_r by the gel filtration method gave a value of 84,000, indicating that the enzyme is dimeric. Amino acid sequence analysis revealed it to have a single N-terminal sequence that has no sequence homology to any other known agarases. The optimum temperature and pH were 30°C and 7.7, respectively. The K_m and maximum rate of metabolism for neoagarobiose were 5.37 mM and 92 U/mg of protein, respectively.

Agarose, a thoroughly characterized polysaccharide that occurs in the cell walls of some red algae, has been shown to have a linear chain structure composed of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose (9).

Hydrolytic enzymes, which degrade agarose to various kinds of oligosaccharides, have been found in some microorganisms (12, 13, 25). These enzymes are classified into two groups according to their mode of action on agarose. One is β-agarase, which cleaves the β-1,4 linkage (6, 7). Several types of β-agarase have been purified and characterized in the past decade: two from *Vibrio* sp. strain JT0107 (M_rs, 107,000 and 105,000) (19–21), one from *Vibrio* sp. strain AP-2 (M_r, 20,000) (1), two from *Pseudomonas atlantica* (M_r, 32,000 and 57,486) (2, 16, 17), and one from *Streptomyces coelicolor* (M_r, 32,000) (4). The other is α-agarase, which cleaves the α-1,3 linkage. Only two α-agarases have been reported (18, 26), one of which has been purified and characterized (18).

Some α- and β-agarases originating from different microorganisms have been investigated with respect to their physiological roles, but those from the same organism have not. If microorganisms utilize agarose as a carbon source, agarose should be degraded to its constitutive monosaccharides, such as D-galactose or 3,6-anhydro-L-galactose. Although neoagarobiose hydrolase (5) and neoagarobiase (24) have been reported to be involved in the degradation of agar by *P. atlantica* and *Cytophaga flevensis*, respectively, it is unclear whether they are the only enzymes involved since the fact that they have been only partially purified has prevented their complete characterization.

Here we describe for the first time the purification and characterization of an enzyme that hydrolyzes oligosaccharides from agarose to monosaccharides. The enzyme, which is not secreted into the culture medium by the marine bacterium *Vibrio* sp. strain JT0107, can hydrolyze the α-1,3 linkages of neoagarooligosaccharides that are smaller than a hexamer, producing 3,6-anhydro-L-galactose and D-galactose. The mode of action and substrate specificity were quite different from those of any other known agarases. Therefore, this enzyme, which has been named α-neoagarooligosaccharide hydrolase (α-NAOS hydrolase), has been classified as a novel enzyme (EC 3.2.1.-). In addition, it is the first time that 3,6-anhydro-L-galactose has been found to take not an aldehydic structure but a pyranose structure.

Furthermore, this report suggests for the first time that strain JT0107 degrades agarose to 3,6-anhydro-L-galactose and D-galactose by using β-agarase (21) and α-NAOS hydrolase.

MATERIALS AND METHODS

Materials. *Vibrio* sp. strain JT0107 was obtained from Sagami Bay in Kanagawa Prefecture, Japan (21). Low-melting-point agarose, neoagarobiose [*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-D-galactose], neoagarotetraose [*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-D-galactose], and neoagarohexaose [*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-D-galactose] were purchased from Sigma Chemical Co. (St. Louis, Mo.) or prepared by our previously reported method by using agarase 0107 (21). Artificial seawater was obtained from Marine Tech (Tokyo, Japan). Bacto Agar, yeast extract, and peptone were from Difco Laboratories (Detroit, Mich.), and all other reagents, including

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molecular markers, were analytical grade and commercially available. Quaternary aminoethyl (QAE)-Toyopearl was obtained from Tosoh (Tokyo, Japan), and Mono-Q, Superdex 200, and Superose 12 were from Pharmacia (Tokyo, Japan). An A-8010G hydroxyapatite column was purchased from Mitsui Toatsu Co. (Tokyo, Japan). Iatrobeads (6RS-8060) for silica gel chromatography was purchased from Yatoron (Tokyo, Japan). Centricon 50 was purchased from Grace Japan (Tokyo, Japan). High-performance silica gel 60 on glass plates (for high-performance thin-layer chromatography [TLC]) was purchased from E. Merck AG (Darmstadt, Germany).

Protein assay. Protein concentrations were determined by the Bradford method (3) with the Coomassie Protein Assay Kit (Pierce Chemical Co., Rockford, Ill.).

