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ORIGINAL PAPER

A new β-agarase from marine bacterium *Janthinobacterium* sp. SY12

Yan-Ling Shi · Xin-Zhi Lu · Wen-Gong Yu

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Abstract An agar-degrading bacterium, strain SY12, was identified as the genus *Janthinobacterium*, which is a member of the class Betaproteobacteria. A β-agarase gene *agaY* was cloned from SY12, and it is the first reported agarase from the Betaproteobacteria. *AgaY* consisted of 1,338 bp encoding 445 amino acid residues, and it was assigned to GH16 family. AgaY has an N-terminal secretary leader peptide preceding a GH16 catalytic domain and a CBM13 carbohydrate binding module. The recombinant agarase AgaY overexpressed in *Escherichia coli* displayed a molecular mass of 50.2 kDa and the optimum temperature and pH for the activity of the enzyme was 40°C and pH 7.0, respectively. It degraded agarose to give neoagarotetraose and neoagarobiose as the main products. Interestingly, in contrast to other agarases of GH16, the enzymatic activity of AgaY is Na^+ and Ca^{2+} independent.

Keywords β-Agarase · *Janthinobacterium* sp. · Cloning · Expression

Introduction

Agar is an abundant biopolymer found in red algae as one of the cell wall component. It is a polysaccharide composed of agarose and agaropectin. Agarose is the neutral fraction of agar, which has a linear chain of alternating residues of 3-O-linked-β-D-galactopyranose and 4-O-linked 3,6-anhydro-α-L-galactose (Duckworth and Yaphe 1971). Agarases are a group of glycoside hydrolases (GH) that catalyze the degradation of agarose. It can be classified into two groups according to the mode of action: α-agarase cleaves the α-1,3-linkages of agarose and β-agarase cleaves the β-1,4-linkages of agarose (Young et al. 1978; Duckworth and Turvey 1969). Most of the reported agarases are β-agarases, which have been grouped into three glycoside hydrolase (GH) families GH-16, GH-50 and GH-86 (http://afmb.cnrs-mrs.fr/CAZY/) based on their similarity in amino acid sequences. α-Agarases have been classified into a new family GH-96 (Flament et al. 2007).

It has been recognized that agarases have potentially wide applications in many areas of industry, such as liberating DNA and other embedded molecules from agarose, preparing protoplasts (Araki et al. 1998) and extracting bioactive or medicinal compounds from algae and seaweed, etc. In addition to these classical applications, it is also a useful tool for generating oligosaccharides from polysaccharides (Kobayashi et al. 1997; Yoshizawa et al. 1995; Li et al. 2007), which are known to possess various new biological and therapeutic properties. Neoagaro-oligosaccharides produced by β-agarase have been reported to facilitate the growth of some bacteria and macrophage stimulating activity (Yoshizawa et al. 1995), and to exhibit moisturizing and whitening effects on melanoma cells (Kobayashi et al. 1997).

In this study, we describe the isolation and identification of an agar-degrading bacterium *Janthinobacterium* sp. SY12 from seawater, and the molecular cloning and characterization of a new agarase AgaY from the isolate.

Materials and methods

Isolation and identification of strain SY12

Strain SY12 was isolated from seawater collected from the coastal sea areas of Qingdao, China. Diluted seawater was

Y.-L. Shi · X.-Z. Lu · W.-G. Yu (✉)
Department of Molecular Biology, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China
e-mail: yuwg66@ouc.edu.cn

plated on solid medium containing 2.5% NaCl, 0.5% KCl, 0.2% NH_4Cl, 0.5% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 0.1% NaH_2PO_4, 0.02% $CaCl_2$, and supplemented with 1.5% agar at 25°C. The colonies were screened out if they showed the agarolytic activity (i.e., they caused clear degrading zone on solid medium). For liquid culturing of the selected bacteria, the concentration of agar was adjusted to 0.2%.

