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A new neoagarobiose-producing agarase from *Vibrio* sp. LA1

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ABSTRACT: An agarolytic bacterium was isolated from the sea coast of Shantou in China and identified as *Vibrio* sp. LA1. The agarase gene *agaA* was cloned from *Vibrio* sp. LA1 by using degenerate oligonucleotide-primed PCR and genome walking technique. Gene *agaA* consists of a 2913 bp open reading frame encoding 970 amino acids; and the predicted molecular mass and isoelectric point were 108 kDa and 4.46, respectively. Based on the amino acid sequence similarity, the encoded protein (AgaA) of gene *agaA* should be an agarase of glycoside hydrolase family GH50 with a catalytic domain of glycoside hydrolase family GH42. Soluble expression of AgaA in *Escherichia coli* was obtained and investigated. The optimal temperature and pH for the activity of the purified recombinant agarase were 35 °C and pH 6, respectively. The reducing reagent β-mercaptoethanol could increase the activity of agarase AgaA by more than 80%. AgaA showed exo-lytic activity on agarose degradation. It decomposed agarose to yield neoagarobiose as the sole product, which was different from the agarase Aga41A from *Vibrio* sp. CN41 that showed 95% identities of amino acid sequence to agarase AgaA. With single end product, purification procedure is easier than that with multi-products. Therefore, agarase AgaA could be a useful tool for producing the bioactive neoagarobiose.

KEYWORDS: agarase, exo-lytic, neoagarobiose, agarose, genome walking

INTRODUCTION

Agar, a component of the cell wall of red algae, is a complex polysaccharide consisting of agarose and agaropectin. Agarose comprises D-galactose and 3,6-anhydro-L-galactose as monomeric units, which are linked by alternative α-l,3- or β-1,4-glycosidic bonds to form polymers [1]. Agarases are glycoside hydrolases (GH) that hydrolyze the α-l,3 bond of agarose (α-agarase) to produce agarooligosaccharides (AOS), and hydrolyze the β-1,4 bond of agarose (β-agarase) to produce neoagarooligosaccharides (NAOS). Marine agar-degrading organisms produce agarases to utilize agar as a convenient carbon source. Therefore, most of the reported agarases were isolated from marine bacteria [2].

According to the homology of amino acid sequences, agarases have been classified into five glycoside hydrolase families: GH16, GH50, GH86, GH96, and GH118, in the Carbohydrate-Active EnZyme database (CAZy) [3]. Among these families, family GH16 included the largest number of agarases which generate neoagrotetraose (NA4) and neoagarohexaose (NA6) as the main products of agarose degradation. In addition, most of the reported agarases were endo-lytic enzymes, while only a few agarases of the family GH50 displayed exo-lytic activity.

Several carbohydrate molecules and derivatives have been reported to exert beneficial biological activities, such as isomaltooligosaccharides [4] and extracellular polysaccharides [5] with prebiotic activity for food use; and arbutin, a glycosylated hydroquinone from plant extract, for medical use [6]. Similarly, NAOS possess diverse biological activities, such as moisturizing effects and whitening effects on skin [7, 8], anti-oxidant activities [9–11], prebiotic effects [11, 12], and potential treatment on type II diabetes [13]. It has been recognized that besides being mainly used in NAOS production, agarases can also be used in recovery of DNA from agarose gel [14, 15], protoplast preparation of from red alga

[16, 17], and the screening of signal peptides [15]. All these applications rely on the search and the large-scale production of potent agarases.

The high application values of NAOS boost researches for novel and unique agarases. This study aims to provide an agarase gene cloning method, using degenerate oligonucleotide-primed PCR and genome walking technique, contributing to the growing agarase resource, and the characterization of a novel neoagarobiose- producing exolytic agarase from the agarolytic bacterium *Vibrio* sp. LA1.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The agarolytic strain LA1 was isolated from seawater samples in seaweed farming area of Shantou sea coast in Guangdong Province, China. Strain LA1 was propagated at 25 °C in ZoBell 2216E medium. *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were used for routine cloning and protein expression, respectively. Vectors pMD19-T (TaKaRa Bio, China) and pET-32a (+) (Merck, Germany) were used for the cloning of agarase gene and preparation of recombinant plasmids, respectively. Agarose (Agarose LE) was purchased from MDbio Inc. (Qingdao, China). Unless otherwise stated, all chemicals used were of analytical grade or higher.