Enzyme assay. Peak areas of high-performance liquid chromatography (HPLC) were used to quantify the amount by which each substrate was reduced after an enzyme reaction. The peak area for various concentrations of each substrate was measured, and calibration curves were made for the various concentrations beforehand. The amount of a substrate remaining after an enzyme reaction was then calculated. In this way, the concentration of each substrate was determined to lie on the appropriate calibration curve. The standard assay used to calculate enzyme activity (unit) was carried out by using neoagarobiose as the substrate.

The activity of the purified enzyme was assayed by the following method. The enzyme (14 mU) was dissolved in 10 μl of 20 mM potassium phosphate buffer (KP buffer) (pH 7.8), and to this was added 90 μl of the same buffer containing 0.1% (wt/vol) neoagarobiose as the substrate. After incubation (30°C, 10 min), the enzyme reaction was stopped by boiling for 1 min. The peak area of the substrate was measured with a Shiseido Capcell pak C-18 column (4.5 by 250 mm) by HPLC (eluent, water; flow rate, 1 ml/min; detector, refractive index), and the amount by which the substrate was degraded was calculated. One unit of enzyme activity was defined as the amount required to hydrolyze 1 μmol of neoagarobiose per min under the above-described conditions.

Substrate specificity. The enzyme solution (15 mU) was dissolved in 10 μl of 20 mM potassium phosphate buffer (KP buffer, pH 7.8) and to this was added 90 μl of the same buffer containing 0.2% (wt/vol) neoagarohexaose, neoagarotetraose, neoagarobiose, or melted low-melting-point agarose as the substrate. After incubation (30°C for 15, 30, 60, 120, 180, and 240 min), the enzyme reaction was stopped by boiling for 1 min. The reduction in the amount of each substrate was measured as already explained in the description of the enzyme assay.

M_r determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed with a 4 to 20% gradient gel or a 10% gel in accordance with the Laemmli method (11), and proteins were detected by staining the gel with silver stain (15). M_r calibration was done with the molecular size markers myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), and carbonic anhydrase (30 kDa). Furthermore, the M_r of the native protein was determined by means of gel filtration on a Superdex 200 column (1 by 30 cm) at a flow rate of 0.4 ml/min with the fast protein liquid chromatography system (Pharmacia). The column buffer was 20 mM KP buffer (pH 7.8) containing 100 mM NaCl. Immunoglobulin G (160 kDa), human serum albumin (67 kDa), β-lactoglobulin (35 kDa), and cytochrome *c* (12.4 kDa) were used as reference markers.

α-NAOS hydrolase purification steps. (i) Bacterial culture. The culture medium was composed of 0.4% agar, 0.4% yeast extract, and 2.0% peptone in artificial seawater and was adjusted to pH 8.0. Strain JT0107 was grown for 20 h in this medium (3 liters) in a 5-liter fermentor at 25°C (600 rpm). The culture was centrifuged (4,500 × *g* for 30 min), and 50 g (wet weight) of cells was obtained.

(ii) Ammonium sulfate treatment. The cells obtained were suspended in 20 mM KP buffer (pH 7.8) and adjusted to 100 ml. The cell suspension was added to 250 ml of glass beads (0.1-mm diameter) and disrupted with a Dyno-Mill KDL apparatus (Willy A. Bachofen AG, Basel, Switzerland) in accordance with the instructions in the manual. All of the following procedures were carried out at temperatures of 0 to 4°C unless 10°C is specified. The suspended cells were centrifuged (20,000 × *g*), and 70 ml of a clear supernatant was obtained. After the supernatant was adjusted to 40% saturation with solid ammonium sulfate, the precipitate was removed and the supernatant was obtained by centrifugation (5,500 × *g*, 30 min). Furthermore, the supernatant was adjusted to 70% saturation with solid ammonium sulfate and left to stand overnight. The precipitate collected by centrifugation (5,500 × *g*, 30 min) was dissolved in 30 ml of 20 mM KP buffer (pH 7.8) and dialyzed against 5 liters of the same buffer.

(iii) QAE-Toyopearl chromatography. For QAE-Toyopearl chromatography, a column (2.2 by 9 cm) of QAE-Toyopearl was equilibrated with 20 mM KP buffer (pH 7.8). The dialyzed solution was applied to the column and washed with 30 ml of equilibrium buffer. The column was eluted with a continuous linear gradient of 0 to 0.5 M NaCl in the same buffer (total volume, 300 ml). The volume of one fraction was 10 ml. The five most active fractions (50 ml) detected by the enzyme assay were collected and pooled for subsequent Mono-Q chromatography.

