Characterization and overexpression of a glycosyl hydrolase family

16 β-agarase Aga0917 from Pseudoalteromonas fuliginea YTW1-15-1

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Yan Wang, Tingwei Liu, Shuai Guo, Peng Zhang, Pengyang Sun, Mengqian Chen, Hong Ming

Synthetic Biology Engineering Lab of Henan Province, School of Life Sciences and Technology, Xinxiang Medical University, 601 Jinsui Avenue, Xinxiang 453003, P. R. China

Author for correspondence:
Yan Wang, School of Life Sciences and Technology, Xinxiang Medical University, 601 Jinsui Avenue, Xinxiang 453003, P. R. China, Tel/Fax: +86-373-3831677, Email: wangyan19820527@163.com
Abstract A gene (aga0917) encoding a putative β-agarase was identified from the genome of Pseudoalteromonas fuliginea YTW1-15-1. The nucleotide sequence analysis revealed that aga0917 had significant homology to the agarase genes of the GH16 family. aga0917 encodes a putative protein of 290 amino acids with an estimated molecular mass of 32.5 kDa, including a 21–amino acid signal peptide. A gene fragment encoding only the putative mature form of Aga0917 (269 amino acids) was overexpressed in Escherichia coli BL21 (DE3) pLysS as a 6×-histine-tagged fusion protein (rmAga0917). The $K_m$, $V_{max}$, and $k_{cat}$ for agarose of rmAga0917 were 39.6 mg/mL, 334 (U/mg) of protein, and 178 (1/s), respectively. According to the results of thin-layer chromatography and mass spectrometry analysis, the main end product from agarose with rmAga0917 was neoagarotetraose, in addition to a small amount of neoagarobiose. Notably, the recombinant protein rmAga0917 showed optimum activity at 60°C and retained approximately 100% agarolytic activity after being kept at 40°C for 1 h and 57% residual activity after incubation at 50°C for 1 h. The rmAga0917 exhibited maximum agarase activity at pH 6.0, and retained more than 80% of activity after incubation over a range of pH 4.0–9.0 for 1 h at 4°C.

Keywords Aga0917  β-Agarase  Characterizations  Neoagarotetraose  Pseudoalteromonas fuliginea  Thermostability

Introduction

Agarose occurs naturally in the form of a pseudocrystalline matrix associated with cellulose in the cell wall of red seaweeds (agarophytes) and represents up to 70% of the cell wall polymers (Pluvinage et al. 2013). Agarose is widely used as a texturing and gelling agent in the food and medical industries as well as for numerous chromatographic and electrophoretic techniques, owing to its jellifying properties (Liu et al. 2014). Agarose is a linear polysaccharide comprising the repeating disaccharide subunits of neoagarobiose [3,6-anhydro-L-galactose-α-(1,3)-D-galactose] that are β-(1,4)-linked (Fig.1) (Fu and Kim, 2010). As one of the most abundant
carbohydrates in the ocean, its hydrolysis is fundamental for carbohydrate cycling and efficient utilization of the marine primary biomass (Pluvinage et al. 2013).

Many marine microbes have evolved multiple enzymes to consume agarose as a source of energy and carbon. Agarases are known to play the most important role in the agarose enzymatic hydrolysis process. Agarose can be specifically hydrolyzed by agarases, which is a kind of glycoside hydrolase (GH). The agarase family is classified into two groups, α-agarases (E.C. 3.2.1.158) and β-agarases (E.C. 3.2.1.81), based on its mode of action. α-Agarases specifically cleave α-1,3 linkages to produce agaro-oligosaccharides with 3,6-anhydro-L-galactose residues at their reducing end, whereas the β-agarases hydrolyze β-1,4 linkages to produce neoagaro-oligosaccharides that have D-galactose at their reducing end (Fig. 1) (Lee et al. 2013).

