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Jingxue Wang · Haijin Mou
Xiaolu Jiang · Huashi Guan

Characterization of a novel β-agarase from marine *Alteromonas* sp. SY37–12 and its degrading products

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Abstract The phenotypic and agarolytic features of an unidentified marine bacteria isolated from the southern ocean of China was studied. The strain was gram-negative, aerobic, and polarly flagellated. It was identified as the genus *Alteromonas* according to its morphological and physiological characterization. In solid agar, the isolate produced a diffusible agarase that caused agar softening around the colonies. An extracellular agarase was purified by the procedure of ammonium sulfate precipitation, gel filtration on Sephacryl S-100HR, and ion-exchange chromatography on diethylaminoethyl-Sepharose. The purified protein exhibited a single band on SDS-PAGE with a molecular mass of 39.5 kDa. The enzyme hydrolyzed the β-1,4-glycosidic linkages of agar, yielding neoagarotetraose and neoagarohexaose as the main products. The optimum reaction temperature of the agarase was 35°C, with a narrow range from 30 to 45 °C. The enzyme activity reached the maximum at pH 7.0 and in the presence of 2% NaCl. Molecular mass and degrading products showed that the agarase from *Alteromonas* sp. SY 37-12 was much different from those previously reported.

Introduction

Agar, as an important food additive and bacterial culture medium, is one of the most well-known marine polysaccharides. Additionally, various potential physiological activities of polysaccharides and oligosaccharides derived from agar have been reported, such as antivirus (Takemoto 1966), antitumor (Fernandez et al. 1989), immune enhancement (Yoshizawa et al. 1993), antioxidation (Wang et al. 2004; Zhang et al. 2003), and elicitor activity on plant (Weinberger et al. 2001).

Agar has been determined to have a linear chain structure composed of alternating residues [*O*-3,6-α-anhydro- L-galactopyranosyl (1→3) *O*-β-D-galactopyranose] combined by β-1,4 bonds (Hamer et al. 1977). It is confirmed that agar oligosaccharide can be attained by many methods, including chemical degradation and enzyme hydrolysis. A special enzyme hydrolyzing the agar, agarase (agarose 4-glycanohydrolase, E.C.3.2.1.81), has been found in certain marine mollusks (Usov and Miroshnikova 1975). However, the most was reported from several bacterial genera, including *Cytophaga* (Van der Meulen et al. 1974), *Vibrio* (Aoki et al. 1990), *Actinomyces* (Stanier 1942), *Alteromonas* (Leon et al. 1992), *Pseudoalteromonas* (Vera et al. 1998), and *Pseudomonas* (Ha et al. 1997), etc. Most of these bacteria were isolated from marine environments, while a few species isolated from rivers (Agbo and Moss 1979), hot spring (Shieh and Jean 1998), soil (Sampietro and Vattuone de Sampletro 1971), and sewage (van Hofsten and Malmqvist 1975) have also been described.

Agarase-producing bacteria can be classified into two groups according to the mode of action on agar: α-agarases cleave the α-1,3 linkage of agarose (Young et al. 1978) and β-agarases cleave the β-1,4 linkage of agarose (Duckworth and Turvey 1969). Some of the agarase have been purified and characterized in the past years. In our laboratory, we have isolated a marine bacterium, *Alteromonas* sp. strain 37-12, which has the high ability to decompose the agar from the southern ocean of China. We describe here the identification of this strain, the characterization of its extracellular β-agarase, and the composition of its degrading products.

Materials and methods

Isolation of strains and cell growth

The agarase-producing strain SY37-12 was isolated in this laboratory from the surface of rotted red algae in the South China Sea coast in Sanya, Hainan Island. The screening

J. Wang · H. Mou (✉) ·
X. Jiang · H. Guan
Ocean University of China,
Qingdao, 266003,
PR China
e-mail: mousun@ouc.edu.cn
Tel.: +86-532-82032290
Fax: +86-532-82894024

was carried out on agar plates in a medium containing 2.0% NaCl, 0.1% K_2HPO_4, 0.1% NH_4Cl, 0.05% $MgSO_4$, 0.01% $CaCl_2$, and 1.5% agar. The plates were incubated at 33°C for 48 h. Colonies that formed pits or clearing zones on agar were picked up and purified further by the same plating method. For liquid culture, 0.2% agar was added before sterilization.

The agar used here was commercial agar powder extracted from *Gracilaria verrucosa* (Mingfu Sea-weed Industry Co., Fujian Province, China).

