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ORIGINAL PAPER

Purification and characterization of agarases from a marine bacterium *Vibrio* sp. F-6

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Abstract Marine bacterium *Vibrio* sp. F-6, utilizing agarose as a carbon source to produce agarases, was isolated from seawater samples taken from Qingdao, China. Two agarases (AG-a and AG-b) were purified to a homogeneity from the cultural supernatant of *Vibrio* sp. F-6 through ammonium sulfate precipitation, Q-Sepharose FF chromatography, and Sephacryl S-100 gel filtration. Molecular weights of agarases were estimated to be 54.0 kDa (AG-a) and 34.5 kDa (AG-b) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH values for AG-a and AG-b were about 7.0 and 9.0, respectively. AG-a was stable in the pH range of 4.0–9.0 and AG-b was stable in the pH range of 4.0–10.0. The optimum temperatures of AG-a and AG-b were 40 and 55 °C, respectively. AG-a was stable at temperature below 50 °C. AG-b was stable at temperature below 60 °C. Zn^{2+}, Mg^{2+} or Ca^{2+} increased AG-a activity, while Mn^{2+}, Cu^{2+} or Ca^{2+} increased AG-b activity. However, Ag^{+}, Hg^{2+}, Fe^{3+}, EDTA and SDS inhibited AG-a and AG-b activities. The main hydrolysates of agarose by AG-a were neoagarotetraose and neoagarohexaose. The main hydrolysates of agarose by AG-b were neoagarooctaose and neoagarohexaose. When the mixture of AG-a and AG-b were used, agarose was mainly degraded into neoagarobiose.

Keywords Purification · Characterization · Agarase · Neoagarooligosaccharide · *Vibrio* sp.

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Introduction

Agarose is an abundant biopolymer present as the main component of cell walls in agarophyte red algae. Agarose is a complex linear polysaccharide consisting of 3-O-linked β-D-galactose units alternating with 4-O-linked 3,6-anhydro-α-L-galactose units and also containing substituent groups such as sulfate, pyruvate, and methoxyl [1]. Owing to its gelling ability, stable property and high viscosity, agarose and its derivatives are widely used in food, cosmetics, and pharmaceutical industries. Agarases are glycoside hydrolases (GH) consisting of α-agarases (EC 3.2.1.158) and β-agarases (EC 3.2.1.81). α-Agarases cleave α-1, 3-linkage of agarose to produce agarooligosaccharides and β-agarases cleave inter-β-1,4-linkage to produce neoagaro-oligosaccharides [2, 3]. β-Agarases are classified into three families of GH-16, GH-50 and GH-86 based on the amino acid sequence similarity (http://www.cazy.org/). There have been reports on agarases from several bacteria genera, including *Pseudomonas* [4–7], *Streptomyces* [8], *Vibrio* [9–11], *Pseudoalteromonas* [12, 13], *Alteromonas* [14, 15], *Microbulbifer* [16], *Agarivorans* [17, 18], *Acinetobacte*r [19], *Thalassonona*s [20], *Zobellia* [21], and *Saccharophagus* [22]. The crude preparation of extracellular agarases isolated from sea mud was useful for isolating protoplasts from red algae, *Bangia atropurpurea* (Rhodophyta) [23] and *Gracilaria verrucosa* (Rhodophyta) [24]. The hydrolysates of agarose by agarases had various special chemical

properties and biological activities such as inhibiting the growth of bacteria, slowing down the degradation of starch, and showing anticancer, antivirus, and antioxidation activities and therefore they are suitable source for physiologically functional foods with protective and immunopotentiating activity [25–29]. In the present study, a marine agarolytic bacterium *Vibrio* sp. F-6 was isolated from marine environment. The agarases (AG-a and AG-b) produced by the strain were purified and characterized. The hydrolysates of agarose by AG-a, AG-b and the mixture of AG-a and AG-b were analyzed.

Materials and methods

Isolation of strains and optimization of culture conditions

Strain F-6 used in this study was one of marine strains isolated from the coastal water in Qingdao, China. The selective medium contained agarose as the single carbon source. The culture medium was composed of 0.7% agar, 2.5% NaCl, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.02% $CaCl_2$, and 0.002% $FeSO_4 \cdot 7H_2O$ (pH 7.5). The plates were incubated at 26 °C for 36 h. Colonies that formed pits or clearing zones were picked up and purified further by the same plating method. In one-factor-at-a-time methodology and orthogonal experiment, the optimal fermentation medium and conditions of the strain F-6 were studied.