Identification of strain LA1

Genomic DNA of strain LA1 was isolated using a Genomic DNA Purification Kit (Guangzhou Dongsheng Biotech, China). The 16S rRNA gene was amplified from the chromosomal DNA by PCR with the universal primers of 27F (forward primer) and 1492R (reverse primer) in Table S1. The PCR products were ligated with the T-A cloning vectors; and, then, the ligation mixtures were transformed into *E. coli* DH5α cells. Subsequently, the recombinant plasmids were purified and sequenced. The 16S rRNA gene sequence was compared with sequences in the GenBank database for primary identification.

Cloning of agarase gene

In order to obtain the agarase gene of strain LA1, a multiple sequence alignment of reported agarases was performed and found two conserved amino acid sequences of agarases of which the sequences were "DPWCVGYFVDNEM" and "VGAHWFQYIDSPITG", respectively. Degenerate primers, DENf and DGEr, (Table S1) were accordingly designed to these two conserved amino acid sequences using the online tool CODEHOP, respectively [18]. The conserved gene fragment of agarase was amplified by degenerate oligonucleotide-primed PCR. Subsequently, in order to obtain the complete structural gene, three rounds of genome-walking PCR (Genome Walker Kit, TaKaRa Bio, China) were run to amplify the upstream and downstream sequences of the conserved gene fragment. Specific primers of Lnsp1–3 (Table S1) were used to amplify the N-terminal sequence of the complete structural gene, while specific primers of Lcsp1–3 were used to amplify the C-terminal sequence. The PCR products were directly ligated to the vector pMD19-T for sequencing.

Bioinformation analysis

The nucleotide sequence of agarase gene and its deduced protein product were analyzed by different programs. The reading frames, theoretical isoelectric point (p*I*), and molecular weight (MW) were predicted by using the DNAstar program. The signal peptide sequence of agarase gene was predicted using the SignalP server [19]. The alignments of protein sequences were conducted with the tblastn program (http://www.ncbi.nlm.nih.gov/BLAST). The prediction of protein domain was performed in the database of InterPro (http://www.ebi.ac.uk/interpro).

Expression of the *agaA* gene and purification of the recombinant AgaA

To amplify the target gene, including the signal peptide, primers, EXPf and EXPr, (Table S1) were designed with restriction enzyme sites of *Nde* I and *Xho* I at the N-terminus and C-terminus of *agaA*, respectively. A poly 6-histidine residue was added to the C-terminus of the protein in order to purify the protein by affinity chromatography. The recombinant plasmid (pET-32a-agaA) constructed based on the expression vector pET-32a (+) was finally transformed into *E. coli* BL21 (DE3) cells. The recombinant *E. coli* BL21 (DE3) cells harboring the agarase gene were inoculated in LB medium and grown to mid-log phase at 37 °C, then they were induced for expression with isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and incubated at 15 °C for an additional 16 h. The cells were collected by centrifugation, suspended in 20 ml of 20 mM Tris-HCl buffer (pH 8.0), and disrupted on ice by sonication. To maintain its bioactivity, the His-tagged agarase was purified with Ni^{2+} affinity column under native conditions according to the recommendations of the manufacturer

(Beijing RuiDaHengHui Co., Ltd, China). The purity and homogeneity of the purified protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Assay of agarase activity

Agarase activity was determined by the enzymatic production of reducing sugars from agarose as described by Lin et al [20] with some modifications. The reaction mixture, containing 200 μl appropriately diluted enzyme and 800 μl of 0.25% agarose in pH 8 Tris-HCl buffer (20 mM) in a test tube, was incubated at 42 °C for 30 min. After incubation, the sample was mixed with 2 ml of 3,5-dinitrosalicylic acid (DNS) reagent and heated in boiling water for 15 min. After cooling the test tube in a cold-water bath, the mixture was diluted to 10 ml with deionized water. The absorbance at 540 nm was subsequently recorded. The amounts of reducing sugars generated were determined using D-galactose as a standard. One unit of the enzyme activity was defined as the amount of enzyme required to produce 1 μmol reducing sugar per minute.