Alteromonas sp. strain 37-12 is stored now in the China Center for Type Culture Collection (CCTCC), with strain number M204009.

Evaluation of agarase activity

Agarase activity was determined by measuring the increase in the concentration of reducing sugar as described by von Borel et al. (1952). The isolated colonies were inoculated into a medium of the same composition as that of isolation medium, except that the agar concentration was lowered to 0.2%. The cells were incubated in an orbital shaker at 150 rpm and 33°C for 24 h. After centrifuging at 7,000×*g* for 15 min to remove bacterial cells and gel residues, a 1-ml culture supernatant was added to 20 ml pH 7.0 phosphate buffered saline solution (PBS) with 0.5% agar substrate and incubated at 33°C for 30 min. Then the 1-ml reaction solution was mixed with 1.5 ml 3,5-dinitrosalicylic acid (DNS) reagent. After being heated at 100°C for 5 min and then cooled, the mixture was diluted to 25 ml with deionized water. Optical density was read at 520 nm, and values for reducing sugars were expressed as D-galactose equivalents. One unit of agarase activity was defined as the amount of enzyme that released 1 μmol reducing sugar (measured as D-galactose) from agar per minute under the above conditions.

Purification of agarase

Unless specified otherwise, all operations were done at 4°C. An overnight culture of strain SY37-12 was prepared in the medium described above and transferred to 250 ml of fresh medium containing 0.2% agar. Incubation was carried out in an orbital shaker at 150 rpm and 33°C for 24 h until the stationary phase, followed by centrifugation at 7,000×*g* for 15 min. The supernatant was brought to 80% saturation with solid ammonium sulfate overnight. The collected enzyme protein was resuspended in 20 ml of 50 mM Tris–HCl (pH 7.5), sealed in dialysis bags (cut-off value, 12,000–14,000 MW), and dialyzed three times against the same buffer at 4°C for 2 days. The dialyzate was loaded onto a diethylaminoethyl (DEAE)-Sepharose column (1.5 cm×20 cm, Pharmacia) equilibrated with 50 mM Tris–HCl (pH 7.5). The flow rate was adjusted to 0.5 ml/min. The protein was eluted from the column with the same buffer containing a linear NaCl gradient (0~1.0 M), and the elute fractions were collected. The eluates were monitored continuously at 280 nm, and fractions were assayed for activity against agar.

Fractions containing agarase activity were pooled and concentrated by polyethylene glycol 20,000, then applied for gel filtration chromatography on Sephacryl S-100HR (1.2 cm×92 cm, Pharmacia) equilibrated with 50 mM Tris–HCl (pH 7.8). The flow rate was adjusted to 0.7 ml/min. Fractions were assayed for protein and agarase activity. The enzyme was lyophilized and stored at −20°C and was stable for more than 2 months.

The purification of the fractions was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in a 0.75-mm slab gel consisting of a stacking gel (5% polyacrylamide) and a separating gel (12.5% polyacrylamide) with 25mM Tris–HCl buffer, pH 7.8. Proteins were stained with Coomassie brilliant blue R-250. Molecular mass standards were phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysosyme (14.4 kDa).

Effects of temperature and pH on enzyme activity and stability

The optimum temperature of agarase was determined at different temperatures (from 20 to 50°C). Temperature stability was determined by measuring the residual activities after incubation at different temperatures. These studies were carried out in sealed Eppendorf tubes that were completely immersed in a water bath at the required temperatures. Samples (1 ml) were removed after incubation at each of the indicated time periods, chilled on ice and then assayed for enzyme activity.

The optimum pH was determined with agar as substrate dissolved in KH_2PO_4–NaOH buffer with different pH values (5.0–9.0). The optimum NaCl concentration in the reaction solution was determined in PBS solution (pH 7.0) with different NaCl concentration (0–5.0%).

Preparation of agar oligosaccharides

Ten grams of agar powder was scattered in 500 ml deionized water. A 50-ml agarase solution (total agarase activity was 90 U) extracted from strain SY37-12 was added to the agar solution. The reaction was carried out at 33°C for 12 h, and then was stopped by heating the solution in boiling water for 10 min. Twofold ethanol was added to the reaction mixture to remove the high-molecular-mass polysaccharides. After centrifugation and concentration, the depolymerized end-products were produced by repeated ethanol fractionation.