Identification of the strain F-6

Strain F-6 was identified according to the criteria described by Bergey's Manual of Systematic Bacteriology [30]. Polymerase chain reaction (PCR) was performed to amplify 16S rDNA coding region of the strain F-6, using the universal primers 27F and 1492R. PCR was carried out in the following program: predenaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 60 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products were subcloned into pMD 18-T vector and sequenced (TaKaRa, Dalian, China). The 16S rDNA gene sequence was compared with sequences in nucleotide database by using the BLAST algorithm at NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree of the strain F-6 was constructed using the biological software of MEGA4.

Assay of agarase activity

Unless indicated, colloidal agarose containing 0.5% (w/v) agarose (Bio-Rad, Hercules, CA, USA) in 50 mM sodium phosphate buffer (pH 7.5) was used as substrate for agarase activity assay. Agarase activity was determined by using the modified dinitrosalicyclic acid (DNS) method [31] to measure the increase in the concentration of reducing sugar. The reaction mixture containing 9 ml of colloidal agar and 1 ml of diluted enzyme solution was carried out at 40 °C for 15 min. Then 1 ml of reaction solution was mixed with 1.5 ml of DNS reagent and heated at 100 °C for 5 min. The mixture was diluted to 25 ml with deionized water. Optical density was read at 520 nm. One unit of agarase activity was defined as the amount of enzyme that released 1 μmol reducing sugar (measured as D-galactose) from agarose per minute under above conditions.

Purification of agarases

Unless stated, all purification steps were carried out at 4 °C.

Ammonium sulfate precipitation. The cultured medium of strain F-6 was fermented at 26 °C for 24 h with agitation at 150 rpm. The cells were removed from medium by centrifugation at 8,000×*g* for 20 min. Solid ammonium sulfate was added to the supernatant to 20% saturation. After standing overnight, the precipitate of mixed proteins was removed by centrifugation at 10,000×*g* for 30 min. Solid ammonium sulfate was added to 80% saturation. The precipitate of crude agarases was collected by centrifugation at 10,000×*g* for 30 min. The crude agarase pellets were dissolved in 40 ml of 20 mM Tris–HCl buffer, pH 7.8 (buffer A) and the agarase solution was dialyzed against buffer A overnight.

Chromatography on Q-Sepharose FF. The dialyzed solution was freeze-dried, and then the crude agarase powder was dissolved in 15 ml of buffer A and loaded on Q-Sepharose FF column (Ø 2.0 × 30 cm; Amersham, Uppsala, Sweden) that had been equilibrated with buffer A. The column was washed with 300 ml of buffer A. Then the column was eluted with 300 ml of linear gradient of 0–2.0 M NaCl in buffer A at the flow rate of 30 ml/h. The fractions of 5 ml of each tube were collected. The two active fractions were separately collected and concentrated.

Gel filtration on Sephacryl S-100. Fractions from each peak of Q-Sepharose FF column were separately dialyzed, freeze-dried, and loaded on Sephacryl S-100 column (Ø 2.0 × 80 cm; Amersham, Uppsala, Sweden) that was equilibrated with buffer A. The column was eluted with buffer A at the flow rate of 18 ml/h, and the active fractions were collected and used as the purified agarases throughout this study.

Protein assay and electrophoresis

Protein concentration was determined by folin phenol reagent, using bovine serum albumin as the calibration standard. Protein marker was purchased from Biolabs Inc. (Hitchin, Herts, UK). During purification steps, the relative

protein content was estimated by absorbance at 280 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the purity and molecular mass of agarases under denaturing conditions described by Laemmli [32]. SDS-PAGE was performed in a 0.75-mm slab gel consisting of a stacking gel (5% polyacrylamide) and a separating gel (10% polyacrylamide) with 25 mM Tris–HCl buffer, pH 7.8. Proteins were stained with coomassie brilliant blue R-250 (Sigma, St. Louis, MO, USA).

