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A β-agarase with high pH stability from *Flammeovirga* sp. SJP92

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ABSTRACT

A novel endo-type β-agarase, AgaB, was cloned from an agar-degrading bacterium, *Flammeovirga* sp. SJP92. The gene *agaB* consists of 2, 550 bp and encodes a protein of 849 amino acids including a 19 amino acids signal peptide. Based on the amino acid sequence similarity, AgaB belongs to the glycoside hydrolase family GH16. The recombinant AgaB was expressed in *Escherichia coli* and exhibited maximal activity at around 45 °C and pH 8.0, with a specific activity of 254.2 U/mg, a K_m of 3.99 mg/ml and a V_{max} of 700 U/mg for agarose. The agarase was stable at neutral to mildly alkaline condition, and remained 85%–90% of activity after treatment for 1 h, a characteristic much more different from other agarases reported. The recombinant enzyme was sensitive to some metal ions (Cu^{2+}, Co^{2+} and Zn^{2+}), but resistant to some denaturants (urea and SDS). It can hydrolyze the β-1, 4-glycosidic linkages of agarose, yielding neoagarotetraose and neoagarohexaose as the main products. These properties could make AgaB has a potential application in the food, cosmetic and medical industries.

1. Introduction

Agar is the main component of cell wall of red alga, which is composed of agarose and agaropectin. Agarose consists of alternating 1-4-linked-3, 6-anhydro-α-L-galactose and 1-3-linked β-D-galactose [1]. And agaropectin has a polysaccharide structure similar to that of agarose, except for its sulfate esters, pyruvate acetal and methyl ethers groups [2]. Agarase is a kind of glycoside hydrolase that catalyzes the hydrolysis of agarose. According to the cleave pattern, agarases are classified into α-agarase (3.2.1.158) and β-agarase (3.2.1.81). α-agarase hydrolyzes agarose at the α-1, 3 linkages and produces agaro-oligosaccharides that have 3, 6-anhydro-L-galactose residues at their reducing ends. While β-agarase hydrolyzes agarose at the β-1, 4 linkages and produces neoagaro-oligosaccharides with D-galactose residues at their reducing ends [3]. Nowadays, (neo)agaro-oligosaccharides have been found to exhibit various biological and physiological functions, such as moisturizing effect on skin [4], anti-inflammation [5], whitening effect on melanoma cells [6], inhibition of bacterial growth, and decrease of starch degradation [7].

Moreover, according to the classification of glycoside hydrolase (GH), β-agarase can be further assorted into GH16, GH50, GH86 and GH118 families [8]. Currently, most agarases, which belong to β-agarase, have been isolated and cloned from different microorganism, such as *Vibrio* sp. AP-2 [9], *Vibrio* sp. Strain V134, [10] *Alteromonas* sp. E-1, [11] *Bacillus* sp. MK03, [12] *Alteromonas* sp. SY37-12, [13] *Zobellia galactanivorans* Dsij [14], *Microbulbifer* sp. JAMB-A94 [4], archaea *Halococcus* sp. 119A [15], *Vibrio* sp. strain PO-303 [16] and so on. While only four α-agarases, encoded in *Pseudoalteromonas* sp. KJ 2–4, *Saccharophagus degradans* 2-40D [17] and *Thalassomonas* sp. JAMB-A33 [18], have been cloned and classified as a new family of GH96 [19].

Flammeovirga is a newly defined bacterial genera belonging to the family *Flammeovirgaceae* of the class Cytophagia. There are five species have been reported in this genera: *Flammeovirga aprica*, *Flammeovirga arenaria* [20], *Flammeovirga yaeyamensis* [21], *Flammeovirga kamogawensis* [22] and *Flammeovirga pacifica* [23]. All of the type strains have a potent ability to degrade marine complex polysaccharides (CPs), including agarose. Previously, a strain with high-producing agarase was isolated and identified as *Flammeovirga* sp. SJP92 in our lab (data no shown). In this study, a novel agarase gene, *agaB*, was successfully cloned from *Flammeovirga* sp. SJP92 and expressed in *Escherichia coli*. The purified recombinant agarase was analyzed and characterized. Owing to its high pH stability, AgaB is a good candidate for industrial application.

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2. Materials and methods

2.1. Bacterial strains and plasmids

Flammeovirga sp. SJP92 was isolated from the intestine of abalone and deposited in our lab. It was incubated in 2216E plate (2% NaCl, 0.3% $MgCl_2{\cdot}6H_2O$, 0.6% $MgSO_4{\cdot}7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.02% $NaHCO_3$, 0.03% $CaCl_2{\cdot}2H_2O$, 0.05% KCl, 0.042% KH_2PO_4, 0.005% NaBr, 0.002% $SrCl{\cdot}6H_2O$, 0.1% yeast extract, 0.5% tryptone) supplemented with 1.5% agar. After incubation at 30 °C for 2 days, colonies with clear zones were picked out and incubated in 2216E at 30 °C overnight. *E. coli* top 10 and *E. coli* BL21 (DE3) were used as the hosts for cloning and expression, which were routinely grown at 37 °C in Luria–Bertani (LB) broth supplemented with kanamycin (60 μg/mL) when required. A modified pET-28m (Novagen) was used for the construction of recombinant plasmid.

2.2. Sequence analysis of the agaB gene

Sequencing and annotation of the *Flammeovirga* sp. SJP92 genome were performed at the Beijing Novogene Bioinformatics Technology Co., Ltd in our preliminary work (data no shown). According to the result of genome analysis, the sequence of *agaB* was obtained. The nucleotide sequence of *agaB* was deposited in GenBank under accession number of KU382734. Gene sequence analysis was performed by the DNAMAN Software (Lynnon, USA). Prediction of signal peptide sequence was performed on the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). The alignment of protein sequences was performed with CLUSTALW (http://www.genome.jp/tools/clustalw/) and conserved domain search was conducted with blastp programs (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was constructed using the neighbor-joining (NJ) method by MEGA 6.0.

