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A Novel Glycosyl Hydrolase Family 16 β-Agarase from the Agar-Utilizing Marine Bacterium *Gilvimarinus agarilyticus* JEA5: the First Molecular and Biochemical Characterization of Agarase in Genus *Gilvimarinus*

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The agarase gene *gaa16a* was identified from a draft genome sequence of *Gilvimarinus agarilyticus* JEA5, an agar-utilizing marine bacterium. Recently, three agarase-producing bacteria, *G. chinensis*, *G. polysaccharolyticus*, and *G. agarilyticus*, in the genus *Gilvimarinus* were reported. However, there have been no reports of the molecular characteristics and biochemical properties of these agarases. In this study, we analyzed the molecular characteristics and biochemical properties of agarases in *Gilvimarinus*. Gaa16A comprised a 1,323-bp open reading frame encoding 441 amino acids. The predicted molecular mass and isoelectric point were 49 kDa and 4.9, respectively. The amino acid sequence of Gaa16A showed features typical of glycosyl hydrolase family 16 (GH16) β-agarases, including a GH16 domain, carbohydrate-binding region (RICIN domain), and signal peptide. Recombinant Gaa16A (excluding the signal peptide and carbohydrate-binding region, rGaa16A) was expressed as a fused protein with maltose-binding protein at its N-terminus in *Escherichia coli*. rGaa16A had maximum activity at 55°C and pH 7.0 and 103 U/mg of specific activity in the presence of 2.5 mM $CaCl_2$. The enzyme hydrolyzed agarose to yield neoagarotetraose as the main product. This enzyme may be useful for industrial production of functional neoagaro-oligosaccharides.

Keywords: *Gilvimarinus*, agarase, neoagaro-oligosaccharides, cloning, overexpression

Introduction

Agar is a complex polysaccharide found in the cell walls of some red algae, and up to 70% of the algal cell wall can be made up of agar polymers [1]. Agarose and agaropectin are major components of agar [2]. Agarose contains D-galactose and 3,6-anhydro-L-galactose as its monomeric units, which are linked by alternating α-1,3- and β-1,4-glycosidic bonds [3]. The hydrolysis of agarose is accomplished by α-agarase (E.C. 3.2.1.158) and β-agarase (E.C. 3.2.1.81). First, the α-1,3 linkage of agarose is cleaved, resulting in agaro-oligosaccharides, and the β-1,4 linkage is then cleaved to generate neoagaro-oligosaccharides [2]. Most agarases that are currently being studied and utilized are β-agarases. On the basis of on amino acid sequence homology, β-agarases are classified into four families: glycoside hydrolase family 16 (GH16), GH50, GH86, and GH118 [4]. The reported biological and physiological functions of neoagaro-oligosaccharides, including their moisturizing effect on the skin [5], whitening effect on melanoma cells [6], macrophage stimulation [7], antitumor activity [8], prebiotic effects [9], and antibacterial activity [10], have increased their value while simultaneously broadening their applications in the food, cosmetic, and medical industries.

The isolation of agar-degrading bacteria has been accomplished with different natural sources such as seawater, marine sediments, marine algae, marine mollusks, fresh water, and soil [2]. In microorganisms, many agarases have been reported from *Agarivorans* sp. [11], *Alteromonas* sp. [12], *Pseudomonas* sp. [13, 14], *Vibrio* sp. [15, 16], *Pseudoalteromonas* sp. [17], *Bacillus* sp. [18, 19], *Microbulbifer* sp. [20, 21], *Saccharophagus* sp. [22], *Acinetobacter* sp. [23], among others. Recently, *Gilvimarinus chinensis* [24], *Gilvimarinus polysaccharolyticus* [25], and *Gilvimarinus agarilyticus* [26] were described, and their agarolytic activity was reported. However, there have been no published reports that include the molecular and functional characterization of agarases from the *Gilvimarinus* genus. Previously, we isolated the agar-degrading bacterium *Gilvimarinus agarilyticus* JEA5 and reported its draft genome sequence [27]. In this study, we report the first molecular and biochemical characterization of agarase from the *Gilvimarinus* genus.