Effects of pH and temperature on agarase stability and activity

The effects of pH values on agarase activity were determined under the standard assay conditions except that pH values of the reaction mixture were changed from pH 3.0 to 10.0 using 50 mM Britton–Robinson's universal buffer. The effects of pH values on agarase stability were determined under the standard assay conditions after preincubating agarase solutions in various pH values using 50 mM Britton–Robinson's universal buffer from pH 3.0 to 10.0 at 4 °C for 6 h. The effects of temperatures on agarase activity were examined under the standard assay conditions except that temperature was varied from 25 to 70 °C. The effects of temperatures on the agarase stability were determined under the standard assay conditions after preincubating agarase solutions in various temperatures from 25 to 70 °C for 30 min. The relative activity was defined as the percentage of activity determined with respect to the maximum agarase activity.

Effects of reagents on agarase activity

The effects of reagents on agarases were examined under the standard assay conditions except that each reagent was added to agarase solutions at a final concentration of 1 or 5 mM, preincubating at 4 °C for 30 min. The relative activity was defined as the percentage of activity determined with respect to that measured under the standard conditions.

N-terminal amino acid sequences of agarases

Proteins from SDS-PAGE were electrotransferred to polyvinylidene–difluoride (PVDF) membrane (Immobilion-P; Millipore, Bedford, CA, USA) using electroblotting apparatus (Bio-Rad, Richmond, CA, USA) with *N*-cyclohexyl-3-aminopropane sulfonic acid (CAPS) transfer buffer (1 mM CAPS and 20% methanol, pH 10.5). *N*-terminal amino acid sequences of agarases were determined by automated edoman degradation using a protein sequencer (model 473A; Applied Biosystems). The results were analyzed using data from the National Center for Biotechnology Information Protein Data Bank.

Analysis of hydrolysates of agarose

Five milliliters of agarase solutions (total agarase activity was 50 U) of AG-a, AG-b and the equivalent amount of AG-a and AG-b were used to hydrolyze 100 ml of 0.5% colloidal agarose, respectively. The reaction was carried out at 40 °C for 10 h, and then stopped by heating the reaction solution in boiling water for 10 min. Fourfold volume of ethanol was added to the reaction solution to remove residues and high-molecular-mass polysaccharides. Then the reaction solution was centrifugated at $8{,}000 \times g$ for 10 min at 20 °C and concentrated by rotatory evaporator. The end-products were precipitated by sixfold volume of ethanol. The hydrolysis pattern of agarose was analyzed by thin-layer chromatography (TLC) on Silica G 60 plates (Merck, Darmstadt, Germany) using *n*-propanol/30% ammonia (3:1, v/v) as the developing solvent. Products were detected by spraying aniline–diphenylamine reagent (4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone, and 30 ml of 85% phosphoric acid) and baking at 120 °C for 10 min. High-performance liquid chromatography (HPLC) (CLASS-VP HPLC System; Shimadzu, Tokyo, Japan) was used to determine the quantification of reaction products as described by Ohta et al. [16]. Standard neoagarooligosaccharides were purchased from Sigma Inc. (St. Louis, MO, USA). Carbon 13 nuclear magnetic resonance (^{13}C NMR) (Varian Unity Inova 500 system; Palo Alto, CA, USA) was used to determine the structure information of the reaction products as described by Ohta et al. [33].

Results

Isolation of agarase-producing bacteria

As a result of screening, 72 agarase-producing bacteria were isolated from seawater samples, Qingdao, China. Agarase activity was compared among culture supernatants of these isolates. Strain F-6 had the highest agarase activity and was selected as a test strain in this study. It was interesting that after being incubated at 26 °C for 48 h, the plates were stained by Lugol's iodine solution (5% I_2 and 10% KI in distilled water), and the staining effect of the strain F-6 was different from the effects of other strains (Fig. 1). That properties of agarases produced by the strain F-6 may be different from those of other strains that may cause the phenomenon. This phenomenon needs to be studied further. Agarases produced from the strain F-6 was inducible because the strain F-6 needed agar or agarose to produce agarases. Strain F-6 also required 1.5–3.5% (w/v) NaCl in culture medium for producing agarases. The optimal fermentation conditions were carried out in a 500 ml of baffle flask with 200 ml of culture medium at 26 °C for 24 h