Biochemical characterization of agarase AgaA

The temperature effects on agarase activity were measured at different temperatures (25–50 °C) in 20 mM Tris-HCl buffer (pH 8). The pH effects on agarase activity were assayed at 35 °C in a pH range of 4–11 using a combination of four different buffer systems: 50 mM sodium citrate (pH 4–6), 50 mM Tris-HCl (pH 7–8), 50 mM Tris-Glycine (pH 9–10), and 50 mM Na_2HPO_4-NaOH (pH 11). Effects of various cations and reagents on the enzyme reaction were assessed, including KCl, $MgCl_2$, $CaCl_2$, ethylene diamine tetraacetic acid (EDTA), β-mercaptoethanol, and urea.

Agarose degradation of agarase AgaA

The products of agarose degradation were analyzed by thin layer chromatography (TLC). After mixing the enzyme solution with 0.2% agarose in 50 mM sodium citrate buffer (pH 6) and incubated at 35 °C for various time periods, the reaction mixtures were applied to a silica gel TLC plate along with the standards, which were neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6), and neoagarooctaose (NA8). An aliquot (10 μl) of the reaction mixture was loaded on a silica gel 60 TLC plate (Shenghai Inc, Qingdao, China) and then developed with a solvent mixture composed of n-butanol/acetic acid/H_2O (2:1:1, by volume). The reaction products (NAOS) on the plate were visualized by spraying with 10% (v/v) H_2SO_4, followed by heating at 85 °C. NAOS with different degrees of polymerization, which were prepared by other studied agarases in our laboratory and confirmed by mass spectrum as described by Lin et al [21], were used as standards and substrates to identify the cleavage pattern of AgaA.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence of strain LA1 and agarase gene nucleotide sequence reported in this study had been submitted to the GenBank database under accession numbers JF682609 and JF461054, respectively.

RESULTS

Identification of the agarolytic strain LA1

The nucleotide sequence of the 16S rRNA gene (1512 bp) was aligned with sequences available in the GenBank database by using the BLASTN search program, and it exhibited maximum homology (99%) with those of *Vibrio* species. The results indicated that the agarolytic strain LA1 was assigned to the genus *Vibrio*.

Cloning and bioinformation analysis of the agarase gene

A PCR product, about 700 bp long, was amplified by degenerate oligonucleotide-primed PCR sing degenerate primers DENf and DGEr). Subsequent sequencing and BLAST (NCBI) search showed that this gene fragment of 700 bp was probably a partial sequence of agarase gene. The upstream and downstream sequences of the 700 bp fragment were obtained by genome-walking PCR. The whole amplified sequence contained 4693 bp, in which a complete open reading frame (ORF) of 2913 bp was found and named *agaA*. The gene *agaA* encodes a novel agarase with an initiation codon of GTG. The upstream sequence of gene *agaA*, including the putative promoter sequence and the ribosome-binding site, is shown in Fig. 1. The -10 region was located 39 bp upstream from the initiation codon. The putative ribosome-binding site (SD sequence) was located 7 bp upstream from the initiation codon. The encoded protein AgaA has a theoretical molecular mass of 108 kDa and an isoelectric point (p*I*) of 4.46. Analysis on the SignalP server revealed that the protein possesses a signal peptide of 23 amino acids (aa). And according to the analysis results in the database of InterPro, there

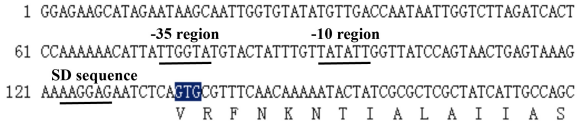

Fig. 1 The upstream sequence of gene *agaA*. The start codon (GTG) was in shadow. The -35 region, -10 region, and SD sequence were underlined. The putative ribosome-binding site (SD sequence) was located 7 bp upstream from the initiation codon.