Materials and Methods

Identification and Molecular Characterization of β-Agarase

Previously, we reported that the agar-degrading bacterium *G. agarilyticus* JEA5 was isolated from Jeju Island, South Korea. We described identification of that strain using 16S rRNA and genome sequencing with next-generation sequencing (NGS) technology [27]. A putative agarase was identified with the Basic Local Alignment Search Tool algorithm [28] and designated Gaa16A.

The signal peptide of Gaa16A was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) [29], and the percentage identity/similarity of this agarase against homologs was calculated with the EMBOSS Pairwise Sequence Alignment Tool (http://www.ebi.ac.uk/Tools/psa/) [30]. Active sites, catalytic sites, and functional domains were identified with the use of the following programs: NCBI Conserved Domains Database (CDD; http://www.ncbi.nlm.nih.gov/cdd/) [31], Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) [32], and the InterPro web program (http://www.ebi.ac.uk/interpro) [33, 34].

Cloning of Gaa16A

To amplify the predicted agarase gene *gaa16a*, a primer set (Gaa16A-F and Gaa16A-R) was designed to bind the pMal-c2x site with >15 bp nucleotide coverage at the cloning site using the pMal-c2x sequence. The primers are shown in Table 1. Polymerase chain reaction (PCR) amplification of the *gaa16a* gene was performed with LA Taq polymerase (TaKaRa, Japan). We removed the predicted signal sequences at the N-terminus of Gaa16A, as well as the RICIN superfamily domain at the C-terminus. The pMal-c2x expression vector (New England Biolabs, UK) was digested, and the amplicons were digested with BamHI (TaKaRa, Japan) and SalI (TaKaRa, Japan) restriction enzymes at 37°C for 2 h, following the manufacturer's instructions. The digested pMal-c2x expression vector was gel purified using the AccuPrep Gel Purification Kit (Bioneer, Korea). The Gaa16A PCR product was cloned into the digested pMal-c2x expression vector with the Ez-Fusion Cloning Kit (Enzynomics, Korea) following the manufacturer's protocol, and the recombinant plasmid was then transformed into *Escherichia coli* DH5α. The clone was purified using the AccuPrep Nano-Plus Plasmid Mini Extraction Kit (Bioneer, Korea) and then again transformed into the expression vector *E. coli* BL21 (DE3). Finally, the nucleotide sequence of the newly constructed recombinant plasmid was confirmed by sequencing (Macrogen, Korea).

Overexpression and Purification of Recombinant Agarase

E. coli BL21 (DE3) containing pMal-c2x-*gaa16a* was inoculated into 5 ml of LB broth supplemented with ampicillin (100 mg/ml) and cultured overnight at 37°C. The overnight culture was transferred into 250 ml of fresh LB broth supplemented with ampicillin (100 mg/ml) and incubated with shaking at 37°C until the culture reached an optical density of 0.6 at 600 nm. Isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM. To overexpress the recombinant protein, the cultures were incubated at 37°C for 3 h. Cells from the 250 ml culture were pelleted by centrifugation at 1,800 ×*g* for 15 min and resuspended in 20 ml of column buffer before storing at −20°C overnight. Frozen cells were thawed on ice, sonicated, and centrifuged (13,000 ×*g*, 20 min, 4°C). rGaa116A was purified from the supernatant using the pMal Protein Fusion & Purification System (New England Biolabs, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the purified protein, and the protein concentration was determined using the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc., USA).

Agarolytic Activity Assay

The amount of reducing sugar was determined by a modified 3,5-dintrosalicylic acid method [17, 35] using D-galactose as the standard. The amount of enzyme required to produce 1 μmol of reduced sugar per minute was defined as one unit of enzyme

Table 1. PCR primer set for amplification of the *gaa16a* gene without the RICIN domain-coding region.