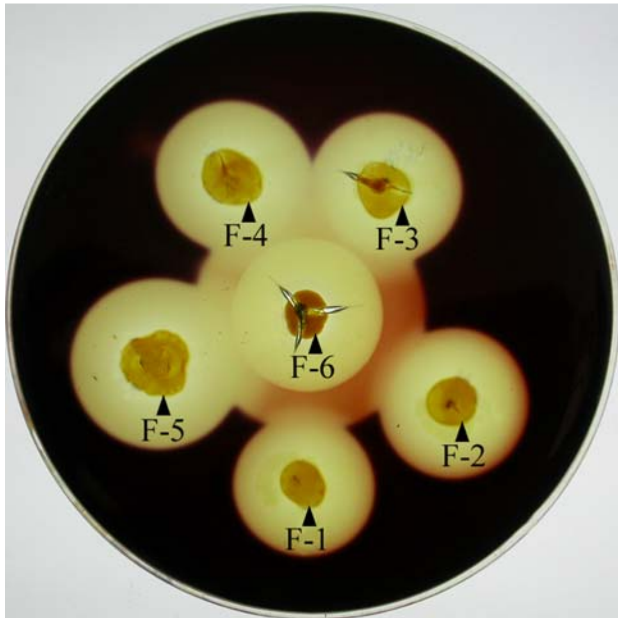

Fig. 1 Results of lugol dyeing of different agarolytic strains

with agitation at 150 rpm. At the optimal fermentation conditions, the maximum agarase activity of fermentation medium of the strain F-6 reached 8.52 U/ml.

Identification of the strain F-6

Strain F-6 was identified according to the criteria described by Bergey's Manual of Systematic Bacteriology. Strain F-6 was gram-negative, rod-shaped and motile bacterium, and had polar flagella and no-sporangium. Results of phenotypic test showed that strain F-6 was positive on catalase and oxidase tests, produced acid but no gas from glucose, and showed fermentation on O–F test. According to characteristics, strain F-6 was found to belong to *Vibrio*. To further study taxonomic position and phylogenetic position of strain F-6, the 1,470-bp fragment of 16S rDNA was amplified by PCR. The 16S rDNA sequence of strain F-6 was used for comparative sequence analysis against known 16S rDNA sequences in the NCBI database. The 16S rDNA gene sequence of strain F-6 showed similarity (95–97%) to that of *Vibrio shilonii* (AY911392), *Vibrio* sp. O2 (DQ235159), and *Vibrio* sp. V134 (EF100710). The phylogenetic tree further suggested that the strain F-6 belonged to *Vibrio* (Fig. 2).

Purification of agarases

Crude enzyme solution prepared by salting out with ammonium sulfate was applied to anion exchange chromatography on Q-Sepharose FF. Two agarase activity peaks of A (major fraction) and B (minor fraction) were separated. The agarases eluted from peak A and peak B were designated as AG-a and AG-b, respectively. Both agarases were purified by successive Sephacryl S-100 gel filtration. The homogeneity of agarases was judged by SDS-PAGE (Fig. 3). The molecular masses of AG-a and AG-b were estimated to be 54.0 and 34.5 kDa, respectively. The results of purification steps from culture medium were summarized in Table 1. The purification procedures yielded a 13.7-fold purified AG-a with 18.7% recovery rate and a 18.3-fold purified AG-b with 21.4% recovery rate. The final specific activities of AG-a and AG-b were 230.1 and 307.4 U/mg, respectively. It should be noted that individual agarase activitie of AG-a or AG-b could not be distinguished in the crude extract, and the recovery rate was defined as the rate of the final total agarase activity of AG-a or AG-b in total activity of culture medium.