Structural analysis of agar oligosaccharides

The molecular mass distribution of the agar oligosaccharides was determined by matrix-assisted laser desorption

ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using LDI-1700 instrument (Linear Scientific, Inc., USA). The instrument was fitted with a pulsed nitrogen laser at 337 nm with 3 ns pulse duration. 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix. Infrared spectroscopy was performed using a Nicolet Nexus 470 spectrophotometer (Thermo Nicolet, Madison, WI, USA). For ^{13}C-NMR spectroscopy analysis, the samples were taken up in $^{2}H_2O$ and processed at 30°C. Spectra were recorded on a JNM-ECP 600 SCM spectrometer (JEOL, Tokyo, Japan).

Results

Strain properties and identification

Bacteria isolated from different areas of the China Sea were screened for a stable and effective agar-decomposing enzyme. A total of 216 strains producing agar-decomposing enzyme were isolated from the soil or seaweed surface, including *Vibrio*, *Alteromonas*, and *Cytophaga*. Strain SY37–12, isolated from the red alga *Gracilaria verrucosa* collected on Hainan Island of China, produced a large quantity of extracellular agarase when incubated in basal salt medium.

The strain SY 37-12 produced soft pits on the agar surface with clear haloes around the bacteria colonies, which was regarded as an obvious mark indicating the high ability of degrading agar. Electron micrograph of the strain showed a polarly flagellated and straight-rod-shaped cell, 0.5–0.6 μm×1.2–1.5 μm. It was gram-negative and oxidase- and catalase-positive. It requires sodium ion for growth, has an oxidative metabolism, and does not accumulate poly-β-hydroxybutyrate (PHB) as an intracellular reserve product. The preliminary identification results showed that the morphologic and physiological characteristics of this strain were in accordance with *Alteromonas*, according to *Bergey's Manual of Systematic Bacteriology* (Holt et al. 1994) (Table 1).

Table 1 Morphologic and physiological characteristics of *Alteromonas* sp. SY37–12

Characteristics tested	Results
Cell shape	Straight rod
Microcysts or endospores	-
Polar flagellum	+
Production of pigments	-
Growth at 4°C	-
Growth at 35°C	+
Growth at 40°C	-
Anaerobic growth	-
Organic growth factors required	-
Oxidase reaction	+
Catalase reaction	+
Argin1ine dihydrolase	-
Accumulation of PHB	-
O/F test	O
Production of H_2S	-
Requirement of sodium for growth	+
Hydrolysis of agar, starch, carrageenan, and gelatin	+
Hydrolysis of alginate, chitin, and cellulose	-
Utilization of D-glucose, D-galactose, D-fructose, sucrose, cellobiose, and D-mannose	+
Utilization of L-arabinose, dulcitol, raffinose, and D-ribose	-

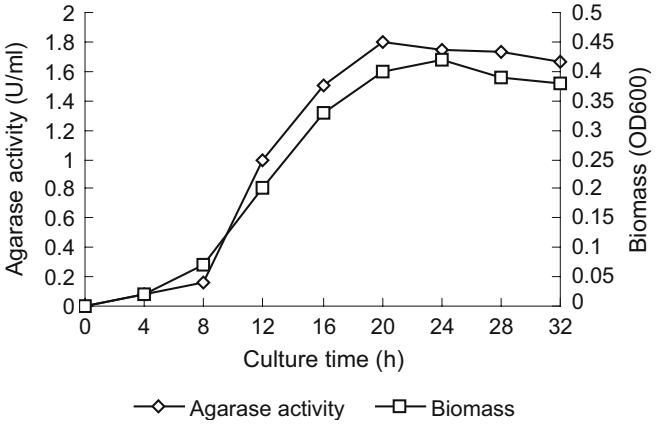

Fig. 1 Growth and agarase activity of *Alteromonas* sp. SY37–12. Fermentation liquids were monitored at 600 nm for biomass (□) and assayed for agarase activity (◊) by measuring the increase in the concentration of reducing sugar

Agarase produced by SY37-12 was shown to be an induced enzyme. In the presence of crude agar, the strain produced extracellular agarase. No detectable agarase activity was found in the culture medium without agar. Furthermore, the addition of carrageenan, alginate, starch, galactose, lactose, and glucose in the absence of agar showed no effects on the production of agarase of SY37-12. When the agar was used as the sole carbon source, the