(iv) Mono-Q chromatography. A column (1 by 9 cm) of Mono-Q that had been equilibrated with 20 mM KP buffer (pH 7.8) was used. The active fractions (50 ml) obtained by QAE-Toyopearl chromatography were diluted to 200 ml with the equilibrium buffer to reduce the NaCl concentration. They were then applied to the column and washed with 24 ml of the same buffer. The elution was carried out with a continuous linear gradient of 0 to 0.5 M NaCl in 20 mM KP buffer (pH 7.8; total volume, 120 ml). The volume of one fraction was 8 ml. After the two active fractions (16 ml) were further purified by rechromatography on the same column and eluted, one active fraction (8 ml) was obtained.

(v) Gel filtration on Superose 12. A column (1 by 30 cm) of Superose 12 that had been equilibrated with 20 mM KP buffer (pH 7.8) containing 0.1 M NaCl was used. The active fraction (8 ml) obtained by Mono-Q chromatography was concentrated to 0.5 ml with a Centricon 50 ultrafiltration kit. This was then applied to the column, and fractions were collected at a flow rate of 0.4 ml/min. The volume of one fraction was 0.4 ml. Two active fractions (0.8 ml) were pooled.

(vi) Hydroxyapatite chromatography on A-8010G. A column (0.8 by 10 cm) of A-8010G that had been equilibrated with 10 mM KP buffer (pH 6.8) was used. The active fraction (0.8 ml) obtained by Superose 12 chromatography was diluted to 4 ml with the same buffer and applied to the column. Elution was carried out with a continuous linear gradient of 10 to 175 mM KP buffer (pH 6.8; total volume, 15 ml). The volume of one fraction was 0.5 ml. Two active fractions (1 ml) were pooled.

(vii) Gel filtration on Superdex 200. Two columns (1 by 60 cm) of Superdex 200 that had been linearly connected and equilibrated with 20 mM KP buffer (pH 7.8) containing 0.1 M NaCl were used. The active sample (1 ml) obtained by hydroxyapatite chromatography was applied, and then fractions were collected at a flow rate of 0.25 ml/min. The volume

of one fraction was 0.5 ml. Three active fractions (1.5 ml) were pooled.

Thermostability analysis. Ten microliters of α-NAOS hydrolase (40 μg/ml) was placed in a water bath at a temperature of 30, 35, 40, or 50°C for 0, 1, 2, 3, 4, 5, 6, 8, 10, 20, 30, 45, 60, 90, or 120 min. The enzyme activity after each treatment was measured at 30°C for 10 min.

pH profile analysis. The pH profile of the activity of α-NAOS hydrolase was obtained with 67 mM potassium phosphate buffer (pH 5.5 to 7.7), 50 mM bicine-NaOH buffer (pH 7.7 to 8.9), and 50 mM glycine-NaOH buffer (pH 9.0 to 9.4). To each buffer, neoagarobiose was added to a concentration of 0.1% (wt/vol). One microliter of α-NAOS hydrolase (40 μg/ml) was added to 9 μl of each substrate solution, and the enzyme activity was measured at 30°C for 10 min.

Kinetic measurements. The K_m and maximum rate of metabolism of neoagarobiose were determined at substrate concentrations ranging from 0.56 to 14 mM.

Amino acid sequence analysis. Twenty micrograms of the purified enzyme was run on SDS–10% PAGE and electrotransferred to a commercial membrane (Problot; Applied Biosystems) (14). The membrane was stained with Coomassie brilliant blue R-250, and then the enzyme band was cut from the membrane and applied to 477A and 120A protein sequencer systems (Applied Biosystems).

Isoelectric point determination. Isoelectrophoresis was performed by isoelectric focusing with pH gradients of 4 to 6.5 by using a Phastsystem (Pharmacia), and the isoelectric point (pI) of α-NAOS hydrolase was estimated with a pI marker kit (Pharmacia).

Identification of enzyme reaction products. (i) TLC. TLC for enzyme reaction products was performed on a high-performance silica gel 60 glass plate and developed with *n*-butanol–ethanol–water (3:1:1 [by volume]). The developed samples were identified with 20% (vol/vol) methanol in concentrated H_2SO_4 or Mo-Ce reagent [15 g of ammonium molybdate tetrahydrate plus 10 g of cerium (IV) sulfate *n*-hydrate in 1 liter of 10% H_2SO_4]. The R_f values of spots were compared with those of authenticated samples (8).