Name	Sequence	Purpose
gaa1-F	5′-TTC AGA ATT CGG ATC GCC GAC TGG GAT AAC AC-3′	Gaa1 amplification without RICIN
gaa1-R	5′-TTG CCT GCA GGT CGA CTA TTG ACG ACC TTT AAT GCT G-3′	Gaa1 amplification without RICIN

activity. Substrate was prepared using SeaKem LE Agarose (Lonza, Switzerland) dissolved in deionized water by boiling, and then cooled to 45°C. The enzyme reaction mixture was prepared in a 200 μl total volume containing 100 μl of 1% agarose (final concentration 0.5%), 95 μl of the proper buffer, and 5 μl of diluted recombinant enzyme. The optimum pH of rGaa16A was determined over a pH range of 3.0–10.0 with intervals of pH 0.5 at 50°C for 20 min. Citrate phosphate buffer (pH 3.0–6.0), phosphate buffer (pH 6.0–8.0), and glycine-NaOH buffer (pH 8.0–10.0) were used for various pH conditions. The optimum temperature for the enzyme activity of rGaa16A was determined by monitoring the relative activity at temperatures ranging (in 5°C intervals) from 40°C to 75°C at the optimum pH of 8.0 for 20 min. The thermostability of rGaa16A was measured at 50°C and pH 8.0 after preincubation at temperatures of 40°C, 50°C, and 60°C for 30, 60, and 90 min. The sensitivities of rGaa16A to various metal ion salts and chelators were determined under optimal pH and temperature conditions in reaction mixtures supplemented with 2.5 mM $CaCl_2$, $CuSO_4$, EDTA, $FeSO_4$, KCl, $MgSO_4$, $MnCl_2$, NaCl, or $ZnSO_4$.

Hydrolytic Product Analysis

The hydrolytic product of agarose from the recombinant enzyme rGaa16A was identified using thin-layer chromatography (TLC). Enzymatic hydrolysis of agarose was carried out at 45°C for 1 h in distilled water containing 10 μl of rGaa16A and 90 μl of 0.5% agarose. The mixture was applied to a silica gel 60 TLC plate (Merck, Germany) and developed with *n*-butanol:acetic acid:dH_2O (2:1:1 (v/v)). Spots were visualized by spraying with an orcinol dip reagent (80 mg of orcine monohydrate dissolved in 160 ml of acetone; 8 ml of sulfuric acid then added), followed by heating at 100°C for 10 min. D-(+)-Galactose (Riedel de Haen, Germany), neoagarobiose (Carbosynth, UK), neoagarotetraose (Carbosynth, UK), and neoagarohexaose (Carbosynth, UK) were used as standards.

Results

Molecular Characterization of Gaa16A

The nucleotide and amino acid sequences of Gaa16A have been submitted to NCBI under accession number KP716979. *gaa16a* has a 1,323-bp open reading frame that encodes 441 amino acids. The molecular mass and isoelectric point were predicted to be 49 kDa and 4.9, respectively. A signal sequence is located in the N-terminal region (amino acid positions 1–22). Conserved GH16 and RICIN superfamily (ricin-type beta-trefoil; carbohydrate-binding domain formed from presumed gene triplication) sequences were identified at amino acid positions 19–289 and 307–440, respectively. We assumed that the catalytic residues of Gaa16A were 145-Glu, 147-Asp, and 150-Glu in the GH16 catalytic domain. The predicted calcium-binding sites were observed at the amino acid positions 20-Asp, 46-Asn, and 283-Asp. Gaa16A showed the highest identity with hypothetical proteins from *G. chinensis* (GenBank Accession No. WP_020208752.1) and *G. polysaccharolyticus* (WP_049721016.1), as well as a predicted agarase from *Pseudomonas* sp. ND137 (BAB79291.1) (Table 2). However, there are no published characterization or overexpression studies on these proteins. These sequences, including Gaa16A, showed less than 71% similarity with characterized agarases, such as those from *Catenovulum agarivorans* YM01 and *Agarivorans* sp. LQ48.

Expression and Purification of Recombinant Agarase

Maltose binding protein (MBP)-tagged recombinant Gaa16A protein (with the RICIN superfamily domain removed) was overexpressed in *E. coli* (BL21) and purified using the pMAL Protein Fusion and Purification System. The purified recombinant protein was identified by the strong band on the SDS-PAGE gel (Fig. 1), which was similar to the predicted molecular mass (33 kDa with 42 kDa of MBP).