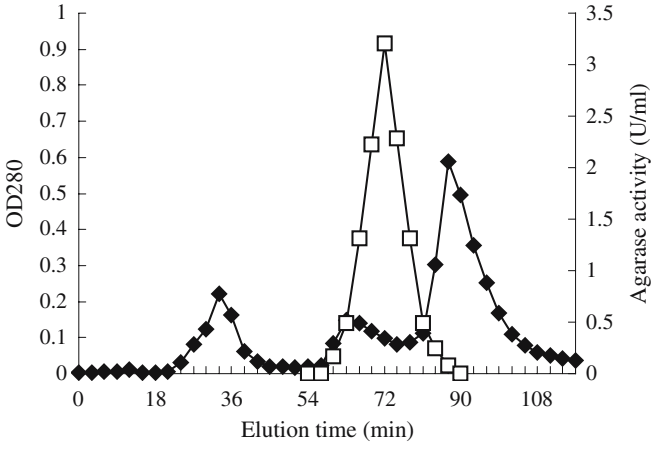

Fig. 2 Ion-exchange chromatography of agarase on DEAE-Sepharose. The Tris buffer containing gradient NaCl rising from 0 to 1.0 M at a flow-rate of 0.5 ml/min was used to wash out the sample. Fractions were monitored continuously at 280 nm for protein content (♦) and assayed for agarase activity (□) by measuring the increase in the concentration of reducing sugar

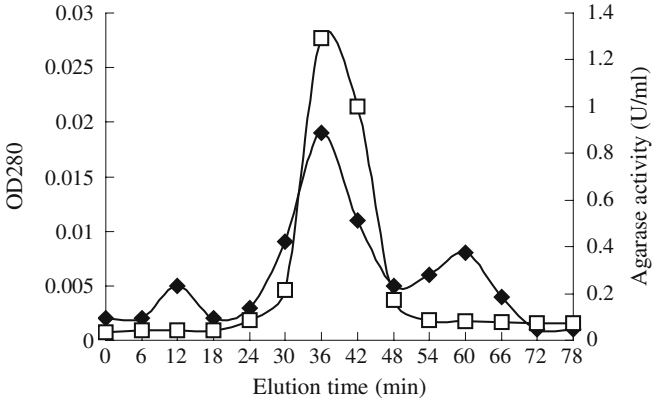

Fig. 3 Gel filtration chromatography of agarase on Sephacryl S-100. The Tris buffer at a flow-rate of 0.7 ml/min was used to wash out the sample. Fractions were monitored continuously at 280 nm for protein content (♦) and assayed for agarase activity (□) by measuring the increase in the concentration of reducing sugar

agarase activity of fermentation medium of SY37-12 reached a maximum of 1.8 U/ml after cultivation in conventional batch culture for 20 h, whereas the biomass peaked at 24 h (Fig. 1).

Purification of agarase

According to the pattern of ion-exchange chromatography on DEAE-Sepharose, several protein peaks were contained in the agarase sample (Fig. 2). Additional purification of the fractions with agarase activity was achieved by gel filtration on Sephacryl S-100 (Fig. 3). Three protein peaks were shown in the chromatography, which was in accordance with the result of SDS-PAGE (Fig. 4). The purified enzyme had a molecular mass of 39.5 kDa, as determined by a comparison with the mobility of protein standards. The purification steps and recovery rate of the agarase is summarized in the Table 2.

Table 2 Purification of agarase from *Alteromonas* sp. SY37-12

Step	Volume (ml)	Agarase (U)	Protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Cell-free medium	1,000	1,818	606.2	3.0	1	100
80% $(NH_4)_2SO_4$ precipitate	45	727	157.8	4.6	1.5	40.0
DEAE-Sepharose	200	642	17.1	37.5	12.5	35.3
Sephacryl S-100 HR	200	259	3.1	83.5	27.8	14.2

Enzyme properties

The optimum reaction temperature of the agarase was 35°C, with a narrow range from 30 to 45°C. At 20°C, the enzyme activity was only 30% in comparison with the maximum. The agarase was heat-labile, with rapid loss of activity when treated at 50°C for 15 min or at 70°C for 1 min (Fig. 5). It even lost above 80% of its original activity after incubation at 90°C for 20 s. The effect of pH on the enzyme activity was determined at 33°C in the pH range 5.0–9.0. The agarase exhibited maximum activity at pH 7.0. Since strain *Alteromonas* sp. SY37-12 came from