2.3. Cloning and expression of the recombinant agarase

The CTAB/NaCl method [24] was used for the extraction of chromosomal DNA of *Flammeovirga* sp. SJP92. The *agaB* gene without the signal peptide sequence was amplified from the genomic DNA of *Flammeovirga* sp. SJP92 by PCR using primers *agaB*-F (5′- CGGGATCCCAGGATTGGAGCGGTATT - 3′) and *agaB*-R (5′-CCGCTCGAGTTAGTTCATTAGTACCACTTT - 3′), which contained *Bam*H I and *Xho* I site (underline), respectively. The PCR-amplified DNA fragment was purified and digested with *Bam*H I and *Xho* I, and then cloned into the pET-28m. The resulting expression plasmid pET-28m-*agaB*, having the *agaB* gene with sumo and his tags at the N-terminus, was transformed into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) cells containing the recombinant plasmid were grown at 37 °C in LB medium supplemented with kanamycin (60 μg/ml) overnight. Then 15 ml culture were added to 1 L LB medium and grown at 37 °C until the cell density reached OD600 = 0.6–0.8. Subsequently the culture was induced with a final concentration of 0.4 mM isopropyl-β-D-galactopyranoside (IPTG). The cultivation was then incubated at 37 °C and 200 rpm for another 4–5 h.

2.4. Purification of the recombinant agarase

The recombinant agarase AgaB-sumo expressed in the inclusion body were harvested from the induced BL21 (DE3) cells by centrifugation at 9000 *g* for 10 min and then disrupted by ultrasonic (200 W, 5 s bursts and 55 s pulses for 5 min). The protein in the inclusion bodies was collected by centrifugation at 13,000 rpm for 10 min at 4 °C. The precipitate was dissolved in the denatured buffer (20 mM soudium phosphate, 0.5 M NaCl, 8 M Urea, 10 mM imidazole, pH 7.4) and purified with a Ni Sepharose™ 6 Fast Flow column (GE Healthcare, USA) according to the manufacturer's instructions. Protein refolding was done in the phosphate buffer (20 mM soudium phosphate, 0.5 M NaCl, pH 7.4) with a gradient decline of the urea concentration (6 M – 4 M – 3 M – 2 M – 1 M – 0.5 M – 0 M) at 4 °C. At last, sumo tag of the recombinant agarase AgaB-sumo was cleaved by sumo protease p19 in buffer A (150 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, pH 8.0) at 16 °C for 2 h. And then the mixture was further purified by the Ni Sepharose™ 6 Fast Flow column to remove the sumo tag.

2.5. Enzyme activity assay

The activity of agarase was assayed using 3, 5-dinitrosalicylic acid (DNS) method [25]. Briefly, 10 μl of the enzyme solution was mixed with 390 μl of 20 mM Tris-HCl buffer (pH 7.4) containing 0.3% (w/v) agarose, and the mixture was incubated at 40 °C for 30 min. Then 400 μl of DNS was added into the mixture and boiled for 10 min. After cooled down, this solution was measured at 540 nm. According to the standard curve of D-galactose, the quality of reducing agarose was obtained. Enzyme activity (1 U) was defined as the amount of enzyme that can liberate 1 μg D-galactose per minute.

2.6. SDS-PAGE and zymogram analysis

SDS-PAGE of the recombinant agarase was performed on 10% gel at 4 °C. After that, the gel was soaked in the Tris-HCl buffer (20 mM, pH 7.0) for 10 min to remove SDS, and the soaking buffer was replaced for 2 times. Then the gel was overlaid onto a sheet containing 1.5% (w/v) agarose in the same Tris-HCl buffer and incubated at 40 °C for 1–2 h. At last, the sheet (the gel was removed and then stained with Coomassie Brilliant Blue R-250) was stained by Lugol's iodine solution (5% I_2 and 10% KI in distilled water) to observe agarase activity.

2.7. Analysis of the properties of enzyme

The standard reaction system contained 10 μl enzyme solution and 390 μl of 20 mM Tris-HCl buffer (pH 7.4) containing 0.3% (w/v) agarose. The effect of temperature on enzyme was measured by incubating the standard reaction system under different temperatures (35 °C – 80 °C) for 30 min. Then the enzyme activity was assayed as mentioned in the Section 2.5.

For the thermal stability, the enzyme solutions were incubated at temperatures of 35 °C–80 °C for 1 h, respectively. After that the residual enzyme activities were measured in the standard reaction system.

The effect of pH on AgaB was assayed at 40 °C in a pH range of 4.5–11.0. In the standard reaction system, Tris-HCl buffer (pH 7.4) was replaced by NaOAc-HOAc buffer (pH 4.5–5.5), NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0–7.5), Tris-HCl buffer (pH 8.0–9.0) and Glycine–NaOH buffer (pH 9.5–11.0), respectively. And the agarase activity was measured as mentioned above.

The pH stability of AgaB was evaluated by incubating enzyme at different pH for 1 h at room temperature. Then the residual enzyme activities of AgaB were measured in the standard reaction system with DNS method.

2.8. Kinetic parameter of AgaB

10 μl of the enzyme solution was added to 390 μl of Tris-HCl buffer (20 mM, pH 8.0) containing different concentrations of agarose (0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 4.0 mg/L and 5.0 mg/L), respectively. Then the mixture was incubated at 45 °C for 15 min,