During the last decade, numerous agarases have been identified from the microorganisms of various sources (Fu and Kim 2010). All agarases that have been characterized to date belong to β-agarases, except for two α-agarases, AgaA from Alteromonas agarlyticus GJ1B and AgaA33 from Thalassomonas agarivorans JAMB-A33, which were included in the glycoside hydrolase family 96 (GH96) in the Carbohydrate-Active EnZymes (CAZy) database (Potin et al. 1993; Ohta et al. 2005). In contrast to α-agarases, β-agarases were identified from microorganisms with taxonomically diverse genera, including Streptomyces (Temuujin et al. 2012), Zobellia (Jam et al. 2005), Pseudoalteromonas (Oh et al. 2010), Pseudomonas (Morrice et al. 1983), Agarivorans (Hu et al. 2009), Alteromonas (Wang et al. 2006), Microscilla (Zhong et al. 2001), Pseudozobellia (Nedashkovskaya et al. 2009), Saccharophagus (Ekborg et al. 2006), Vibrio (Fu et al. 2008), and Catenovulum (Cui et al. 2014), etc. On the basis of the amino acid sequence similarity, β-agarases are classified into four distinct families of GH16, GH50, GH86, and GH118 in the CAZy database (Chi et al. 2012). At the time of writing, the GH50, GH86, and GH118 families consisted of β-agarases with 476, 94, and 8 members, respectively, of which 22, 8, and 3 members have been characterized (http://www.cazy.org). The GH16
family is the most abundant and contains more than 6025 members, including 207
categorized members, which are functionally heterogeneous. e.g., endo-glucanase,
licheninase, κ-carrageenase, endo-galactosidase, xyloglucanase, and
xyloglucosyltransferase.

Agarases have been successfully applied in biotechnology for DNA purification
and algae protoplast preparation (Fu and Kim, 2010). Recently, it has been proposed
that agarases have potential applications in food and nutraceutical industries for the
industrial production of agar-derived oligosaccharides, which exhibit various
physiological and biological activities beneficial to the health of human beings (Wang
et al. 2004). Thus, seeking agarases with high stability is of importance for
commercial purposes.

The agarase-producing bacterium *Pseudoalteromonas fuliginea* YTW1-15-1 was
isolated from seawater of the Yellow Sea, China. In the present study, a putative
agarase gene (*aga0917*) from *P. fuliginea* YTW1-15-1 was cloned and heterologously
expressed in *Escherichia coli*. The purified recombinant agarase was also
characterized.

**Material and methods**

*Bacterial strains and growth conditions.* The agarase-producing bacterium *P. fuliginea* YTW1-15-1 was isolated from seawater and cultured at 28°C in the marine
broth 2216 (Becton Dickinson, USA). The vectors of cloning and expression were
pMD19 (Simple) (TaKaRa, Japan) and plasmid pET28a (+) (Novagen, USA),
respectively. The hosts for cloning and expression, *E. coli* DH 5α (New England
Biolabs, USA) and *E. coli* BL21 (DE3) pLysS (Novagen), were routinely grown at
37°C in the Luria-Bertani (LB, Becton Dickinson) broth supplemented with
ampicillin or kanamycin (100 µg/mL) when required.

*Isolation and identification of agarolytic bacterium P. fuliginea YTW1-15-1.*
Seawater sample (collected from a coastal region of the Yellow Sea, China, in November 2013) was spread on marine agar 2216 (MA; Becton Dickinson) and incubated at 28°C. Strain YTW1-15-1 was purified by streaking three times on MA. Cultures were maintained at 28°C on MA plates and stocks were preserved in sterile 0.85% (w/v) saline supplemented with 15% (v/v) glycerol at -80°C.

Strain YTW1-15-1 was identified according to the phylogenetic framework based on analysis of the nucleotide sequence of the small ribosomal subunit RNA. Polymerase chain reaction (PCR) was performed to amplify the 16S rDNA coding region of strain YTW1-15-1, using the universal primers (B8F, 5’-AGAGTTTGATCCTGGCTCAG-3’; B1510, 5’-GGTTACCTTGTTACGACTT-3’). PCR was carried out according to the following program: predenaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The PCR products were subcloned into a pMD-18T vector (TaKaRa) and sequenced. The 16S rDNA gene sequence was aligned with available sequences in the NCBI GenBank using BLAST. The phylogenetic tree of strain YTW1-15-1 was constructed using the biological software MEGA7 (Kumar et al. 2016).