Enzymatic Characterization of Recombinant Agarase

The recombinant agarase rGaa16A showed its highest agarolytic activity at 55°C, and over 80% of its activity remained at 65°C (Fig. 2A); however, its activity was dramatically decreased to less than 20% at 70°C. The

Table 2. Identity and similarity of rGaa16A compared with amino acid sequences of other predicted agarase sequences and characterized agarases.

	Identity (%)	Similarity (%)	Gap (%)	Accession No.	Remark
Pseudomonas sp. ND137	97.7	98.6	0.0	BAB79291.1	Uncharacterized
Gilvimarinus chinensis	94.8	97.5	0.0	WP_020208752.1	Uncharacterized
Gilvimarinus polysaccharolyticus	91.2	96.1	0.0	WP_049721016.1	Uncharacterized
Catenovulum agarivorans YM01	54.4	70.5	4.8	AGU13985.1	Characterized
Agarivorans sp. LQ48	40.6	57.7	8.9	ACM50513.1	Characterized
Bacteroides plebeius DSM 17135	34.9	43.3	36.9	EDY95404.1	Characterized
Microbulbifer agarilyticus	32.7	44.0	34.4	BAE06228.1	Characteriaed

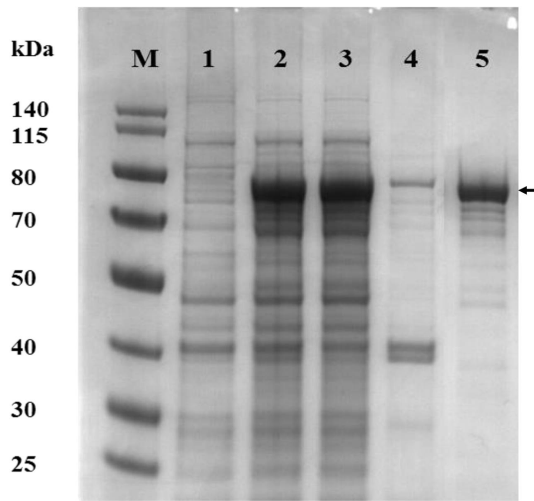

Fig. 1. SDS-PAGE analysis of purified rGaa16A.

M: Molecular weight marker; Lane 1: whole-cell lysates before induction; lane 2: whole-cell lysates after induction; lane 3: soluble fraction after cell lysis; lane 4: insoluble fraction after cell lysis; lane 5: purified rGaa16A.

optimum pH of rGaa16A was 7 (Fig. 2B). The activity of the enzyme increased gradually from pH 5 to 7, and it then dramatically decreased at pH 10. The thermostability of rGaa16A is shown in Figs. 2C and 2D; rGaa16A was stable with over 80% of its activity after pre-incubation at 50°C for 60 min. However, it showed low stability, retaining less than 50% of its activity at 55°C after preincubation for 30 min. Interestingly, the thermostability of rGaa16A was improved in the presence of $CaCl_2$. The effects of metal ions, salts, and chelators on rGaa16A activity are shown in Fig. 3. The activity of rGaa16A was significantly inhibited by $CuSO_4$, and $ZnSO_4$, whereas its activity was enhanced by $CaCl_2$, $MnCl_2$, NaCl, and $FeSO_4$.

The specific activity of rGaa16A is shown in Table 3. rGaa16A exhibited specific activity toward agarose at 103.5 U/mg. rGaa16A maintained its activity well in the presence of $CaCl_2$. In the absence of $CaCl_2$, rGaa16A activity was only 63 and 67 U/mg at 50°C and 60°C, respectively. Meanwhile, it was 101.5 and 84.3 U/mg at 50°C and 60°C, respectively, in the presence of $CaCl_2$.