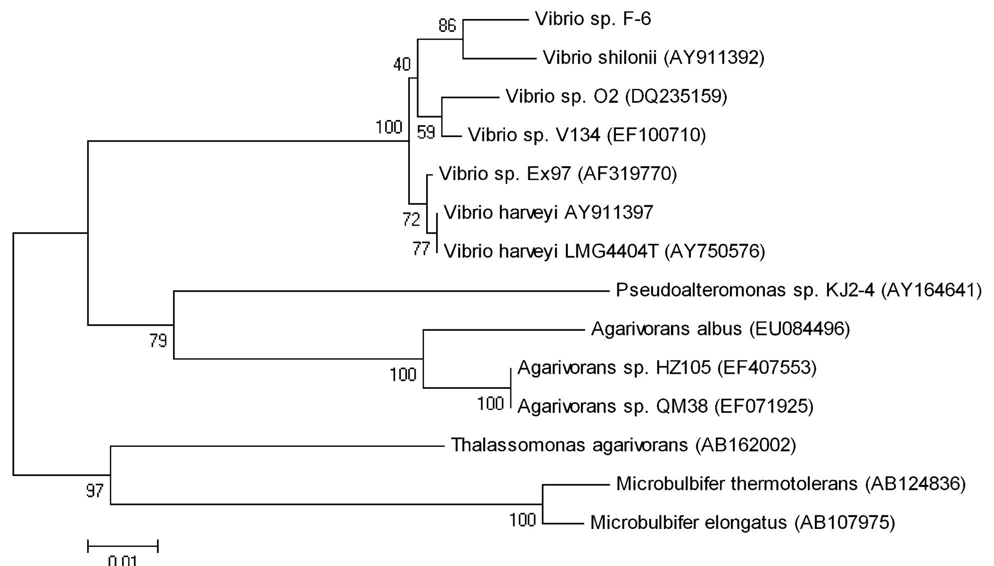

Fig. 2 Neighbor-joining tree based on 16S rDNA sequences showing relationships among strain F-6 and other related genera of *vibrio* and agarolytic bacteria. *Numbers* at nodes are levels of bootstrap support (%)

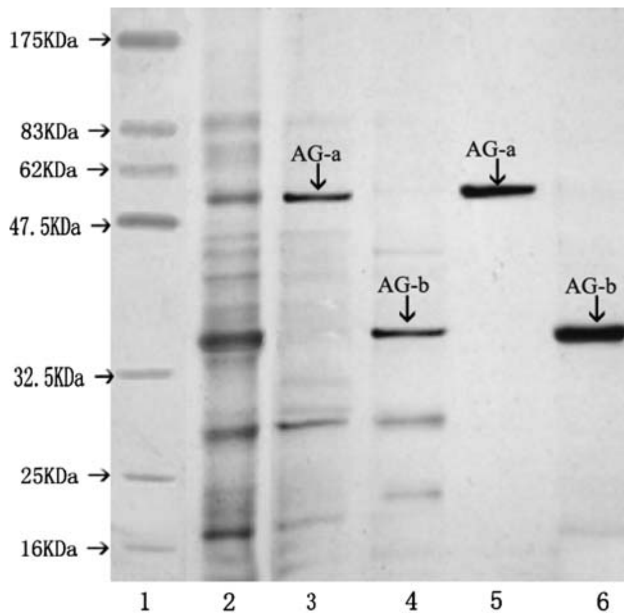

Fig. 3 SDS-PAGE analysis of agarases. *Lane 1* standard molecular mass markers, *Lane 2* crude agarases precipitated from ammonium sulfate, *Lane 3* AG-a purified from Q-Sepharose FF chromatography, *Lane 4* AG-b purified from Q-Sepharose FF chromatography, *Lane 5* AG-a purified from Sephacryl S-100 gel filtration, and *Lane 6* AG-b purified from Sephacryl S-100 gel filtration

Effects of pH and temperature on agarase activities

Figure 4 shows the effects of pH on AG-a and AG-b activities and stabilities. The results showed that the optimum pH values for AG-a and AG-b were about 7.0 and 9.0, respectively. AG-a was stable in the pH range of 4.0–9.0 and AG-b was stable in the pH range of 4.0–10.0. Figure 5 shows the effects of temperature on AG-a and AG-b activities and stabilities. The results showed that the optimum temperatures of AG-a and AG-b were 40 and 55 °C, respectively. AG-a was stable at temperature below 50 °C. AG-b was stable at temperature below 60 °C. AG-a lost 8% agarase activity and AG-b lost 5% agarase activity during storage in phosphate buffer (pH 7.5) at 4 °C for 6 months (data not shown), so both agarases are very stable enzymes.