**Sequence analysis of the Aga0917 gene.** The amino acid sequence was predicted using the software BioEdit (Ibis Biosciences, USA). The homology searches of the full-length amino acid sequence were performed using the Protein Basic Local Alignment Search Tool (BLASTP) available on the National Center for Biotechnology Information (NCBI) website. The signal peptide of Aga0917 was predicted by the SignalP 4.0 server (Petersen et al. 2011). The ClustalW tool was used as multiple alignments of agarase amino acid sequences from different species (Sievers et al. 2011).

**Construction of recombinant plasmids with Aga0917 gene.** *P. fuliginea* YTW1-15-1, which can degrade agar, was isolated from seawater of the Yellow Sea, China. A putative agarase gene (*aga0917*) was identified in the draft genome of *P.*
fuliginea YTW1-15-1. Genomic DNA was extracted from the strain YTW1-15 using a Bacterial Genomic DNA Extraction Kit (TaKaRa). The Aga0917 gene (GenBank accession No. KY026618) excluding its signal sequence was amplified by polymerase chain reaction (PCR) using the primer sets F0917 (5′-CGCGGATCCGCAGATTGGGACGCATATAG-3′) and R0917 (5′-CCGCTCGAGTTAGTTTGCTTTGTAACACG-3′), containing BamHI and XhoI sites (underline), respectively. The PCR products with an appropriate size (810 bp) were gel purified using the TaKaRa agarose gel DNA purification kit and subsequently ligated into the pMD19 (Simple) vector. The plasmid was transformed into E. coli DH 5α competent cells. The cloned Aga0917 gene was sequenced for confirmation.

The recombinant plasmid, pMD19/rmAga0917, was digested with BamHI and XhoI restriction endonucleases, and the product was purified and inserted into the linearized plasmid, pET-28a (+), generating pET-28a (+)/rmAga0917 for the expression of a fusion protein with an N-terminal 6-His tag. Subsequently, pET-28a (+)/rmAga0917 was transformed into E. coli BL21 (DE3) pLysS competent cells.

Overexpression and purification of the recombinant agarase rmAga0917. The transformant BL21 (DE3) pLysS/pET-28a (+)/rmAga0917 was cultured at 37°C in the LB broth supplemented with kanamycin (100 µg/mL). When cultures reached the mid-exponential stage of growth, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.05mM and further incubated at 16°C, 170 rpm for 16 h.

The induced cells were harvested by centrifugation at 5000 rpm for 15 min and disrupted by sonication on the ice, then again centrifuged at 12,000 rpm for 30 min. The His-tagged agarase was purified by nickel-affinity chromatography (6-His FastFlow, GE, USA). The eluent was dialyzed using 50mM Tris-HCl, pH 8.0, for 12 h. The protein concentration was determined by the Bradford method, with bovine serum as the standard.

Zymogram analysis. The purified rmAga0917 sample was analyzed using sodium
dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For zymogram analysis, an unboiled rmAga0917 sample was loaded onto the gel. After electrophoresis, the gel was soaked in 2.5% (v/v) Triton X-100 for 0.5 h and washed three times with distilled water to remove the SDS. Then, the gel was overlaid onto a plate containing 2% agar, incubated at 37°C for 5 h, and subsequently stained with 2% (w/v) iodine solution. The agarase activity was visualized as a clear zone on a brown background. Moreover, another copy of the gel was stained with Coomassie Brilliant Blue R-250 to visualize the protein bands. Finally, the agarase activity of rmAga0917 was determined by comparing the zymogram and the gel.