Biochemical characteristics of strain SY12 were determined using Biolog (BIOLOG, USA). The 16S rRNA gene of strain SY12 was amplified using the eubacteria specific primers 27F and 1522R, and sequenced. The sequence was compared with those 16S rRNA sequences deposited in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/).

Cloning of the *agaY* gene

All DNA manipulations and *E. coli* transformations were carried out using the protocols described by Sambrook and Russell (2001). Genomic DNA of strain SY12 was isolated using Wizard™ Genomic DNA Extraction Kit (Promega, USA) following manufacturer's instructions. Genomic DNA was digested with *Hin*dIII. The digestion products were inserted into the *Hin*dIII site of pBluescript II KS(+) and transformed into *E. coli* DH5α. A clone, pBS-Y, showing agarolytic activity was selected out. The insert of pBS-Y was sequenced and analyzed using Predict Protein software (http://www.embl-heidelberg.de/predictprotein/), and SignalP 3.0 Server (http://www.cbs.dtu.dk/services/signalp).

Expression and purification of the recombinant agarase AgaY

The *agaY* gene including its signal sequence was PCR-amplified from chromosomal DNA of *Janthinobacterium* sp. SY12 using the forward primer, 5′-GGAATTCCATATGAAAAATAACCTTTTGTTG-3′ and the reverse primer, 5′-CCGCTCGAGCTCAATCAGCTGAAAACGTTG3′, containing *Nde*I and *Xho*I sites (underlined), respectively. The PCR product was purified and digested by *Nde*I and *Xho*I, and cloned into the pET-24a (+) expression vector linearized with the same restriction enzymes. The resulting pET-24a-*agaY* was transformed into *E. coli* BL21(DE3) competent cells. The *E. coli* BL21(DE3) cells carrying the agarase gene (pET-24a-*agaY*) were cultured at 37°C to $OD_{600} = 0.6$ in LB medium supplemented with kanamycin (30 μg/ml), and then at 25°C for 24 h in the presence of 0.2 mM IPTG.

All procedures for enzyme purification were carried out at temperatures below 4°C. The culture supernatants were brought to 70% saturation with solid ammonium sulfate, and stirred overnight. The precipitate formed was resolved in 20 mM phosphate buffer (pH 7.0), loaded onto Sephadex™ G-25 column (HiTrap™ Desalting 5 × 5 ml, Amersham Biosciences, Sweden). The elution fractions with the enzyme activity were combined and loaded onto an immobilized metal ion affinity chromatography column (Ni Sepharose™ High Performance, HisTrap™ HP 5 × 1 ml, Amersham Biosciences, Sweden) pre-equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl (buffer A). After washing the column with the same buffer until the A_{280} absorbance reached the baseline, the enzyme was eluted with a 30-min linear gradient of 0–150 mM imidazole in buffer A. The fractions containing agarase activity were collected, and used as the final preparation of purified enzyme throughout the experiments. SDS-PAGE was performed to detect the purified enzyme as described by Laemmli (1970).

Enzyme activity assays

The activity of agarase was assayed using DNS (3,5-dinitrosalicylic acid) method (Miller 1959) with minor modifications as described by Ma et al. (2007). One unit of enzyme was defined as the amount of enzyme that produces an increment of 1 μmol of reducing ends per minute at 42°C and pH 7.0. Protein concentration was measured with Bradford method using bovine serum albumin (Sigma) as the standard.

Enzymatic properties of the recombinant AgaY

The optimum temperature for the activity of the recombinant enzyme was assayed with 0.25% agarose at pH 7.0 and different temperatures for 10 min. The optimum pH for the activity of AgaY was assayed with 0.25% agarose at 40°C and various pH for 10 min. The effects of metal ions and chelators on AgaY activity were examined by determining the activity at 25°C for 30 min in the presence of 2 mM of various ions or chelators. In addition, the substrate speciality of AgaY was detected at 40°C and pH 7.0 using 0.25% of agarose, agar, neoagaro-oligosaccharides, κ-carrageenan, ι-carrageenan, λ-carrageenan as the substrates, respectively.