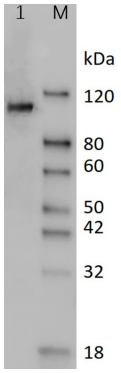

Fig. 2 SDS-PAGE of the recombinant AgaA overexpressed in *E. coli*. Lane M: protein markers; Lane 1: purified proteins by histidine-tag affinity chromatography.

was an N-terminal CBM-like domain (246–418 aa CBM: carbohydrate-binding module) and a catalytic domain of glycoside hydrolase family 42 (677–769 aa) in the encoded protein. In the conserved domain database of GenBank, the amino acid sequence of AgaA showed highest identities to reported agarases of glycoside hydrolase family GH50: 95% to agarase Aga41A of *Vibrio* sp. CN41 [22], 50% to agarase HZ2 from *Agarivorans* sp. HZ105 [21], and 50% to agarase AgaB from *Vibrio* sp. JT0107 [23]. Besides, the Carbohydrate-Active EnZyme database had assigned agarase AgaA to family GH50. Therefore, agarase AgaA should be a member of family GH50.

Expression of *AgaA* gene and purification of the recombinant AgaA

The structural gene of AgaA was cloned into the pET-His expression system and conditionally expressed as a C-terminally His-tagged recombinant protein. When the transformed organism *E. coli* BL21 (DE3) harboring pET-32a–AgaA was inoculated in LB agar plate, there was no pits or clear zone-forming-around colonies. After IPTG induction at the temperature of 15 °C, agarase activity of the recombinant protein was detected in the supernatant of the cell lysates. The recombinant protein was present mostly in a soluble form, instead of expressing as inclusion bodies. The observed molecular weight of recombinant AgaA was about 109 kDa in the SDS-PAGE including the 6-histidine tag, and it was in agreement with the theoretical molecular mass of the mature protein (Fig. 2).

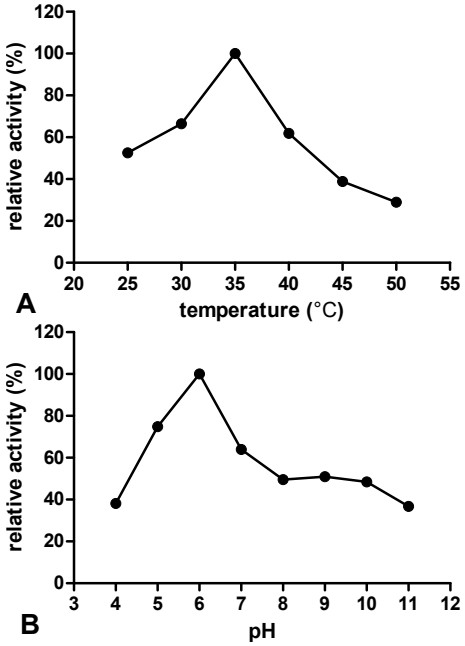

Fig. 3 Effects of temperature (A) and pH (B) on activity of AgaA. Temperature profiles were measured at different temperatures (25–50 °C) in 20 mM Tris-HCl buffer (pH 8). The agarase activity determined at 35 °C was 1.44 U/mg, and it was set as 100% relative activity. pH profiles were assayed at 35 °C in a pH range of 4–11 in which a combination of four different buffer systems was used: 50 mM sodium citrate (pH 5–6), 50 mM Tris-HCl (pH 7–8), 50 mM Tris-Glycine (pH 9–10), and 50 mM Na_2HPO_4-NaOH (pH11). The agarase activity determined at pH 6.0 was 2.63 U/mg, and it was set as 100% relative activity.