(ii) Silica gel chromatography. To separate D-galactose and 3,6-anhydro-L-galactose, silica gel chromatography was carried out. A column (1.5 by 84 cm) of silica gel (Iatrobeads; Yatoron) was used. One-hundred-milligram samples of reaction products were applied to the column, and elution was carried out with a continuous linear gradient from solution A (chloroform-methanol-water, 70:28:2 [by volume]) to solution B (chloroform-methanol-water, 53:45:2 [by volume]). The total eluent volume was 960 ml.

(iii) HPLC. HPLC was performed to isolate some of the reaction products. The reaction solution was applied to a Capcell pak C-18 column and eluted with water (flow rate, 1 ml/min). The eluate was then fractionated according to the elution peak.

(iv) Spectrometric analyses. A ^1H nuclear magnetic resonance (NMR) spectrum was measured at 300 or 500 MHz (at 288K) with a Bruker AC-300P or AM-500.

Fast atom bombardment mass spectra (FAB-MS) were measured under sodium-additive conditions with a Kratos Concept IIH apparatus.

Optical rotation of 3,6-anhydro-L-galactose. 3,6-Anhydro-L-galactose was dissolved in 2 ml of distilled water and left to stand overnight. Its optical rotation, $[\alpha]_D^{27}$, was then measured with a JASCO DIP-370 digital polarimeter.

Acetylation of 3,6-anhydro-L-galactose. Fifty milligrams of 3,6-anhydro-L-galactose was treated with 1 ml of acetic anhydride in 1 ml of dry pyridine at room temperature for 15 h. The reaction mixture was applied to a silica gel column (2 by 24 cm) for chromatography. After elution with a continuous linear gradient from solution A (hexane-ethyl acetate, 80:20 [by volume]) to solution B (hexane-ethyl acetate, 10:90 [by volume]), 3,6-anhydro-1,2,4-tri-*O*-acetyl-L-galactose was eluted with hexane-ethyl acetate (30:70) and further purified by HPLC with a YMC-pak column (Yamamurakagaku, Kyoto, Japan) under isocratic conditions (solvent, hexane-ethyl acetate [60:40, by volume]). The highly purified 3,6-anhydro-1,2,4-tri-O-acetyl-L-galactose (9 mg) was then analyzed with a 300 MHz ^1H NMR spectrometer.

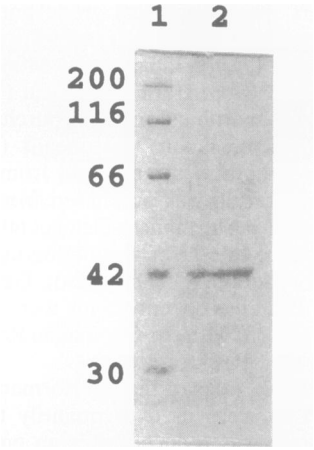

FIG. 1. SDS-PAGE of α-NAOS hydrolase. The purified enzyme was subjected to electrophoresis on a 10% polyacrylamide gel at pH 8.0 in Tris-glycine buffer. Lanes: 1, molecular mass standards (ca. 0.5 μg of each); 2, α-NAOS hydrolase (1.2 μg). The numbers to the left are molecular masses in kilodaltons.

RESULTS

Purification of α-NAOS hydrolase. α-NAOS hydrolase activity was found mainly in the disrupted cell fraction, and hardly any was found in the supernatant of the culture medium when strain JT0107 was cultured by our previously reported method (21). Therefore, JT0107 was grown as fast as possible by modifying the composition of the medium. The enzyme activity of the disrupted cell solution was 117 U/50 g (wet weight) of cells.

The disrupted-cell solution was treated with ammonium sulfate, and the precipitate obtained was dialyzed and purified twice by anion-exchange column chromatography. The active fractions eluted at 0.3 to 0.35 M NaCl were then subjected to gel filtration on Superose 12. Activity was found in the fractions where proteins with molecular weights of 75,000 to 110,000 were eluted. The most active two fractions were further purified by hydroxyapatite column chromatography with A-8010G and then by gel filtration with Superdex 200. The highly purified protein gave one band on SDS-PAGE with an estimated molecular mass of 42 kDa (Fig. 1). The isoelectric point was estimated to be 4.6. The overall yield from the disrupted cells was 3.6%, for a purification of 383-fold (Table 1).