**Agarolytic activity assay of rmAga0917.** The agarolytic activity of rmAga0917 was determined by the modified 3,5-dinitrosalicylic acid (DNS) method (Temuujin et al., 2011). The standard assay conditions were as follows: citric acid–sodium citrate buffer (0.1 M, pH 6.0) containing 0.25% (w/v) agarose (molecular mass of 10 kDa; Regular Agarose G-10, Biowest) was used as a substrate; 900 µL of substrate warmed to 40°C was mixed with 100 µL of diluted enzyme solution. After incubating at 40°C for 30 min, 1 mL of the DNS reagent solution was added to the reaction mixture and boiled for 7 min. The test tube was cooled for terminating the reaction, and 1 mL of distilled water was added. The absorbance was measured at 540 nm against a reagent blank, in which 100 µL of heat-inactivated enzyme was used instead of the enzyme solution. D-galactose was used as the reference reducing sugar for preparing the standard curve. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol of D-galactose per minute under standard assay conditions. All enzymatic assays were performed in triplicate.

**Biochemical and kinetic characterization of rmAga0917.** The optimal temperature of rmAga0917 was determined over a temperature range of 30°C–80°C for 30 min. The thermostability was determined by monitoring the residual agarase activity of rmAga0917 after incubating the enzyme at temperatures ranging from 4°C to 70°C for 1 h. The optimal pH of rmAga0917 was assayed with a pH range of 4.0–10.0 (at 1.0 interval). The buffers, 100 mM citric acid/sodium citrate (pH 4.0–6.0), 20 mM
Tris-HCl (pH 6.0–9.0), and 50mM Tris-glycine (pH 9.0–10.0), containing 0.25% (w/v) agarose were used as substrates. The pH stability of rmAga0917 was determined by preincubating rmAga0917 in the buffers with a pH range of 4.0–10.0 at 4°C for 1 h, followed by measuring the residual enzymatic activity. The maximum agarolytic activity was considered 100% when calculating the relative activities.

Various metal ions and agents with the final concentrations of 1mM and 10mM were added to the reaction mixture to assess the effect of metal ions, chelators, and denaturants on the enzyme activity of rmAga0917. The agarase activity of rmAga0917 was measured after incubating for 30 min. The relative activity in the absence of any additives was taken as 100%. All the aforementioned characterization assays for rmAga0917 were carried out in triplicate.

The $K_m$ and $V_{max}$ values for rmAga0917 were determined with various amounts of agarose ranging from 0.5 to 5 mg/mL and determined by linear regression analysis of Lineweaver–Burk double-reciprocal plots with initial velocity data obtained under the aforementioned standard conditions.

**Identification of hydrolysis products from rmAga0917.** The hydrolysis reactions were conducted at 40°C in 20mM Tris-HCl buffer (pH 6.0) containing rmAga0917 and 0.5% agarose. Aliquots of samples from the reaction were withdrawn at different time intervals (5 min, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h). The reactions were terminated by heating the mixture in boiling water for 5 min. Subsequently, the reaction solution was loaded onto the silica gel 60 thin-layer chromatography (TLC) plates (Merck, Germany). The plates were developed using $n$-butanol/acetic acid/water (2:1:1, v:v:v) as a solvent system. The oligosaccharide spots were visualized by spraying a modified diphenylamine–aniline reagent and heating at 100°C for 10 min (Lu et al. 2009).

The reaction mixtures were incubated for 24 h and then centrifuged (12,000 rpm, 10 min, 4°C) to remove the remaining agarose to identify the final agarose degradation products by rmAga0917. The supernatant was loaded onto silica gel 60
TLC plates. One quarter of the plate was visualized as mentioned earlier. The unstained regions corresponding to the hydrolysis products spots were scraped out from the TLC plates and dissolved in the mixture of hexanenitrile and 1mM NH$_4$HCO$_3$ (1:1, v:v). The molecular mass of the products was determined by an ion trap mass spectrometer (Agilent 6530 Accurate-Mass Q-TOF, Agilent, USA).