Biochemical characterization of agarase AgaA

The optimum temperature of agarase activity of the recombinant AgaA was determined to be around 35 °C. And the agarase activity decreased sharply at temperatures higher than 45 °C. The optimum pH for the activity was 6.0, but more than 50% of the maximal activity was retained over a broad range of pH 5–9 (Fig. 3). The results in Table 1 showed that the agarase activity of AgaA was conspicuously inhibited by Ca^{2+} ion as well as EDTA, especially at the concentration of 100 mM. K^+, Mg^{2+}, and urea

Table 1 Effects of cations and reagents on AgaA activities. Standard enzyme assays were performed at 35 °C in 50 mM sodium citrate buffer (pH 6) supplemented with the tested metal ions and reagents at two different concentrations. The agarase activity of control without adding any chemical was 2.63 U/mg, and it was set as 100% relative activity.

Reagent	Relative activity (%) of AgaA Reagent concentration	
	10 mM	100 mM
KCl	109.3	96.1
$MgCl_2$	98.0	98.5
$CaCl_2$	83.9	14.6
Urea	95.7	93.8
β-mercaptoethanol	189.2	182.0
EDTA	85.7	0
None	100	100

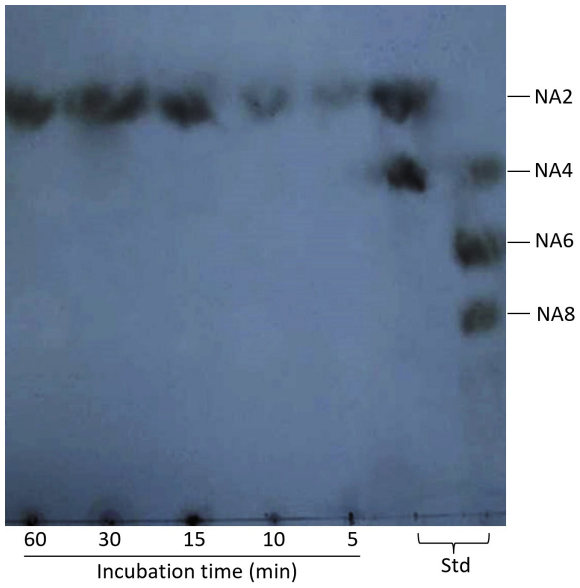

Fig. 4 TLC analysis of agarose degradation by agarase AgaA. Assays were performed in 80 μl reaction mixtures containing 40 μl of purified agarase and the equivalent volume of agarose substrate (0.2%, w/v, prepared in 50 mM sodium citrate buffer pH 6) at 35 °C for a series of incubation times: 5 min, 10 min, 15 min, 30 min, and 60 min. Neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6), and neoagarooctaose (NA8) were used as standards (Std).

barely had negative effects on the agarase activity even at 100 mM. However, β-mercaptoethanol, a reducing reagent, was found to increase the activity of AgaA by more than 80% at both 10 mM and 100 mM.

Agarose degradation of agarase AgaA

As shown in Fig. 4, the enzyme hydrolyzed agarose to generate NA2 as the sole product during incubation for 5–60 min without any other reaction intermediates, such as NA4 or NA6. As exo-agarases cleave dimers from the end of agarose chains to yield NA2; therefore, AgaA should be an exo-agarase.

DISCUSSION

The N-terminal CBM-like domain found in agarase AgaA in this study has been described in the exo-agarase Aga50D from the marine bacterium *Saccharophagus degradans* [24]. A loop in the CBM-like domain is involved in forming the roof of the active site channel. The contribution of the CBM-like domain to formation of the active site of the enzyme supports a role in substrate recognition explaining the exo-mode of beta-agarase action.

Agarases are generally classified in the CAZy database into five glycoside hydrolase families: GH16, GH50, GH86, GH96, and GH118. To date, a few agarases of family GH50 have been reported, and some are exo-agarases. The endo-agarases of family GH50 generally degrade agarose to yield NA4 and NA2 as the main products. For example, agarase Aga41A from *Vibrio* sp. CN41, which shows 95% identities of amino acid sequence to agarase AgaA, decomposes agarose to yield NA4 as the end product [22]; and agarase HZ2 from *Agarivorans* sp. HZ105, which shows 50% identities of amino acid sequence to agarase AgaA, produces NA4 as the main product [21]. However, the exo-agarases of family GH50 generally hydrolyze agarose to produce NA2 as the sole product, including the agarase AgaA in this study. Therefore, the agarase AgaA should be assigned to family GH50 based on amino acid similarity and the mode of agarose degradation. Besides, agarase AgaA also carries conserved amino acid sequences of family GH42, as distinct from other reported agarases.