M_r and N-terminal sequence of the enzyme. The M_r of the purified enzyme was approximately 84,000 as determined by the gel filtration method but 42,000 as determined by SDS-PAGE. Therefore, it was concluded that this enzyme is a dimer. The first 23 amino acid residues of the N-terminal

TABLE 1. Purification of α-NAOS hydrolase from *Vibrio* sp. strain JT0107

Purification step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Purification (fold)
Disrupted cells	1,285	117	0.09	100	1
Ammonium sulfate preparation	432	109	0.25	93.2	2.78
Dialyzed sample	364	104	0.29	88.9	3.22
QAE-Toyopearl fraction	93.7	49.7	0.53	42.5	5.89
First Mono-Q fraction	22.3	19.3	0.87	16.5	9.67
Second Mono-Q fraction	11	15.1	1.37	12.9	15.2
Superose 12 fraction	1.55	6.5	4.19	5.56	46.6
A-8010G fraction	0.387	4.91	12.7	4.20	141
Superdex 200 fraction	0.121	4.17	34.5	3.56	383

sequence were determined to be SGTGSKLSLASKIAIEX GYDKKG, where X is an unknown residue.

Properties of α-NAOS hydrolase. (i) Effect of pH. The pH profile of α-NAOS activity is shown in Fig. 2. The optimum pH was determined to be pH 7.7 in 50 mM bicine-NaOH buffer. Furthermore, the activity was drastically reduced when the pH fell below 6.2 or rose above 8.9.

(ii) Effect of temperature. α-NAOS hydrolase retained 60 and 30% of its initial activity when stored at 4°C for 3 weeks and 2 months, respectively, in 20 mM KP-buffer (pH 7.8), but it retained 60% of its initial activity when stored at −20°C for 2 months in 20 mM KP buffer (pH 7.8) containing 30% (vol/vol) glycerol. α-NAOS hydrolase did not lose its activity when heated at 30°C for 120 min but lost it gradually at 35°C and rapidly at temperatures over 40°C (Fig. 3).

(iii) Identification of reaction products and substrate specificity. When neoagarobiose was hydrolyzed, two reaction products were observed on TLC (R_f values, 0.46 and 0.72) and separated by silica gel chromatography. One reaction product was identified as D-galactose on the basis of its ^{1}H NMR spectrum and its R_f value of 0.46. The other product showed a molecular ion at an *m/z* of 185 $(M+Na)^+$ on FAB-MS, and its NMR spectrum was suggestive of that of a derivative of galactose, as shown in Fig. 4A. The NMR spectrum of the acetylated derivative of the compound is shown in Fig. 4B. Since acetylation chemically shifted H-1, H-2, and H-4 to lower magnetic field strengths while other protons were little affected, this acetylated product was identified as 3,6-anhydro-1,2,4-tri-*O*-acetylgalactose. On the basis of these results, the enzyme reaction products from neoagarobiose were determined to be D-galactose and 3,6-anhydro-L-galactose, which has an $[\alpha]_D^{27}$ of −12.7° (c = 0.43, H_2O). Therefore, this enzyme must hydrolyze the α-1,3 linkage of neoagarobiose.

When neoagarotetraose was used as the substrate, three reaction products were observed on TLC. Two were identified as D-galactose and 3,6-anhydro-L-galactose on the basis of their identical *Rf* values of 0.72 and their FAB-MS spectra. As FAB-MS revealed the third (R_f, 0.27) to have a molecular ion at an *m/z* of 509 $(M+Na)^+$, it was identified as agarotriose [*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-D-galactose]. When neoagarohexaose was used as the substrate, it yielded one major product with three minor compounds. FAB-MS showed the predominant product to be agaropentaose [*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-D-galactose] [*m/z*, 815 $(M+Na)^+$] and the minor products to be agarotriose [*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose] [*m/z*, 509 $(M+Na)^+$], agarobiose [*O*-β-D-galactopyranosyl(1→4)-3,6-anhydro-L-galactopyranose] [*m/z*, 347 $(M+Na)^+$], and 3,6-anhydro-L-galactose (*m/z*, 185 (M+