Hydrolysis Product of rGaa16A

The enzyme reaction products of rGaa16A were identified using TLC, which revealed that rGaa16A catalyzed the rapid degradation of agar to neoagarotetraose (NA4),

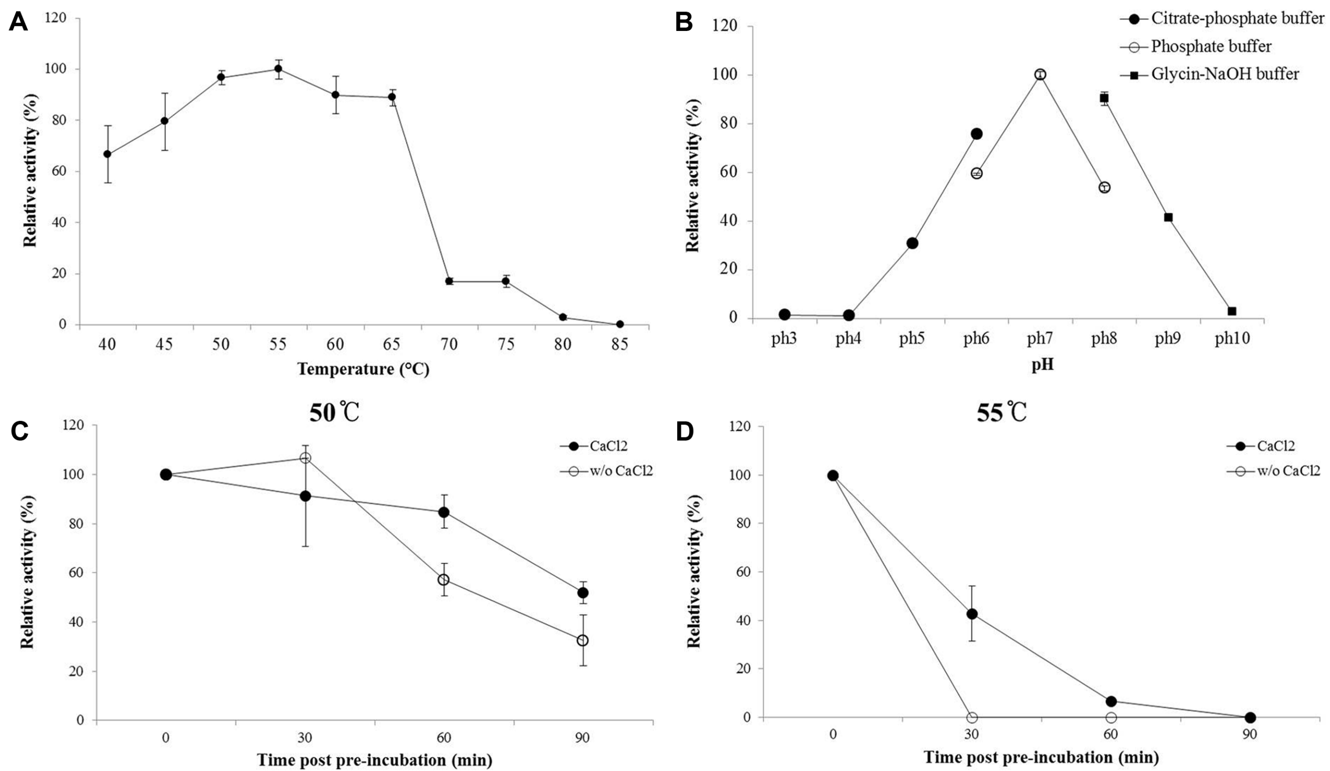

Fig. 2. Biochemical properties of rGaa16A.

Optimum temperature (**A**), optimum pH (**B**), and thermal stability at 50°C (**C**) and at 55°C (**D**).

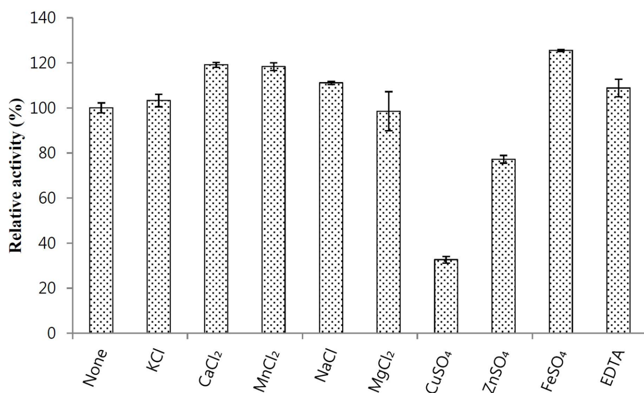

Fig. 3. Effects of chemicals and metal ions on the agarolytic activity of rGaa16A.

neoagarohexaose (NA6), neoagaro-oligosaccharides, and neoagaro-oligosaccharides larger than NA8 as products of the early stage of the reaction (Fig. 4A). After 60 min of incubation, NA4 and NA6, as well as trace amounts of compounds larger than NA8, were present. Finally, only NA4 was observed after overnight incubation (Fig. 4B).