Fluorophore assisted carbohydrate electrophoresis (FACE)

FACE was performed as described by Jackson (1990) and Yu et al. (2002) with some modification. Briefly, the products of agarose degraded by the agarase AgaY was modified by mixing 6 μl carbohydrate (10 μg) with 6 μl 0.2 mol/l ANDS (dissolved in 15% acetic acid) and 6 μl 1 mol/l $NaCNBH_3$ followed by incubation at 37−40°C for 10 h. The labeled oligosaccharide was mixed with an equal volume of 50% sucrose and separated on polyacrylamide gel buffered with the discontinuous Tris–glycine/Tris–HCl system.

Results

Identification of agar-degrading marine bacteria

Among the agar-degrading bacteria, strain SY12 was selected for further use because it showed the strongest agarolytic activity (i.e., it formed large and clear degrading zone around colony). SY12 exhibited 16S rDNA similarity levels of 99.52%, 99.39% and 99.33% to *Janthinobacterium lividum* DSM1522^T (Y08846), *Janthinobacterium lividum* (AF174648) and *Janthinobacterium lividum* GA01 (DQ473538), respectively. In addition, the substrate utilization of SY12 showed homology of 53% to that of *J. lividum* (DSM1522^T) and 46% to that of *J. agarcidamnosum* (DSM9628^T) (data not shown). Accordingly, the bacterium was identified as *Janthinobacterium* sp. SY12, which was the first reported agar-degrading bacterium of beta proteobacteria. The sequence of the 16S rDNA of *Janthinobacterium* sp. SY12 was deposited in GenBank with the accession number of EF455530.

Cloning of *agaY* gene from *Janthinobacterium* sp. SY12

A genomic DNA library of *Janthinobacterium* sp. SY12 was constructed and screened for agar-degrading recombinants. Positive colonies were selected according to the clear degrading zone around them on solid LB agar medium. The 5.3 kb *Hin*dIII fragment from positive clone pBS-Y was sequenced and an ORF of 1,338 bp was identified. The ORF encoded a protein of 445 amino acids with a predicted molecular weight of 50.2 kDa. The protein contained a 19 amino acid signal peptide, a β-agarase domain belonging to the GH16 family and a ricin-type domain belonging to carbohydrate binding module 13. An amino acid linker rich in proline and hydroxyamino acids formed a flexible hinge joining the two domains. Accordingly, the ORF was named as *agaY* (agarase encoding gene, GenBank accession number EF455531). Based on analysis of sequence similarity, this agarase (EC 3.2.1.81) was assigned to GH16 family. The catalytic domain showed 77% similarity to that of AgaA from *Pseudoalteromonas* sp. CY24 and that of AgaV from *Vibrio* sp. V134. The carbohydrate binding module showed 47% similarity to the CBM13 domain of AgaA from *Pseudoalteromonas* sp. CY24 and 46% to that of AgaD from *Vibrio* sp. PO-303.

Expression of and purification of the recombinant AgaY

The agarase AgaY with 6-His tag was overexpressed in pET-24a(+)/*E. coli* BL21(DE3) system. The recombinant enzyme was purified from the culture supernatant using affinity chromatography, with a specific activity of 837.11 U (mg purified protein)$^{-1}$ and a final yield of 19.77% (Table 1). Approximately 1.0 mg of recombinant AgaY was obtained from 1 l culture supernatant (The agarase detected in this experiment was not intracellular but extracelluar. It is because the intracellular agarase expressed mostly as inactive inclusion bodies in *E. coli*

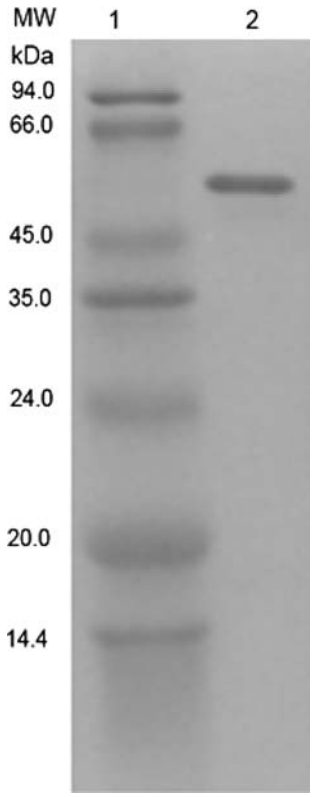

Fig. 1 SDS-PAGE analysis of the purified AgaY Lane 1, low molecular mass standards (in kDa). Lane 2, purified AgaY