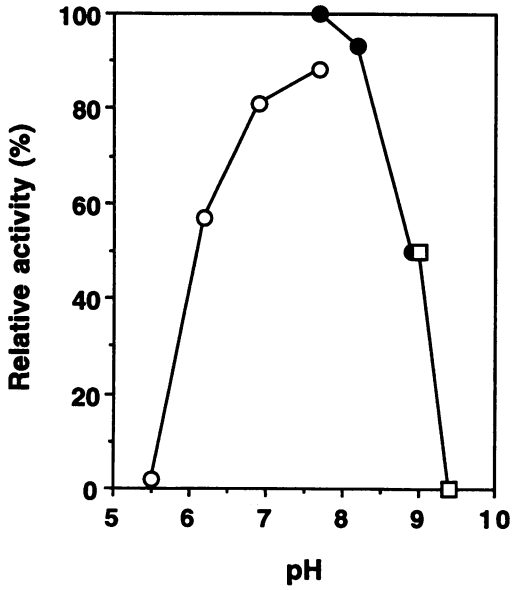

FIG. 2. pH profile of the activity of α-NAOS hydrolase. Symbols: ○, 67 mM potassium phosphate buffer (pH 5.5 to 7.7); ●, 50 mM bicine-NaOH buffer (pH 7.7 to 8.9); □, 50 mM glycine-NaOH buffer (pH 9.0 to 9.4).

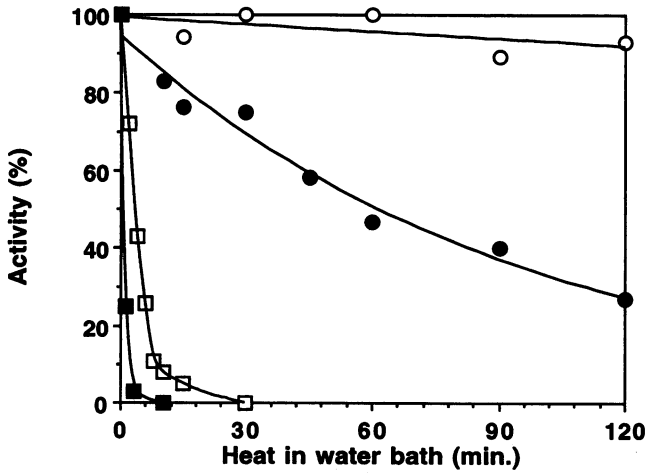

FIG. 3. Thermostability of α-NAOS hydrolase. Enzyme activities remaining after heating at 30°C (○), 35°C (●), 40°C (□), and 50°C (■) are shown.