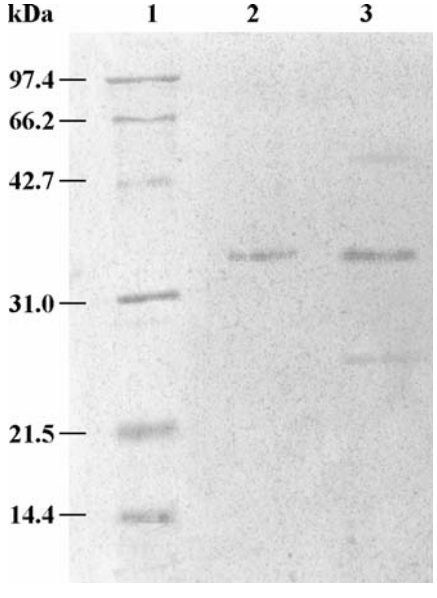

Fig. 4 SDS-PAGE of purified agarase. *Lane 1* molecular mass standards, *lane 2* purified agarase by Sephacryl S-100 (only one band with a molecular mass of 39.5 kDa), *lane 3* partly purified agarase by DEAE-Sepharose (three bands exist in the sample)

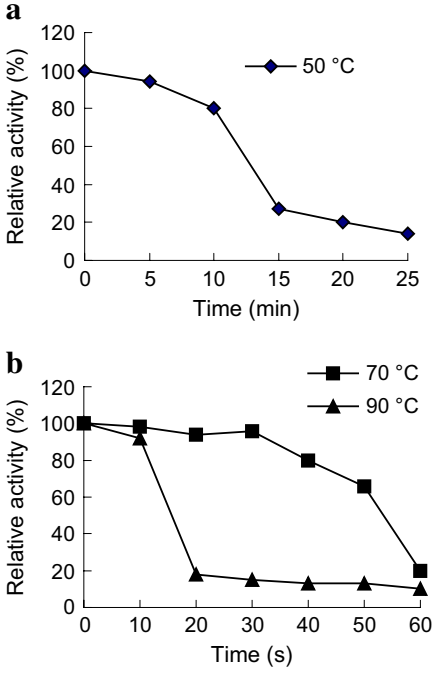

Fig. 5 Effect of temperature on stability of agarase

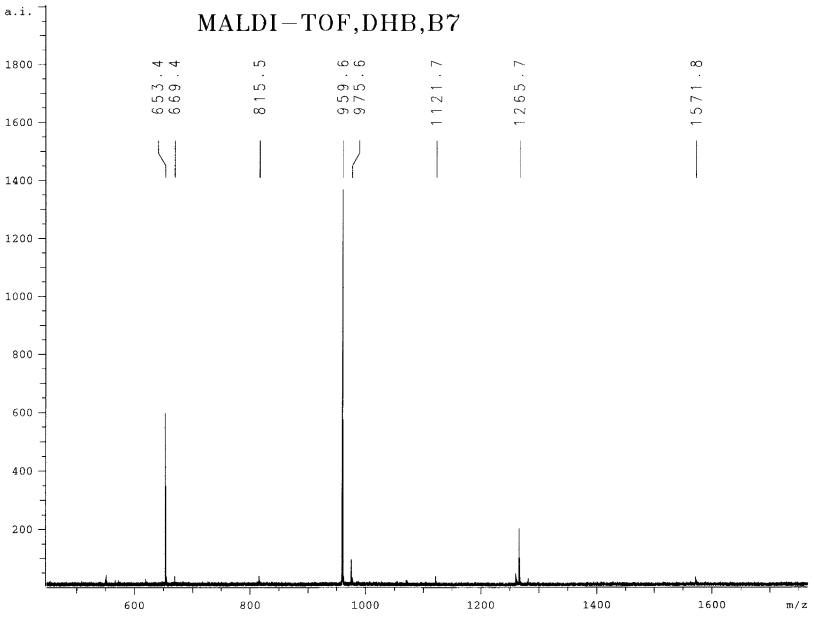

Fig. 6 MALDI-TOF mass spectrum of agar hydrolysis fragments

the ocean, high NaCl concentration was necessary for its growth and enzyme production. When enzyme activity was measured in the presence of salt, a significant elevation could be observed. The enzyme activity reached the maximum in the presence of 2% NaCl, and then declined when the NaCl concentration continued to increase.