**Results**

**Isolation and identification of agarolytic bacterium *P. fuliginea* YTW1-15-1**

A marine bacterium, designated as YTW1-15-1, which formed apparent soft pits on the agar plates was purified. To identify this bacterium, PCR was employed to amplify the 16S rDNA coding region using the universal primers B8F and B1510. The PCR product was cloned into a pMD-18T vector. The nearly complete 16S rDNA sequence (1497 nt) of strain YTW1-15-1 was submitted to GenBank with nucleotide accession number KU995300. A BLASTN search using 16S rDNA sequence of YTW1-15-1 placed it among members of the family *Pseudoalteromonas*. The closest phylogenetic neighbour of strain YTW1-15-1 was the newly reclassified species *P. fuliginea*, which showed 99.7% 16S rDNA sequence similarity with YTW1-15-1. The phylogenic relationship between strain YTW1-15-1 and representatives of bacterial species in the genus *Pseudoalteromonas* was reconstructed using the neighbor-joining (NJ) method by analyzing their 16S rDNA sequences. Strain YTW1-15-1 formed a distinct cluster with *P. fuliginea* (Fig.2). On the basis of phylogenetic inference, strain YTW1-15-1 is assigned to *P. fuliginea*.

**Sequence analysis of the Aga0917 gene**

On the basis of gene prediction, an 873-bp open reading frame (GenBank accession No. KY026618, designated as *aga0917*), encoding a putative protein composed of 290 amino acids with an estimated molecular mass of 32.5 kDa and a putative isoelectric point of 6.30, was identified in the genome of *P. fuliginea* YTW1-15-1.
A putative signal peptide of 21 amino acids, with a cleavage site between Ala21 and Ala22, was detected with the SignalP 4.0 server. Sequence analysis using the BLASTP search in the NCBI database showed that Aga0917 was placed in the β‐agarase group. It showed 85.9% and 85.2% identity to agarase AgrP from *Pseudoalteromonas* sp. AG4 (ADD60418) and agarase I from *P. atlantica* (AAA91888), respectively; both enzymes belonged to the GH16 family. The conserved domain analysis demonstrated that the deduced protein only contained a 268–amino acid catalytic module of the GH16 family, immediately following the signal peptide, without the carbohydrate-binding module (CBM).

**Expression and purification of the recombinant Aga0917**

A gene fragment encoding a mature form of the protein without the signal peptide sequence (Met1–Ala21) (referred to as rmAga0917) was cloned into the plasmid pET-28a (+) and overexpressed in *E. coli* BL21 (DE3) pLysS cells as a 6×histine-tagged fusion protein. After induction with IPTG, the agarolytic activity and a strong protein band with an approximate molecular mass of 32 kDa (corresponding to the estimated size of 6×histine-tagged Aga0917 fusion protein) were detected in the supernatant of the cell lysate. The 6×histine-tagged rmAga0917 fusion protein was purified on a nickel-affinity column. The purified enzyme was then subjected to SDS-PAGE and a parallel zymogram analysis. A single band and a clear zone, corresponding to rmAga0917 of approximately 32 kDa, were detected in SDS-PAGE and zymogram analysis, respectively (Fig. 3). This suggested that the purified protein was an agarase.

**Biochemical and kinetic characterization of rmAga0917**

The effects of temperature and pH on the activity of rmAga0917 were evaluated by measuring the relative activity. The rmAga0917 exhibited activity over a broad temperature range from 30°C to 70°C with the optimum at 60°C (Fig. 4A). Although Aga0917 was from the marine environment, rmAga0917 exhibited excellent thermostability at medium-high temperatures. It retained approximately 100%
agarolytic activity after being kept at 40°C for 1 h and 57% residual activity after incubating at 50°C for 1 h (Fig. 4A).

The rmAga0917 showed a medium pH profile, exhibiting maximum agarase activity at pH 6.0, and retained more than 80% of activity after incubation over a range of pH 4.0–9.0 for 1 h at 4°C (Figs. 4B and 4C). However, the relative activities rapidly declined at both acidic (pH 4.0) and basic (pH 10.0) conditions.