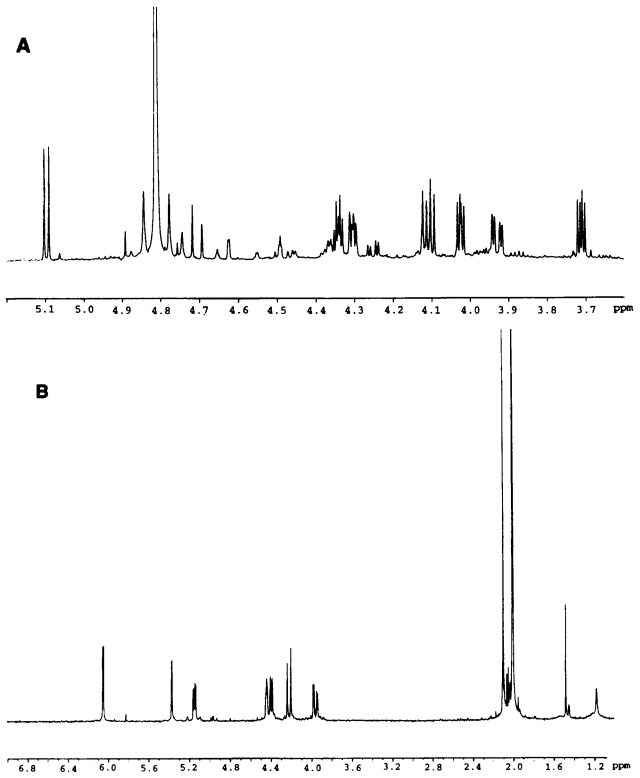

FIG. 4. (A) NMR spectrum suggestive of that of a derivative of galactose with δ (D_2O) 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (sodium salt) as the internal standard: 3.71 (1H dd, $J_{1,2}$ = 6.2 Hz, $J_{2,3}$ = 3.4 Hz, H-2), 3.93 (1H dd, $J_{5,6}$ = 3.1 Hz, $J_{6,6'}$ = 10.0 Hz, H-6), 4.02 (1H dd, $J_{2,3}$ = 3.4 Hz, $J_{3,4}$ = 4.9 Hz, H-3), 4.11 (1H dd, $J_{5,6'}$ = 4.9 Hz, $J_{6,6'}$ = 10.00 Hz, H-6'), 4.30 (1H dd, $J_{3,4}$ = 4.9 Hz, $J_{4,5}$ = 3.1 Hz, H-4), 4.34 (1H dt, $J_{4,5}$ = 3.1 Hz, $J_{5,6}$ = 3.1 Hz, $J_{5,6'}$ = 4.9 Hz H-5), 5.10 (1H d, $J_{1,2}$ = 6.2 Hz, H-1). (B) NMR spectrum of the acetylated derivative of compound A with δ ($CDCl_3$) tetramethysilane as the internal standard: 2.02, 2.03, 2.11 (9H, each s, Ac), 3.96 (1H dd, $J_{5,6}$ = 2.9 Hz, $J_{6,6'}$ = 10.6 Hz, H-6), 4.22 (1H d, $J_{6,6'}$ = 10.6 Hz, H-6'), 4.39 (1H d, $J_{2,3}$ = 5.5 Hz, H-3), 4.44 (1H br s, H-5), 5.15 (1H dd, $J_{1,2}$ = 2.9 Hz, $J_{2,3}$ = 5.5 Hz, H-2), 5.37 (1H d, $J_{4,5}$ = 1.8 Hz H-4), 6.05 (1H d, $J_{1,2}$ = 2.9 Hz H-1).

Na)$^+$]. No products were detected by TLC or HPLC when agarose was used as the substrate.

The reduction in the amount of each substrate (neoagarobiose, neoagarotetraose, or neoagarohexaose) as a function of reaction time is shown in Fig. 5. According to these data, the conversion rates of the substrates decreased in the order neoagarobiose-neoagarohexaose-neoagarotetraose.

***K_m* and maximum rate of metabolism.** Both the K_m and the maximum rate of metabolism of neoagarobiose were calculated from an S/V plot. The values were 5.37 mM and 92 U/mg of protein, respectively.

DISCUSSION

α-NAOS hydrolase had a molecular mass of 84 kDa as determined by gel filtration, although it gave a single band with a molecular mass of 42 kDa on SDS-PAGE. As the enzyme had one single N-terminal sequence and a single isoelectric point, it was probably a homodimer, although the possibility of a heterodimer could not be ruled out if the pI values and N-terminal sequences of two subunits were completely identical. The N-terminal sequence of α-NAOS hydrolase was not

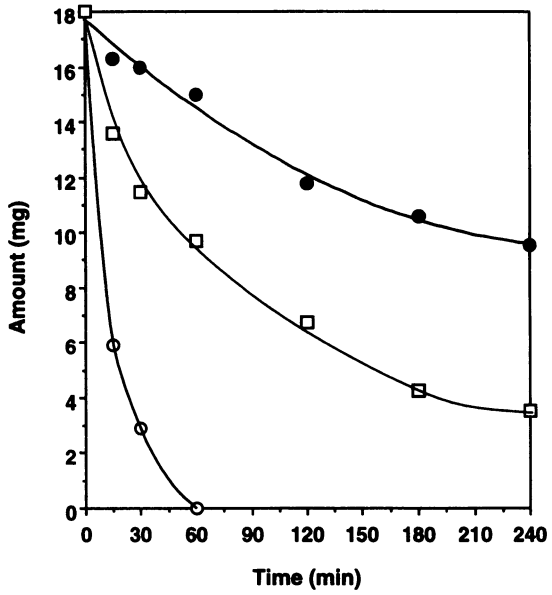

FIG. 5. Time courses of hydrolysis of the substrates neoagarobiose (○), neoagarotetraose (●), and neoagarohexaose (□).

homologous with those of any other known agarases, including the β-agarases from strain JT0107 reported previously (19, 20).

The enzyme seems to exert its activity under rather limited conditions compared with agarase 0107; i.e., it has an optimum pH region in neutral or slightly alkaline conditions and is not very thermoresistant. This may reflect the fact that it is an intracellular enzyme that does not need to adapt to the easily changeable outer environment.

The process by which oligosaccharides are hydrolyzed by the enzyme is summarized in Fig. 6. When neoagarohexaose was used as a substrate, this enzyme cleaved its α-1,3 linkage at the nonreducing end, producing 3,6-anhydro-L-galactose and agaropentaose, which was further hydrolyzed to agarobiose and agarotriose. With neoagarotetraose, it gave predominantly agarotriose and 3,6-anhydro-L-galactose. If this enzyme had recognized the α-1,3 linkage at the reducing end of neoagrohexaose, the reaction products would have been D-galactose and agaroneopentaose [*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-*O*-β-D-galactopyranosyl(1→4)-3,6-anhydro-L-galactopyranose] (molecular weight, 774). However, the latter compound was not detected by FAB-MS, TLC, or HPLC. In the case of neoagarotetraose, agarotriose [*O*-β-D-galactopyranosyl(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-D-galactose] was detected but agaroneotriose [*O*-3,6-anhydro-α-L-galactopyranosyl(1→3)-*O*-β-D-galactopyranosyl(1→4)-3,6-anhydro-L-galactopyranose] was not. From these results, it is concluded that α-NAOS hydrolase is not an endo-type enzyme but recognizes the α-1,3 linkage between D-galactose and 3,6-anhydro-L-galactose at the nonreducing end. Therefore, this enzyme has been classified as a novel kind of α-L-galactosidase. This is the first report of an α-L-galactosidase, although some reports of α-D-galactosidases have been published (22, 23).