MALDI-TOF-MS of the agarooligosaccharides

After sufficient hydrolysis and repeated fractionation by ethanol precipitation, the main depolymerized end-products were collected. The MALDI-TOF mass spectrum of the sample was shown in Fig. 6. According to the mass spectrum, the sample was an oligosaccharide mixture with polymerization degree from 4 to 10. No sulfate group was linked in the sugar ring according to the molecular mass assignment. This speculation could be confirmed further by IR spectrum analysis and chemical determination (data not shown). The main composition was tetrasaccharide (MM-630 Da) containing two galactopyranose residues (G-units) and two 3,6-anhydrogalaxtopyranose residues (An-units), hexasaccharide (MM-936 Da) containing three G-units and three An-units, and octosaccharide (MM-1242 Da) containing four G-units and four An-units. Furthermore, several minor peaks could be found in the mass spectrum, which were attributed to pentasaccharide (MM-792 Da) containing three G-units and two An-units, heptasaccharide (MM-998 Da) containing four G-units and three An-units, and decasaccharide (MM-1548 Da) containing five G-units and five An-units.

^{13}C-NMR of agarooligosaccharides

MALDI-TOF mass analysis of the hydrolysis products of agar generated by agarase from *Alteromonas* sp. SY 37-12 showed the presence of agarotetraose and agarohexaose as the main products. These products were further analyzed by NMR to determine the specificity of the cleavage. Anomeric carbons usually give downfield signals in ^{13}C-NMR spectrum. The configuration of anomeric carbons can be determined according to the downfield shifts. The ^{13}C-NMR spectrum of the oligosaccharide mixture showed a typical pattern for neoagarooligosaccharide (Fig. 7). The neoagarooligosaccharide series is typically produced by the cleavage of β-(1,4) linkages by β-agarase. Resonances at about 97 and 93 ppm are characteristic for the β and α anomeric forms, respectively, of galactose residues at the reducing end of the neoagarooligosaccharides, indicating that the cleavage occurs at the β-(1,4) linkages. No peaks were present at around 90.4 ppm corresponding to hydrolyzed α-(1,3) linkages. Thus, the ^{13}C-NMR spectrum confirmed that agarase from strain SY 37-12 is a β-agarase, specifically hydrolyzing the β-(1,4) glycosidic linkage between D-galactose and 3,6-anhydro-L-galactose.

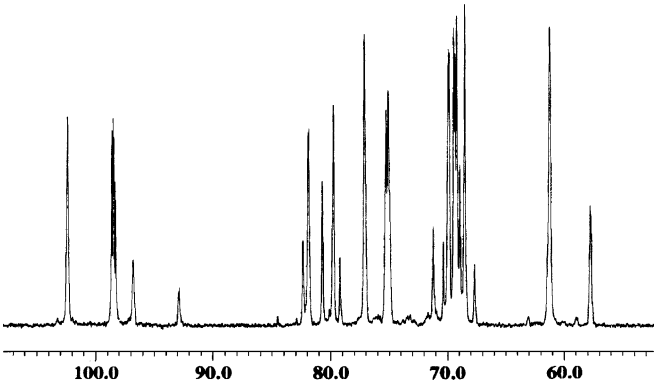

Fig. 7 ^{13}C-NMR spectrum of the hydrolysis products of agar produced by agarase from *Alteromonas* sp. SY37–12

Discussion

We describe here the characterization of a new agarolytic bacterium isolated from the southern ocean of China. This strain was identified as *Alteromonas* sp. according to its morphologic and physiological characteristics. The purified agarase extracted from *Alteromonas* sp. SY 37-12 has a molecular mass of 39.5 kDa, as indicated by SDS-PAGE. This value is close to those reported for β-agarase from *Pseudoalteromonas* sp. N-1 (33 kDa) (Vera et al. 1998), *Pseudomonasatlantica* (32 kDa) (Morrice et al. 1983), and *Vibrio* sp. AP-2 (34 kDa) (Aoki et al. 1990). This enzyme acts as an endoenzyme, which decreased rapidly the viscosity of agar substrate at the initial reaction stage.

Agarases (E.C.3.2.1.81) are classified into two groups depending upon their specificity of the degradation on the agar: α-agarases cleave the α-1,3 linkage of agar, yielding oligosaccharides with 3,6-anhydro-L-galactose at the reducing end and D-galactose at the nonreducing end, and β-agarase cleave the β-1,4 linkage of agar, yielding oligosaccharides with D-galactose at the reducing end and 3,6-anhydro-L-galactose at the nonreducing end (Vera et al. 1998). From the experiment result on analyzing the oligosaccharides by ^{13}C-NMR, it could be deduced that the agarase from the *Alteromonas* sp. SY37-12 was β-agarase, which produced the 3,6-anhydro-L-galactose as the nonreducing end and D-galactose as the reducing end.