The effects of various additives, including metal ions, on the activity of rmAga0917 are summarized in Table 2. Among the 1mM additives tested, the agarase activity was enhanced or slightly enhanced by Na⁺, K⁺, Ca²⁺, Zn²⁺, Co²⁺, Mg²⁺, and EDTA, while it was moderately inhibited by SDS and strongly inhibited by Fe³⁺. Among the 10mM additives tested, the agarase activity was strongly inhibited by Fe³⁺, Zn²⁺, and SDS, while it was moderately inhibited by Ca²⁺ and Co²⁺. Thus, some metal ions, such as Ca²⁺, Co²⁺, and Zn²⁺, increased the agarase activity at a low concentration (1mM) but showed a negative effect at a high concentration (10mM) (Table 1).

All enzymatic reactions for calculating $K_m$ and $V_{max}$ values were performed under optimal conditions, for example, in citric acid–sodium citrate buffer (pH 6.0) at 60°C. The $K_m$, $V_{max}$, and $k_{cat}$ for agarose were 39.6 mg/mL, 334 (U/mg) of protein and 178 (1/s), respectively.

Analysis of hydrolysis products

TLC analysis was performed to separate the time-dependent hydrolysates to investigate the hydrolysis modes of rmAga0917. As shown in Fig.5, rmAga0917 could hydrolyze agarose to generate products with different degrees of polymerization at the first five minutes. As the reaction time progressed, the large oligosaccharides disappeared, and the number of small oligosaccharides increased. After hydrolysis for 12 h, spot 1 was observed. After 24 h, two major spots, 1 and 2, were detected. The decline of the polymerization degree of products over the time course, which is a characteristic feature of endo-agarase, implied that rmAga0917 was an endo-type
β-agarase. The hydrolysis products of the 24-h enzymatic reaction were further analyzed using mass spectrometry to identify the final products. The result revealed that the main product spot 2 has a molecular ion at $m/z$ of 629.1 [M–H]− and 665.0 [M+Cl]−, which was assigned to be neoagarotetraose (DP4). Moreover, spot 1 was assigned to be as neoagarobose (DP2) ($m/z = 359.0$ [M+Cl]−). The results of TLC and mass spectrometry analysis suggested that the main end product from agarose with rmAga0917 was DP4, in addition to a small amount of DP2, which was similar to other β-agarases (Oh et al., 2010; Han et al., 2013).

**Discussion**

In this study, a β-agarase gene *aga0917*, that encoded a 290-amino-acid protein, was cloned and expressed. The deduced protein only contained a 268–amino acid catalytic module of the GH16 family, immediately following the signal peptide, without the carbohydrate-binding module (CBM). Although most of the agarase members from all the mentioned families carried a CBM (Boraston et al., 2004), some β-agarases without CBM have also been reported, such as Aga4436 from the *Flammeovirga* sp. OC4 and β-agarase I from the *Microbulbifer thermotolerans* JAMB-A94 (Chen et al. 2016; Alkotaini et al. 2016). CBM was reported to bring the catalytic domain into close contact with the polysaccharide structure (Boraston et al. 2004). According to the kinetic characterizations, the $K_m$ value of rmAga0917 was remarkably higher than that of other known β-agarases, which might be explained by the absence of CBM.

Eleven conserved active residues and three catalytic residues were identified in the catalytic module, which was consistent with agarases belonging to the GH16 family (Chen et al. 2016; Cui et al. 2014; Allouch et al. 2003). In the three conserved catalytic residues, Glu148 and Glu153 act as nucleophile and acid residues, respectively, while the acidic residue Asp150 is probably important to maintain the charges in the environment of catalytic amino acids (Allouch et al. 2003). BLASTP search results showed that Aga0917 showed the highest amino acid sequence
similarities with agarase AgrP from *Pseudoalteromonas* sp. AG4 (ADD60418) and agarase I from *P. atlantica* (AAA91888). Both of these enzymes belonged to the GH16 family. Thus, on the basis of the amino acid sequence and conserved domain analysis, Aga0917 represents a β-agarase belonging to the GH16 family.