It is worth noting that in the NMR spectrum of 3,6-anhydro-L-galactose obtained from neoagarobiose, a signal assigned to the anomeric proton was observed at δ 5.10 with a coupling constant of 6.2 Hz, indicating that 3,6-anhydro-L-galactose took a pyranose structure instead of an aldehydic structure

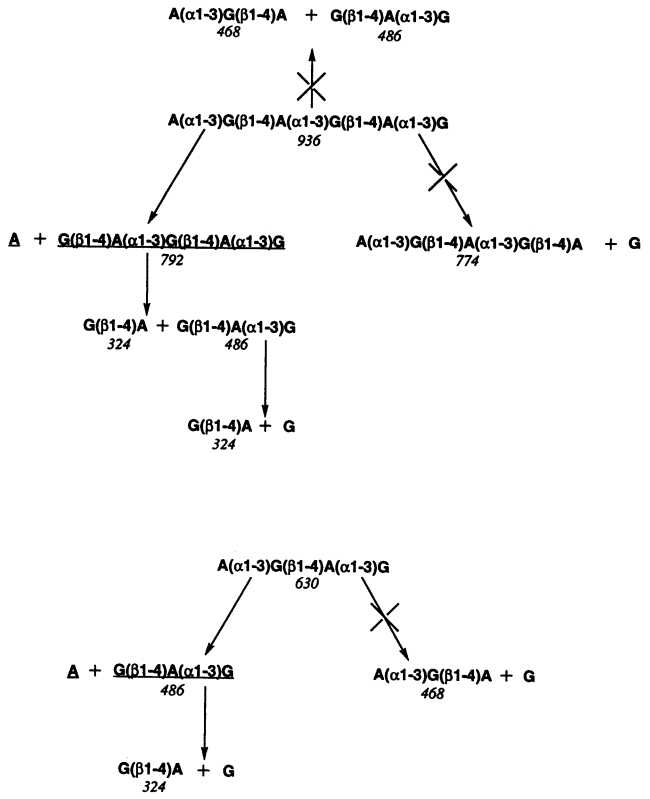

FIG. 6. Scheme of the mode of action on neoagarohexaose and neoagarotetraose. The left end of each oligosaccharide is defined as the nonreducing end, and the right end is defined as the reducing end. A, 3,6-anhydro-L-galactose; G, D-galactose; (β1-4), β-1,4 linkage; (α1-3), α-1,3 linkage. The arrows show the flow of the enzymatic reaction. The symbol × indicates that the reaction does not proceed. The underlined reaction products are the main products. An italic number is the molecular weight of each oligosaccharide.

(10) and existed as the α-anomer under our experimental conditions.

α-NAOS hydrolase degraded neoagarobiose with the highest conversion rate among the substrates tested. This characteristic is very important in explaining how *Vibrio* sp. strain JT0107 utilizes agarose as a carbon source. This strain degrades agarose to neoagarobiose with some secreted β-agarases (20, 21), and the neoagarobiose permeates into the cell, where it decomposes into monosaccharides (i.e., D-galactose and 3,6-anhydro-L-galactose). It must utilize 3,6-anhydro-L-galactose, as well as D-galactose, as a carbon source, because the former compound was detected in neither the supernatant nor the disrupted cell suspension after the culture of strain JT0107. Therefore, to survive in the marine environment, strain JT0107 has acquired the ability to metabolize L-anhydrogalactose, which has been found only in marine red algae.

In conclusion, we purified and characterized α-NAOS hydrolase, which is a novel enzyme cleaving the α-1,3 linkage, and proposed a possible scheme for the hydrolysis of agarose to monosaccharides by strain JT0107 for the first time. This scheme seems to pave the way for elucidation of the hydrolyzing systems of other heteropolysaccharides, such as carrageenan, porphyran, and so on.

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