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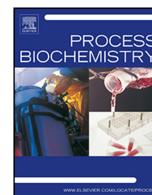

Expression and characterization of a thermostable and pH-stable β-agarase encoded by a new gene from *Flammeovirga pacifica* WPAGA1

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ABSTRACT

A novel β-agarase gene *aga4383* was cloned from *Flammeovirga pacifica* WPAGA1. The gene consists of 2898 bp and encodes a protein, designated as AgaP4383, with 965 amino acids. The DNA sequence of aga4383 has no significant sequence similarity with any known proteins, including all glycoside hydrolases. AgaP4383 shares a highest amino acid sequence homology of 41% with a putative β-agarase from *Agarivorans albus*. Phylogenetic analysis showed that AgaP4383 belongs to family 86 of glycoside hydrolases (GH86). The agarase gene was expressed in *Escherichia coli* and purified by affinity chromatography. The purified AgaP4383 showed endolytic activity on agar degradation, yielding neoagarotetraose and neoagarohexaose as the end products. The K_m values for agar and *Gracilaria lemaneiformis* were 8.53 and 32.41 mg mL^{-1}. The optimal temperature and pH for the recombinant AgaP4383 were 50 °C and pH 9.0, respectively. Notably, the enzyme exhibited good thermostability. No activity loss was observed after incubation at 50 °C for 10 h. The recombinant AgaP4383 showed a wide range of pH stability, retaining 90% of activity at pH 5.0–10.0 for 24 h at 30 °C. These beneficial characteristics of the enzyme provide some advantages for potential application in industry.

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1. Introduction

Agar, which is extracted from marine red algae such as *Gracilaria* and *Gelidium*, is a type of hydrophilic colloid. Because it shows a good performance pertaining to gelling, agar is widely used as gelling substance in scientific research and an additive in the food industry. Agar is a complex molecule consisting of agarose and agaropectin [1]. Agarose, which is the major gelling component of agar, is a neutral polysaccharide comprising a linear chain of alternating 3-O-linked β-D-galactopyranose and 4-O-linked 3, 6-anhydro-α-L-galactopyranose. Agaropectin is constituted by the same framework as agarose, except that some hydroxyl groups are substituted by sulfate, methoxyl, or pyruvate residues [2].

Agarases are the hydrolytic enzymes that catalyze the hydrolysis of agar. Based on their cleavage pattern, agarases are classified into α-agarase (EC 3.2.1.158) and β-agarase (EC 3.2.1.81). The α-agarase cleaves the α-L-(1,3) linkages of agarose to produce oligosaccharides of the agarobiose series with 3,6-anhydro-L-galactopyranose at the reducing end, whereas the β-agarase cleaves the β-D-(1,4) linkages of agarose to produce neoagarooligosaccharides with D-galactopyranose residues at the reducing end [3]. Until now, there have only a few α-agarases been reported [4–7]. However, a number of β-agarases have been isolated from microorganism, particularly, from marine bacteria such as *Pseudomonas* [8,9], *Pseudoalteromonas* [10,11], *Alteromonas* [12], *Cytophaga* [13], *Catenovulum* [14], *Bacillus* [15], *Agarivorans* [16], *Vibrio* [17], and *Flammeovirga* [18]. Most of the known β-agarases are grouped into glycoside hydrolase (GH) families 16, 50, 86, and 118 on the basis of the homology of amino acid sequences (http://www.cazy.org/).

The hydrolysis products of β-agarases were mainly neoagarooligosacchrides, which were reported to possess diverse physiological and biological functions and have potential applications in food, cosmetic, and medical industries [19–21]. In addition, β-agarases are useful tools for isolation of protoplasts from algae [22] and recovery of DNA from agarose gels [23]. Although dozens of β-agarases have been well studied, few of them have been commercially used. New β-agarases with superior properties have continuously attracted attention for commercial and research purposes.

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In our previous study, an agar-degrading bacterial strain of *Flammeovirga pacifica* WPAGA1 was isolated from the deep-sea sediment of the western Pacific Ocean [24]. To the author's knowledge, all the species identified within the genus of *Flammeovirga* are agarolytic bacterium. There have been two studies about agarase from *Flammeovirga*, which were AgaYT from *Flammeovirga yaemensis* strain YT [18] and agarase from *Flammeovirga* sp. MY04 [25]. However, only AgaYT, a GH16 β-agarase, was expressed and analyzed. In the current study, we cloned a novel β-agarase gene from *Flammeovirga pacifica* and expressed it into *E. coli*, which exhibited noticeable thermostability and a broad range of pH stability.

2. Materials and methods

2.1. Construction of the aga4383 recombinant strain

Flammeovirga pacifica WPAGA1 isolated from deep-sea sediment was cultured as described previously [24] and used for the extraction of genomic DNA. The *aga4383* gene was amplified from the genomic DNA of *F. pacifica* by using primers *aga4383*-F (5′-TCATCATATGCAAGATTGGGCACAAATTCC-3′) and *aga4383*-R (5′-CGACAAGCTTTTATTCTTTGATAATCCTCTG-3′). The underlined nucleotides in *aga4383*-F and *aga4383*-R indicate *Nde*I and *Hin*dIII sites, respectively. The PCR-amplified DNA fragment was then ligated into the pMD-18T vector (Takara, Japan) and transformed into *E. coli* DH5α. The resulting plasmid, pMD-*aga4383*, was subjected to DNA sequencing by Invitrogen Co. Ltd. China. Plasmid pMD-*aga4383* was further digested with *Nde*I and *Hin*dIII and the restricted fragment was subcloned into the pColdII expression vector. The resulting expression plasmid pColdII-*aga4383*, having the *aga4383* gene with His-tag at the N-terminus, was transformed into *E. coli* BL21 (DE3).