Table 1 Summary of a typical purification of AgaY

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield[a] (%)	Purification (-fold)
Culture supernatant	134.77	4248.12	31.52	100	1.00
70% Ammonium sulfate	19.91	2937.91	147.55	69.16	4.68
Sephadex G-25	16.81	2832.14	157.89	66.67	5.01
Ni Sepharose HP	1.003	840.22	837.10	19.77	26.56

[a] Yield was defined as the recovery ratio of the initial activity of the enzyme

BL21(DE3)). The purified AgaY exhibited a single band with an apparent molecular weight of 50.2 kDa on SDS-PAGE (Fig. 1). This is in good agreement with the calculated value.

Enzymatic properties of the recombinant AgaY

The optimal temperature for activity of AgaY was around 40°C, as shown in Fig. 2. It retained its activity when the temperature was between 4 and 30°C for 30 min. The optimal pH of AgaY was pH 7.0. It was stable in the pH range from pH 6.0 to 8.0. The activity of AgaY was not affected by EDTA and some metal ions (Na^+, K^+ and Ca^{2+}) (Table 2). However, its activity was strongly inhibited by heavy metal ions, such as Pb^{2+}, Cu^{2+}, Hg^{2+}, Sn^{2+} and Zn^{2+}.

As shown in Fig. 3, AgaY hydrolyzed agarose to yield neoagarotetraose and neoagarobiose as the final products. AgaY did not degrade κ-, ι- and λ-carrageenans, which have the same backbones as agarose with substituted sulfoxy groups (data not shown). In addition, it could hydrolyze neoagaro-oligosaccharides ([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-galacto pyranosyl(1→3)-D-galactose]) comprising at least six sugars (data not shown).