Discussion

The purpose of this study was to molecularly characterize and analyze the biochemical properties of a new β-agarase identified in *G. agarilyticus* JEA5, the first report of such an enzyme isolated from the genus *Gilvimarinus*. The genomic DNA sequence was obtained from *G. agarilyticus* JEA5 using NGS technology. The amino acid sequence of Gaa16A has features typical of GH16 family β-agarases, such as a GH16 domain and RICIN superfamily domain, known to be a carbohydrate recognition region. The presence of RICIN improves functional capabilities, such as enzymatic activity, inhibitory toxicity, and signal transduction. This domain has been shown in some instances to bind simple sugars, such as galactose or lactose [36, 37]. Most GH16 β-agarases consist of two main functional domains GH16 catalytic domain and carbohydrate-binding module 6, but the RICIN superfamily in the C-terminal region also has been reported from some GH16 family β-agarases, such as

Table 3. Specific activity of rGaa16A.

Temp	Specific activity (Unit/mg, μmol/mg/min)	
	rGaa16A	rGaa16A + $CaCl_2$
50°C	63.1 ± 7.5	101.5 ± 5.1
55°C	103.5 ± 5.9	102.7 ± 3.7
60°C	67.9 ± 6.8	84.3 ± 0.7

The specific activity was analyzed at 50°C, 55°C, and 60°C in the presence and absence of $CaCl_2$.

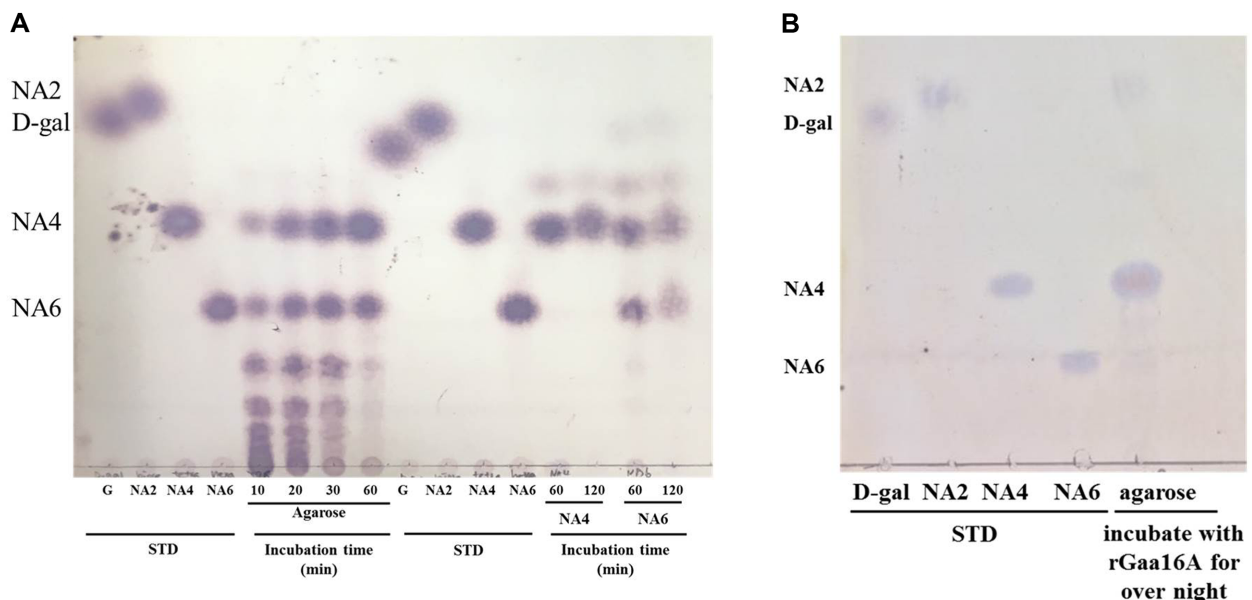

Fig. 4. TLC analysis of the rGaa16A enzyme hydrolysis products from agarose and neogaro-oligosaccharides.
The hydrolytic products of rGaa16A activity toward agarose for 10, 20, 30, and 60 min, and neoagaro-oligosaccharides for 60 and 120 min (**A**) and hydrolytic product after overnight (**B**) were developed in silica gel 60 plate. D-Galactose (G), neoagarobiose (NA2), neoagarotetraose (NA4), and neogarohexaose (NA6) were used as standards (STD).