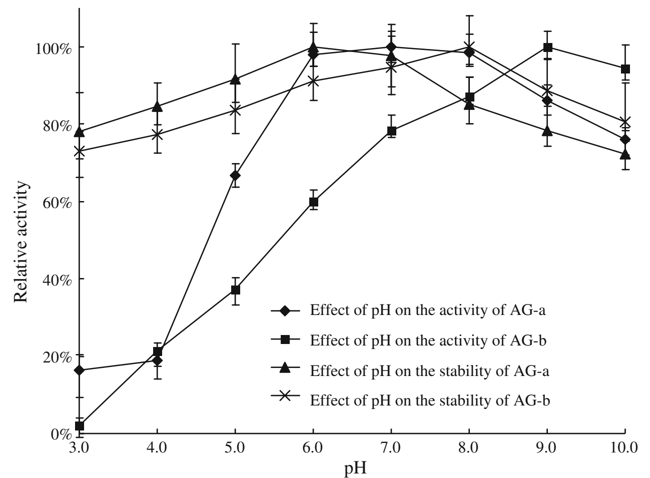

Fig. 4 Effects of pH on the activities and stabilities of AG-a and AG-b

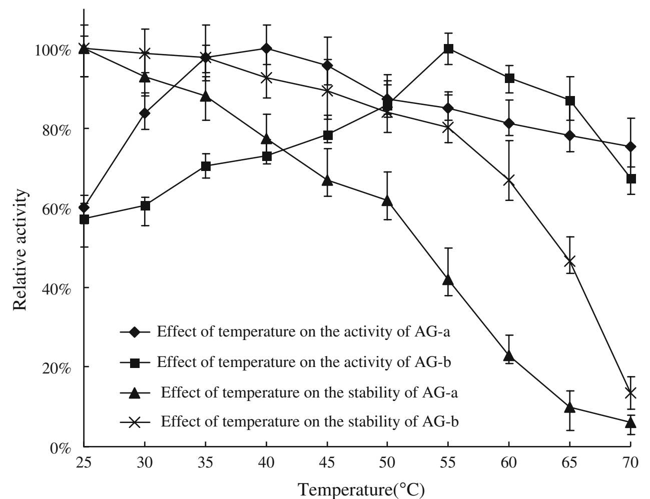

Fig. 5 Effects of temperature on activities and stabilities of AG-a and AG-b

Effects of reagents on agarase activity

The effects of reagents on agarases were presented in Table 2. Although the strain F-6 needed NaCl in culture medium to

Table 1 Results of purification of AG-a and AG-b from the strain F-6

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification folds (%)	Yield (%)
Crude extract	7360.4	438.6	16.8	1	100
20–80%$(NH_4)_2SO_4$	5873.6	206.4	28.5	1.7	79.8
AG-a					
Q-Sepharose FF	2090.4	12.6	164.6	9.8	28.4
Sephacryl S-100	1376.4	6.0	230.1	13.7	18.7
AG-b					
Q-Sepharose FF	2458.4	11.9	206.6	12.3	33.4
Sephacryl S-100	1575.1	5.1	307.4	18.3	21.4

Table 2 Effects of reagents on the activities of AG-a and AG-b

Reagents	AG-a relative activity (% ± SD)		AG-b relative activity (% ± SD)	
	1 mM	5 mM	1 mM	5 mM
Control	100 ± 0.02	100 ± 0.04	100 ± 0.05	100 ± 0.03
NaCl	103 ± 0.08	105 ± 0.10	100 ± 0.07	106 ± 0.11
$AgNO_3$	42 ± 0.21	0 ± 0.0	38 ± 0.18	0 ± 0.0
$CuSO_4$	86 ± 0.21	78 ± 0.24	121 ± 0.10	138 ± 0.13
$BaCl_2$	102 ± 0.16	105 ± 0.13	100 ± 0.18	92 ± 0.16
$MnSO_4$	110 ± 0.32	106 ± 0.21	138 ± 0.19	153 ± 0.36
$ZnCl_2$	110 ± 0.20	138 ± 0.34	122 ± 0.31	118 ± 0.23
$MgSO_4$	106 ± 0.21	120 ± 0.24	116 ± 0.45	106 ± 0.28
$PbCl_2$	83 ± 0.35	74 ± 0.42	86 ± 0.16	51 ± 0.30
$HgCl_2$	43 ± 0.20	19 ± 0.12	31 ± 0.34	0 ± 0.33
$CaCl_2$	120 ± 23	110 ± 0.25	123 ± 0.31	118 ± 0.15
$CdCl_2$	92 ± 0.32	87 ± 0.22	140 ± 0.25	132 ± 0.37
$FeCl_3$	46 ± 0.43	29 ± 0.31	45 ± 0.36	37 ± 0.19
$CrCl_3$	94 ± 0.25	91 ± 0.23	75 ± 0.26	69 ± 0.34
EDTA	92 ± 0.37	83 ± 0.18	94 ± 0.29	85 ± 0.34
SDS	96 ± 0.45	93 ± 0.23	97 ± 0.39	84 ± 0.41
β-Me	103 ± 0.28	99 ± 0.35	110 ± 0.24	106 ± 0.25
Urea	105 ± 0.42	103 ± 0.36	102 ± 0.28	97 ± 0.29