It was reported that a conserved calcium-binding site existed in GH16 β-agarase and the calcium ions could stabilize the enzyme (Allouch et al. 2004; Keitel et al. 1994). A conserved calcium-binding site (Asp 23, Asn 49, and Asp 282) was also identified in the Aga0917 amino acid sequence by sequence analysis. In addition, the agarase activity of rmAga0917 was enhanced by Ca$^{2+}$ with a low concentration (1mM), suggesting that Ca$^{2+}$ stabilized the structure of rmAga0917.

To date, few of the reported agarases have been characterized to be most active up to 60°C, except YM01-3 from *Catenovulum agarivorans* YM01$^T$ (Table 1) (Cui et al. 2014). Thus, the optimal temperature of rmAga0917 was significantly higher than that of most other reported agarases. Recently, attention has been focused on neoagaro-oligosaccharides because of their physiological and biological activities (Chi et al. 2012). However, the industrial production of neoagaro-oligosaccharides is limited by the easily jellifying properties of agarose. The application of thermostable agarase to produce oligosaccharides from agar or marine algae make it possible to conduct the process at a temperature higher than the gelling temperature of agar (around 40°C) (Ohta et al. 2004). The higher reaction temperature will accelerate the mixing and pumping by improving the solubility of the substrates (Lin et al. 1998). Thus, the thermostable agarase rmAga0917 can be vital in the industrial production of oligosaccharide.

In conclusion, a β-agarase gene *aga0917* was cloned, expressed, and characterized. The gene belonged to the GH16 family of agarases with 873 nucleotides and encoded a 290-amino-acid protein. Although the agarase Aga0917 showed a similarity to several other known agarases, such as AgaP from *Pseudoalteromonas* sp. AG4 (Oh et al. 2010) and β-agarase I from *P. atlantica* (Morrice et al. 1983), whose highest optimal temperatures for the agarolytic activity were 55°C and 30°C, respectively, the
optimal temperature of the agarase Aga0917 was 60°C. Thus, they were different agarases. This study highlighted that the high optimum temperature and good thermostability of rmAga0917 strongly recommends it for the industrial production of the biologically active neoagaro-oligosaccharides.

Acknowledgements

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Table 1 Effect of various reagents on agarolytic activity of rmAga0917

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Relative activity (%)</th>
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<th>Relative activity (%)</th>
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<td></td>
<td>1mM</td>
<td>10mM</td>
<td>1mM</td>
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<td>Control</td>
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Fig. 1. Structure of agarose
Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain YTW1-15-1 and representatives of bacterial species in the genus *Pseudoalteromonas*. Percent bootstrap values above 50 (1000 replicates) are shown at branch nodes. Scale bar = 0.002 substitutions per nucleotide position.
Fig. 3. SDS-PAGE and zymogram analysis of purified rmAga0917

Notes: Lane M, protein marker. Lane 1, purified rmAga0917. Lane 2, zymogram analysis of purified rmAga0917. Molecular weights of proteins were indicated at left side.
Fig. 4. Effects of temperature and pH on rmAga0917

Notes: (A) Temperature effects on the activity and stability of rmAga0917. Temperature profiles were measured at different temperatures (4-80 °C) in citric acid–sodium citrate buffer (0.1M, pH 6.0). (B) pH effects on activity of rmAga0917. (C) pH effects on stability of rmAga0917. pH profiles were measured at 40 °C in different buffers. For all of the above plots, values are presented as percentages of the maximum activity of (taken as 100%). All data shown are the mean values from at least three replicate experiments.

Fig. 5. TLC analysis of hydrolysis products.

Notes: Lane 1: D-galactose. Lane 2-9: products after incubation of rmAga0917 with 0.25% agarose in 0.1 M citric acid-sodium citrate buffer at 40 °C for 5 min, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h and 24 h, respectively.