YM01-3 from *Catenovulum agarivorans* YM01T [38], AgaH92 from *Pseudoalteromonas* sp. H9 [39], and AgaV from *Vibrio* sp. V134 [40].

Full-length rGaa16A without the signal peptide and two truncated derivatives (including the carbohydrate-binding module and a sequence without RICIN) were expressed in *E. coli*. The carbohydrate-binding region (RICIN domain) of recombinant agarase exhibited low agarolytic activity (data not shown), whereas high agarolytic activity was observed when recombinant agarase was overexpressed without the RICIN region. This result is similar to the behavior of agarase AgaD from the marine bacterium *Vibrio* sp. strain PO-303 [41]. rAgaD containing only the catalytic module without the carbohydrate-binding module (CBM) also showed higher agarolytic activity than CBM-containing recombinant AgaD.

The optimum temperature of rGaa16A was observed at 55°C, which was higher than the gelling temperature of agar (>40°C), and the activity of rGaa16A was retained at 50°C after preincubation for 90 min. The ability of an enzyme to convert agar into oligosaccharide while maintaining high catalytic activity and thermostability at temperatures above the gelling temperature may be advantageous for different applications. Tightly arranged bundles of gelled agar obstruct enzymatic degradation; therefore, other known agarases also show optimum temperatures that are higher than the gelling temperature [4, 19]. The optimum pH of rGaa16A was neutral. This result is similar to previous reports for marine-derived agarases. Although a few agarases, such as AgaC from *Vibrio* sp. PO-303 and AgaB from *Pseudoalteromonas* sp. CY24, have been reported to have optimum activity at pH 6.5 and 5.5, respectively [42, 43], most agarases exhibit maximum activity at a neutral or a weakly alkaline pH [2]. It is well known that natural seawater has a weakly alkaline pH. For these reasons, marine-derived agarases typically exhibit optimal activity under neutral or weakly alkaline conditions.

The activity of rGaa16A increased in the presence of 2.5 mM $CaCl_2$. Additionally, in the specific activity test, we found that rGaa16A maintained its activity well at a high temperature of 60°C (Table 3) in the presence of $CaCl_2$. The amino acid sequence of Gaa16A contains three predicted calcium-binding sites. Several studies have reported that $CaCl_2$ is not involved in the catalytic machinery, but it has been shown to have a stabilizing effect in family GH16. However, in our study, the presence of calcium ions had a significant effect on rGaa16A activity (approximately 19% increase) and strongly enhanced the thermostability of rGaa16A.

Agarases belonging to the same GH family exhibit similar patterns of agarose degradation. The end product or main product is NA4 for most reported GH16 β-agarases [2]. rGaa16A also hydrolyzed agarose to NA4, similar to results reported previously for GH16 family agarases. NA4 exhibited several bioactivities, such as a whitening effect [44], antioxidative activity, a prebiotic effect [45], and an anti-inflammatory effect [46].

In conclusion, a GH16 family β-agarase gene (*gaa16a*) was isolated from the draft genome sequence of the marine bacterium *G. agarilyticus* JEA5. The recombinant β-agarase (rGaa16A) was overexpressed and purified, and its biochemical properties were analyzed, representing the first agarase characterized from the genus *Gilvimarinus*. rGaa16A was stable at temperatures below 50°C, and the optimum temperature was higher than the gelling temperature of agar. Moreover, its activity and stability were improved in the presence of $CaCl_2$. rGaa16A hydrolyzed agarose to NA4, which has diverse physiological and biological activities with great economic value. Therefore, this enzyme can be used for different industrial applications, such as in the cosmetic, pharmaceutical, and food industries.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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