produce agarses, the concentration of NaCl did not remarkably affect AG-a or AG-b activity. $HgCl_2$, $AgNO_3$ or $FeCl_3$ at a concentration of 1.0 mM remarkably inhibited AG-a and AG-b activities, and at a concentration of 5.0 mM strongly inhibited AG-a and AG-b activities. In contrast, $ZnCl_2$, $MgSO_4$ or $CaCl_2$ at a concentration of 1.0 or 5.0 mM remarkably increased AG-a activity. $CaCl_2$, $CuSO_4$ or $MnSO_4$ at a concentration of 1.0 or 5.0 mM remarkably increased AG-b activity. EDTA or SDS at a concentration of 5.0 mM inhibited AG-a and AG-b activities, while β-mercaptoethanol or urea slightly increased AG-a and AG-b activities.

Determination of N-terminal sequences of agarases

The N-terminal sequences of the first 15 amino acid residues of AG-a and AG-b were ALVTSSHKAPWYSNS and TLASAEDSTYEIPPR, respectively. Similarity search using BLAST algorithm in the NCBI database showed AG-a was related to a family of GH-50, and AG-b was related to a family of GH-16. There was a little homology among amino acid sequences between AG-a and AG-b. The N-terminal sequence of AG-a was some homology (40–60%) with GH-50 family agarases such as those from *Agarivorans* sp. JA-1 (EF100136), *Agarivorans* sp. QM38 (EF051475), and *Vibrio* sp. JT0107 (D14721). The N-terminal sequence of AG-b was of low homology (30–45%) with GH-16 family agarases such as those from *Agarivorans albus* YKW-34 [18], *Microbulbifer* sp. JAMB-A3 (AB158516), *Microbulbifer* sp. (AB124837), *Pseudoalteromonas* sp. CY24 (AY150179).

Analysis of the reaction products

The hydrolysis pattern of agarose by the purified AG-a, AG-b and the equivalent amount of AG-a and AG-b were analyzed by TCL (Fig. 6). Quantification of reaction products by agarases was performed by gel filtration chromatography using CLASS-VP HPLC system. The composition (mol%) of the hydrolysates of AG-a was neoagarotetraose (48%), neoagarohexaose (50%), and neoagarobiose (2%). The composition (mol%) of the hydrolysates of AG-b was neoagarohexaose (67%), neoagarooctaose (30%), and neoagarodecaose (3%). The composition (mol%) of the hydrolysates of the mixture of AG-a and AG-b was neoagarobiose (92%) and neoagarotetraose (8%). NMR spectroscopy was used for the structure determination of the different oligosaccharides. A typical patterns (about 97 and 93 ppm) for neoagarooligosaccharides produced from AG-a or AG-b were observed according to the NMR spectrum (data not shown). Resonances at about 97 and 93 ppm were characteristic of β and α anomeric forms of galactose residues at the reducing end of the neoagarooligosaccharides, respectively. So, AG-a and AG-b were classified as β-agarases.

Discussion and conclusion

In the present study, a marine agarase-producing bacterium F-6 was isolated. According to Bergey's Manual of Determinative Bacteriology [30] and 16S rDNA gene sequence of the strain F-6, strain F-6 was identified as *Vibrio*. The