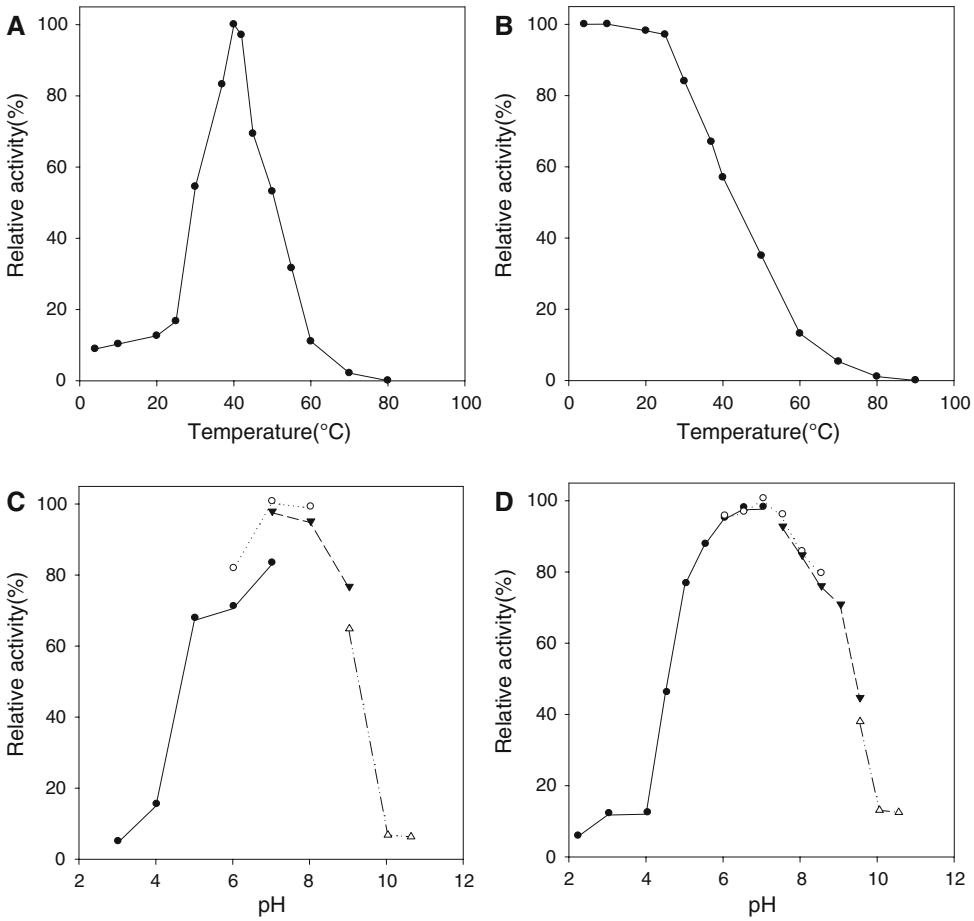

Fig. 2 Effect of temperature and pH on the enzyme activity and stability of AgaY (**a**) Effect of temperature on the enzyme activity of AgaY. (**b**) Effect of temperature on the enzyme stability of AgaY. (**c**) Effect of pH on the enzyme activity of AgaY. (**d**) Effect of pH on the enzyme stability of AgaY. The values on the ordinate are shown as percentages of the enzyme activity (100%) observed at 40°C and pH 7.0 after incubated for 30 min. AgaY used for the assays of the effect of temperature and pH on the enzyme stability was preincubated at various temperature and pH for 30 min, respectively. The optimum pH was determined with agarose as substrate dissolved in various buffers. The buffers were sodium phosphate-citric acid buffer, pH 2.2–7(closed circles), sodium phosphate buffer, pH 6–8(open circles), Tris–HCl buffer, pH 7.1-8.9(closed triangles), and Glycine–NaOH buffer, pH 8.6–10.6(open triangles)

Table 2 Effects of various reagents on the activity of AgaY

Reagents (2 mM)	Relative activity (%)	Reagents (2 mM)	Relative activity (%)	Reagents (2 mM)	Relative activity (%)
$AgNO_3$	12.30	$FeCl_3$	21.75	NaCl	100.13
$AlCl_3$	19.89	$FeSO_4$	74.58	$NiCl_2$	76.32
$BaCl_2$	92.98	$HgCl_2$	17.67	$PbCl_2$	13.77
$CaCl_2$	101.25	KCl	99.13	SDS	54.04
$CuCl_2$	13.82	$MgCl_2$	102.67	$SnCl_2$	23.42
EDTA	92.12	$MnCl_2$	59.71	$ZnCl_2$	13.34

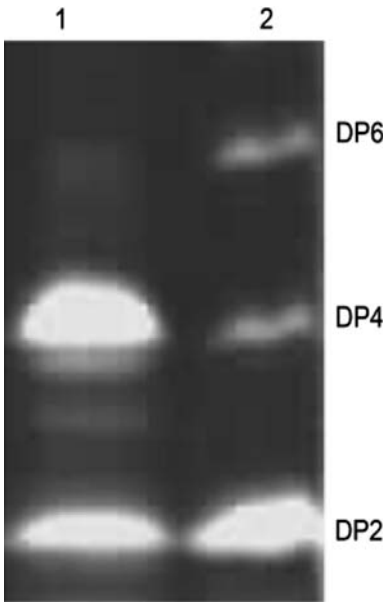

Fig. 3 FACE analysis of hydrolytic products of agarose by AgaY Lane 1, the products of agarose degraded by AgaY. Lane 2, neoagarobiose (DP2), neoagarotetrose (DP4), -hexaose (DP6) (Sigma) as the standard. The degrading product of agarose by AgaY was mainly examined with 0.04 U ml^{-1} of AgaY incubated at 37°C for 24 h