According to the assignment of MALDI-TOF-MS, the main composition of hydrolytic products was neotetrasaccharide (An-G-An-G), neohexasaccharide (An-G-An-G-An-G), and neooctosaccharide (An-G-An-G-An-G-An-G). In addition, pentasaccharide (G-An-G-An-G), heptasaccharide (G-An-G-An-G-An-G), and decasaccharide (An-G-An-G-An-G-An-G-An-G) could also be found in the products. It did not release detectable amounts of agarobiose and agarotriose. With future experiments, the enzyme was confirmed to be not capable of hydrolyzing agarotetrose, agaropentose, agarohexose, and agaroheptose. It could be speculated that the minimal polymer degree of the agar substrate depolymerized by agarase from *Alteromonas* sp. SY 37-12 is eight. The agarase produce mainly agarooligosaccharides with even degree of polymerization, with D-galactose as the reducing end. As an endoenzyme, it cleaves the β-1,4 linkage of agar at random and produces a large quantity of low-molecular-mass fragments during the hydrolysis. Therefore, at the end of the hydrolytic reaction, the oligosaccharides with odd degree of polymerization,

Table 3 Molecular mass and depolymerized products of characterized agarases

Group	Strain	Molecular mass (kDa)	Products	Reference
α-Agarase	*Alteromonas* GJIB	360 (bipolymer)	Agarotetraose	Potin et al. 1993
	Vibrio JI0107	84 (bipolymer)	Agaropentaose, agarotriose, agarobiose, 3,6-anhydro-galactose and D-galactose	Sugano et al. 1994
	Bacillus MK03	320 (octamer)	Agaropentaose, agarotriose, 3,6-anhydro-galactose and D-galactose	Suzuki et al. 2002
β-agarase	*Cytophaga flevensis*	26	Neoagarotetraose, neoagarobiose	Van der Meulen et al. 1974
	Pseudoalteromonas N-1	33	Neoagarotetraose, neoagarohexaose	Vera et al. 1998
	Pseudomonas-like	I[a]: 210	Neoagarohexaose, neoagarotetraose, neoagarobiose	Malmqvist 1978
		II: 63	Neoagarotetraose	
	Pseudomonas atlantica	I: 32	Neoagarotetraose, neoagarobiose	Morrice et al. 1983
		II[b]: N/A	Neoagarobiose	
	Vibrio AP-2	I: 34	Neoagarotetraose	Aoki et al. 1990
		II: 20	Neoagarobiose	
		III: 18	Neoagarotetraose	
	Vibrio JT0107	I: 107	Neoagarotetraose, neoagarobiose	Sugano et al. 1993, 1995
		II: 72	Neoagarotetraose, neoagarobiose	
	Vibrio PO-303	I: 87.5	Neoagarohexaose, neoagarotetraose	Araki et al. 1998
		II: 115	Neoagarobiose	
		III: 57	Neoagarooctaose, neoagarodecaose	
	Bacillus MK03	92	Neoagarotetraose	Suzuki et al. 2003
	Bacillus cereus ASK202	90	Neoagarobiose	Kim et al. 1999
	Alteromonas E-l	82	Neoagarobiose	Kirimura et al. 1999
	Alteromonas C-1	52	Neoagarotetraose	Leon et al. 1992
	Alteromonas SY37-12	39.5	Neoagarotetraose, neoagarohexaose	This work

[a]There are several fractions with agarase activity produced by the strain
[b]Data not available

especially pentasaccharide and heptasaccharide, are unavoidably formed in the products.

Molecular mass and depolymerized products of characterized agarases were given in Table 3, which showed that the molecular mass of agarase from *Alteromonas* sp. SY 37-12 is different from the others, especially that from *Alteromonas* sp. E-1 (Kirimura et al. 1999) and *Alteromonas* sp. C-1 (Leon et al. 1992). The main depolymerized products produced by this enzyme are unique in comparison with the reports. Therefore, a conclusion was drawn here that the agarase produced by *Alteromonas* sp. SY 37-12 might be a novel enzyme. Further characterization of this enzyme will be discussed in the near future.

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