2.2. Expression and purification of the recombinant AgaP4383

The recombinant cells were grown at 37 °C in LB medium supplemented with 100 μg mL^{-1} ampicillin. Recombinant cells were induced at 0.4–0.6 OD_{600} with 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG). After a further incubation at 16 °C for 10 h, the culture was centrifuged at 8000 × *g* for 10 min, and the precipitate was re-suspended in 20 mM PBS buffer (pH 7.4) and disrupted by ultrasonic (290 W, 2.5 s bursts and 7.5 s pulses for 10 min). The supernatant was collected as crude enzyme by centrifugation at 15,000 × *g* and 4 °C for 30 min. The soluble fraction was subjected to purification under non-denaturing conditions with Ni^{2+}-NTA resin according to the manufacturer's protocol (Thermo scientific, Rockford, USA), and the purification was examined by SDS-PAGE. The concentration of purified proteins was measured using the BCA Protein Assay Kit (Thermo Scientific, USA), employing bovine serum album (BSA) as standard.

2.3. Native-PAGE and zymogram analysis

Native-PAGE of the purified recombinant agarase solution was performed on 6% gel at 4 °C. The description of the zymogram analysis of the agarase after native-PAGE is described below. The gel was soaked in Tris–HCl buffer (50 mM, pH 7.0) for 5 min. Then the gel was overlaid onto a sheet containing 1.5% (*w*/*v*) agar in Tris–HCl buffer (50 mM, pH 7.0) and incubated for 30 min at 37 °C. The gel was removed from the agar sheet and stained for proteins with Coomassie Brilliant Blue R-250. The agar sheet was flooded with Lugol's iodine solution to visualize agarase activity.

2.4. Enzyme activity assay

The activity of agarase was assayed by detecting the release of the reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method [26] with a slight adaptation. The standard reaction contained 100 μL enzyme solution and 900 μL of PBS buffer (pH 7.4) containing 0.2% (*w*/*v*) melted agar (BIOSHARP, Japan). After incubation at 40 °C for 5 min, the reaction was stopped by immersion in boiling water for 5 min. The heat-inactivated enzyme was used as a negative control. Following this, 250 μL of the reaction solution was mixed with 750 μL of DNS reagent and heated for 10 min in a boiling water bath and then cooled. The absorbance of reducing sugar was measured at 550 nm and compared with the standard curve of D-galactose. Enzyme activity (*U*) was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute under the assay conditions.

2.5. Characterization of agarase AgaP4383

The characteristics of the recombinant AgaP4383 below were determined using agar as a substrate. Temperature effects were studied at PBS buffer (pH 7.4) in the range of 0–80 °C. The effects of pH on agarase activity were assayed at 50 °C in citric acid–Na_2HPO_4 (pH 3.0–8.0), 50 mM Tris–HCl buffer (pH 7.1–9.0) and 50 mM glycine–NaOH (pH 8.6 to 10.6). The optimal temperature and pH were set as standard conditions.

The thermostability of AgaP4383 was determined by measuring the residual activities of enzyme that was pre-incubated at 20–60 °C with the interval of 10 °C at the optimal pH within or without 10 mM Ca^{2+} for different periods of time. The pH stability was evaluated by pre-incubating the agarase solution in the aforementioned buffers for 24 h at 30 °C, and the residual enzyme activity was assessed under the standard conditions.

The effects of various metal ions, chelators, denaturants, and reducing reagents on the activity of AgaP4383 were determined by pre-incubating the enzyme with each reagent at 4 °C for 30 min. Following this, the residual enzyme activity was assessed under the standard conditions.

Kinetic parameters of AgaP4383 toward agar were determined under the standard conditions. K_m and V_{max} for the substrate were calculated using a Lineweaver–Burk double reciprocal plot.

2.6. Hydrolysis products analysis of recombinant AgaP4383

The hydrolysis products of recombinant AgaP4383 were analyzed using thin-layer chromatography (TLC) as described by Ohta et al. [27]. The purified agarase was incubated with 0.2% agar in Tris–HCl buffer (50 Mm, pH 7.0) at 40 °C. Samples from the reaction were taken at different time points and enzyme activity was stopped by heating in a boiling water bath for 5 min. The polysaccharides in the mixture were removed by ethanol precipitation and centrifugation at 4 °C for 30 min. Following this, the supernatants were concentrated and applied to Silica Gel 60 TLC Plates (Merck, Darmstat, Germany). The plates were developed using a solvent system composed of *n*-butanol/acetic acid/water (2:1:1, *v*/*v*/*v*). The spots were visualized by spraying 10% (*v*/*v*) H_2SO_4 and heating at 100 °C. Neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose were used as standards.

2.7. Sequence analysis tools and accession number

The *aga4383* gene was analyzed by a Basic Local Alignment Search Tool (BLAST) server and the conserved domain search was performed by the NCBI Conserved Domain Database. Multiple sequence alignment was performed using the biological software

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