Discussion

Various agar-degrading bacteria of the phylum proteobacteria have been reported to date. Most of them belonged to the class gamma-proteobacteria, while only one belonged to alpha-proteobacteria (Hosoda and Sakai 2006). The majority of agarase-producing gamma-proteobacteria were isolated from marine environments, such as the species in genus *Pseudomonas* (Groleau and Yaphe 1977; Ha et al. 1997), *Alteromonas* (Leon et al. 1992; Kirimura et al. 1999), *Pseudoalteromonas* (Vera et al. 1998; Schroeder et al. 2003; Ma et al. 2007), *Vibrio* (Aoki et al. 1990; Sugano et al. 1993b) and *Microbulbifer* (Ohta et al. 2004a, b), etc.

In this study, *Janthinobacterium* sp. SY12 was isolated and identified, and it belonged to Betaproteobacteria. To the best of our knowledge, it is the first report of the agar-degrading bacteria of genus *Janthinobacterium* and AgaY is also the first reported agarase from Betaproteobacteria. AgaY of *Janthinobacterium* sp. SY12 displayed a modular structure composed of a catalytic module and one carbohydrate-binding module. The catalytic domain shares the conserved catalytic motif (E [ILV] D [IVAF] [VILMF] (0, 1) E) and the calcium binding sites with the agarases of GH-16. The recombinant agarase AgaY overexpressed in *Escherichia coli* displayed a molecular mass of 50.2 kDa and the optimum temperature and pH for the activity of the enzyme was 40°C and pH 7.0, respectively. It degraded agarose to give neoagarotetraose and neoagarobiose as the main end products.

The full-length deduced amino acid sequence of AgaY showed similarity to other known agarases that are members of the family GH-16. Agarase AgaY has identity of 63% and 62% with agarases from *Vibrio* sp. V134 and *Vibrio* sp. PO303 respectively, 37% with agarase from *Microbulbifer* sp. JAMB-A7, and 43% with AgaA from *Microbulbifer* sp. JAMB-A94, 41% and 42% with the two agarases from *Pseudomonas* sp. ND137. In comparison of the products of agarose hydrolysis by these agarases, agarase AgaV (51.7 kDa) from *Vibrio* sp. V134 degraded agarose to yield neoagarohexaose and neoagarotetraose as the final products (Zhang and Sun 2007), while AgaD (50.8 kDa) from *Vibrio* sp. PO303 degraded agarose to yield neoagarotetraose and neoagarobiose as the main end products (Dong et al. 2007). Agarase AgaA7 (48.9 kDa) from *Microbulbifer* sp. JAMB-A7(Ohta et al. 2004a) and AgaA (48.2 kDa) from *Microbulbifer* sp. JAMB-A94 (Ohta et al. 2004b), both degraded agarose and produced neoagarotetraose as the final product. In addition, the optimal pH for the enzyme activity of these agarases was ranged from pH 7.0 to 7.5, and the optimal temperature of the activity of the agarases was ranged from 40°C to 55°C. However, unlike the known agarases of GH-16, Ca^{2+} was not essential for the activity of AgaY. This distinguished AgaY from the known members of GH-16. Interestingly, in contrast to other agarases of GH-16, the enzymatic activity of AgaY is Na^{+} and Ca^{2+} independent. Moreover, agarase AgaA from *Vibrio* sp. JT0107 with a high molecular mass of 105.2 kDa showed maximal activity at pH 8.0 and 30°C and hydrolyzed not only agarose but neoagarotetraose to yield neoagarobiose (Sugano et al. 1993a). Based on the amino acid sequence similarity, agarase AgaA from *Vibrio* sp. JT0107 belonged to the family GH-50, its deduced amino acid sequence showed no homology to that of AgaY of GH-16 from *Janthinobacterium* sp. SY12. Above all, AgaY from *Janthinobacterium* sp. SY12 is new and unlike the other agarases reported earlier. Therefore, *β*-agarase AgaY will become a useful material for studying the structure, function and evolution of agarases.

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