In most cases, agarase activity is inhibited by β-mercaptoethanol (β-Me) or dithiothreitol (DTT). However, the agarase activity of AgaA in this study was stimulated by 82–89% in the presence of β-Me, suggesting the possible existence of thiol in the catalytic site. The stimulatory effects of β-Me have also been displayed by several other agarases belonging to different glycoside hydrolase families, such as agarase HZ2 of family GH50 from *Agarivorans* sp.

HZ105 [21], agarase AgaXa of family GH118 from *Catenovulum* sp. X3 [25], and β-agarase of family GH16 from *Microbulbifer* sp. Q7 [26]. This indicates that the stimulatory effect of β-Me on the agarases is not glycoside hydrolase family-dependent. The same phenomenon has also been found on the negative effects of Ca^{2+} on the activities of agarases that belong to different glycoside hydrolase families, such as agarase AgaA of family GH50 in this study, β-agarase Aga4436 of family GH16 from *Flammeovirga* sp. OC4 [27], and agarase AgaJ11 of family GH16 [28]. This suggests that the enzyme activities of those agarases do not require Ca^{2+}, unlike the early reported agarases of family GH16 which have Ca^{2+} ion-binding sites and of which the enzyme activities are dependent upon the presence of Ca^{2+} [29]. The protein denaturant urea generally has negative effects on enzyme activity. However, in this study it showed that the agarase AgaA was resistant to 100 mM urea. Actually, there were also some other reported agarases, of which the activities were not affected by urea. For example, agarase ID2563 could resist to 5 mM urea [26] and agarases YM01-1 and YM01-3 were resistant to 10 mM urea [30, 31].

NA2 is a rare reagent showing both moisturizing and whitening effects with low cytotoxicity [32]. Nowadays, almost all agarases degrade agarose to yeild at least two end products, except the exo-agarases of family GH50 hydrolyze agarose to produce NA2 as the sole product. With single end product, purification procedure is easier than that with multi-products. Therefore, agarase AgaA in this study could be a useful tool for producing the bioactive neoagarobiose.

In conclusion, the agarase gene *agaA* of 2913 bp was cloned from the agarolytic bacterium *Vibrio* sp. LA1. The encoded protein (AgaA) of gene *agaA* should be an agarase of glycoside hydrolase family GH50 with a catalytic domain of glycoside hydrolase family GH42. The optimal temperature and pH for the activity of the purified recombinant agarase were 35 °C and pH 6, respectively. AgaA showed exo-lytic activity on agarose degradation. It decomposed agarose to yield neoagarobiose (NA2) as the sole product.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2021.045.

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Appendix A. Supplementary data

Table S1 Primer sequences.

Primer	Sequence (5′ – 3′)
27F	AGAGTTTGATCCTGGCTCAG
1492R	TACGGCTACCTTGTTACGACTT
DENf	CAACTATGGATAAAAATTATTTAGTAGGTTTAGTAGATvarttyggnca
DGEr	AGTATCAGTTACAGATACAAATCCTACATTATAAttytcnccrtc
Lnsp1	GCTACCTTCATCTCAATGCGGACTTC
Lnsp2	ATAAGAACCCGACTCTGGGCGAAAC
Lnsp3	GGTGTGTGTGTTGGCGGTTTTTGATA
Lcsp1	CAGCAGCTCGCTATGTTGATGTGATG
Lcsp2	GGTTTGTTCCACCCTGGCATTATCA
Lcsp3	CATGGATTCACCAGTAACAGGGCGT
EXPf	GGGGGGTC***CATATG***CGTTTCAACAAAAATAC
EXPr	CCG***CTCGAG***ATTCAAAGAACCAAA