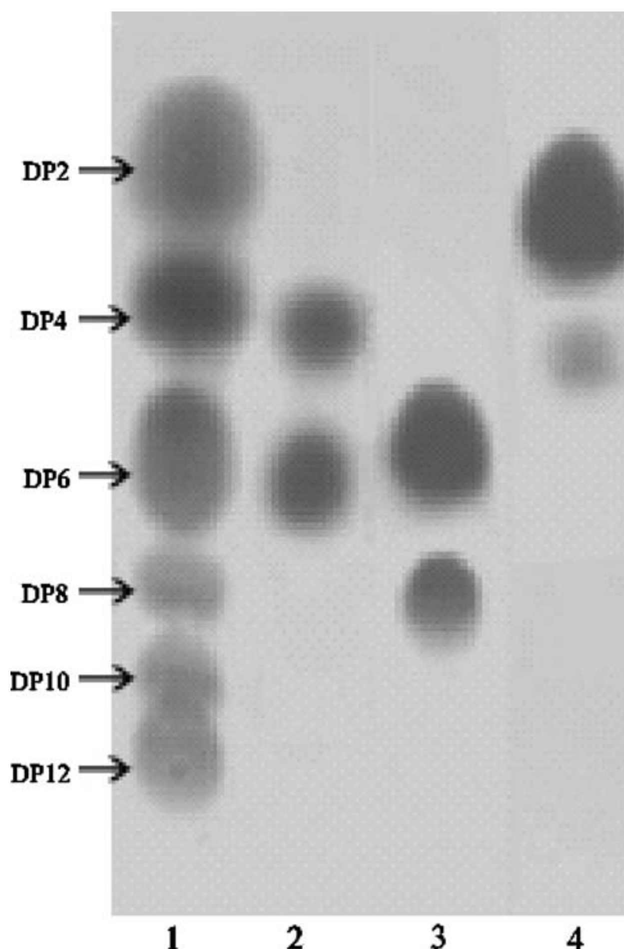

Fig. 6 TLC analysis of neooligosaccharides after treatment of AG-a, AG-b and the mixture of AG-a and AG-b. *Lane 1* standard neoagarooligosaccharides, *Lane 2* result of hydrolytic products of AG-a, *Lane 3* result of hydrolytic products of AG-b, and *Lane 4* result of hydrolytic products of AG-a and AG-b

phylogenetic tree suggested that the isolate may be a new species in the genus *Vibrio* (Fig. 2). Further taxonomic analyses should be performed. It was noteworthy that agarase activity of *vibrio* sp. F-6 (8.52 U/ml) was higher than that of *Alteromonas* (2.32 U/ml) [12], *Vibrio* sp. QJH-12(1.72 U/ml) [34], *Alteromonas* sp. SY37-12 (1.8 U/ml) [15], and *A. albus* YKW-34 (about 1U/ml) [18]. So, strain F-6 had promising potential to be used to produce high activity agarases for future research and application.

Two agarases, AG-a and AG-b, were purified to a homogeneity from the culture medium of the strain F-6 through ammonium sulfate precipitation, Q-Sepharose FF chromatography, and Sephacryl S-100 gel filtration. The molecular masses of AG-a and AG-b were 54.0 and 34.5 kDa, respectively, and were different from those of reported agarases to the best of our knowledge. Similarity search using BLAST algorithm in the NCBI database showed AG-a was related to a family of GH-50, and AG-b was related to a family of GH-16. But N-terminal sequences of AG-a and AG-b had little similarity with other reported agarases [5, 15–17] and even differed from agarases from *Vibrio* sp. [10, 11, 35], so the two agarases may be new kinds of agarases. Further identification of AG-a and AG-b based on the nucleotide sequences of their genes is underway.

The optimum pH values for AG-a and AG-b were 7.0 and 9.0, respectively. The optimum temperatures of AG-a and AG-b were 40 and 55 °C, respectively. Compared with temperature stabilities of agarases reported by Kirimura et al. [3], Suzuki et al. [36], and Ohta et al. [33], both agarases were stable in high temperature, especially AG-b (stable up to 60 °C). The property of high-temperature stablity of agarases will benefit industrial application. Because at low temperature of 40 °C, agarose will easily gel and gelled agarose will hinder agarase activity. AG-a was stable in the pH range of 4.0–9.0 and AG-b was stable in the pH range of 4.0–10.0. Compared with pH stabilities of agarases reported by Kirimura et al. [3] and Suzuki et al. [36], both agarases were stable in a wide pH range. The pH stabilities of both agarases were similar to those of agarases from *Thalassomonas* sp. JAMB-A33 [33] and *A. albus* YKW-34 [18].

From the results described above, we know that the hydrolysis products of agarose by AG-a, AG-b and the mixture of AG-a and AG-b were different. In experiments of different hydrolytic times and amounts of agarases, the proportion and composition of hydrolysis products were different (data not shown). So, AG-a and AG-b may be used to produce different polymerization degrees of neooligosaccharides which may provide advantages in applications [28, 29].

From the viewpoint of biotechnologic potentiality and application, agarases from *Vibrio* sp. F-6 had potentially wide applications in many areas of industry and scientific research, such as generating simple neooligosaccharides from complex polysaccharides, liberating DNA and other embedded molecules from agarose, and extracting bioactive or medicinal compounds from red algae. The nucleotide sequences encoding for both agarases are under investigation and will be published soon.

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