Molecular Cloning, Overexpression, and Enzymatic Characterization of Glycosyl Hydrolase Family 16 β-Agarase from Marine Bacterium Saccharophagus sp. AG21 in Escherichia coli

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Introduction

Agar is a gelatinous substance produced by red algae that provides structural support to the cell wall. Owing to its low melting temperature and rapid but stable solidification, agar is used extensively as a common gelling source in microbiological culture media and as an ingredient stabilizer and thickening agent in the food industry [47]. The two main components of agar are agaropeptin and agarose, the latter of which is composed of alternating residues of 3-O-linked β-D-galactopyranose and 4-O-linked 3,6-anhydro-α-L-galactose [11]. The hydrolysis of agar is catalyzed by agarases, which have been divided into two classes, α-agarase and β-agarase, based on mode of action. The α-agarases hydrolyze α-1,3-linkages of agarose to produce agaroligosaccharides [46], whereas the β-agarases hydrolyze β-1,4-linkages in agarose to produce neoagarooligosaccharides [10].

Most of the agarases that have been isolated and characterized to date belong to the β-agarases. Moreover, this class has been subdivided into three glycoside hydrolase (GH) families (GH16, GH50, and GH86), based on amino acid (aa) sequence homology [18]. The GH16 family is the most abundant and contains more than 1,700 functionally heterogeneous members, including endogalactosidases, endoglucanases, κ-carragenases, lichenases, and xyloglucanases [41]. In general, the GH16 β-agarases degrade agarose molecules that are composed of at least six sugars, to yield...
neoagarotetraose as the main product [3]. GH16 β-agarase orthologs have been isolated from a number of marine bacteria, including *Agarivorans* [38], *Janthinobacterium* sp. SY12 [38], *Microbulbifer* [34], *Pseudoalteromonas* sp. AG52 [35], *Saccharophagus* [12], *Vibrio* [9], and *Zobellia* [21]. In contrast, the GH50 and GH86 families consist of β-agarases only, and presently contain 74 and 32 members, respectively (http://www.cazy.org).

The neoagaro-oligosaccharide product has been successfully developed into several commercial applications, including uses as a whitening agent, an antioxidant, and a moisturizer [22, 43, 45]. Moreover, β-agarases have been used in bioethanol production with red seaweeds [25], and DNA recovery from agarose gel [17]. Further applications have been proposed in various other industries, including cosmetics, food, and reagents.

In this study, a novel β-agarase gene was identified in a *Saccharophagus* sp. AG21 isolate from the Jeju Island coastal environment, designated as *agy1*. Subsequent cloning and expression of the recombinant β-agarase (rAgY1) was analyzed to determine its biochemical properties, such as specific activities and optimum reaction conditions, as well as to characterize its enzymatic product.

**Materials and Methods**

**Isolation of Agarolytic Bacteria Strains**

Agarolytic bacteria were isolated from red seaweed, *Gelidium amansii*, collected from the south coast of Jeju Island, Republic of Korea. After crushing, the seaweed samples were spread on 1.5% agar in seawater plates (SWA; natural seawater, 1.5% agar). Positive colonies showing pits were picked and re-streaked. The pure colonies were selected by repeat streaking under the same conditions and inoculated in marine broth including 0.2% agar for incubation at 30°C. Genomic DNA (gDNA) was extracted from the isolates, and a polymerase chain reaction (PCR) was performed to amplify the 16S rDNA sequence with universal primers (forward (F): 16S-27F, and reverse (R): 16S-1492R; Table 1). The sequences were analyzed using the National Center for Biotechnology Information (NCBI) nucleotide BLASTN program and DNAAssist program. Furthermore, the partial 16S rDNA sequence from *Saccharophagus* sp. AG21 was aligned with the corresponding 16S rDNA partial sequences of *S. degradans* 2-40, available in the GenBank database, in order to perform a phylogenetic analysis.

**PCR Amplification of the agy1 from *Saccharophagus* sp. AG21**

To amplify the partial agarase gene from the identified strain, agy1-F1 and agy1-R1 primers were designed using sequences from other known *Saccharophagus* sp. agarase genes published in the NCBI database. The PCR mixture (50 μl) included 5 μl of 10× ExTaq polymerase buffer, 4 μl of 2.5 mM dNTPs, 20 pmol of each primer, 400 ng of gDNA as template, and 3 units of Ex Taq DNA polymerase (Takara, Japan). The thermal cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 45 sec, 55°C for 45 sec, and 72°C for 3 min. The final extension step was carried out at 72°C for 5 min.

The complete agy1 was cloned using a long and accurate (LA)-PCR in vitro cloning kit (Takara, Japan), according to the manufacturer’s instructions. Briefly, gDNA was digested with restriction enzymes, BamHI and EcoRI, and the digested products were ligated to a BamHI and EcoRI cassette, which was then used as a template for the LA-PCR. The agy1-LA51, agy1-LA52, agy1-LA31, and agy1-LA32 primers were designed to identify the reverse and forward sequences from the known partial sequence of *agy1*, respectively. Amplification of the sequences upstream or downstream of the known sequence was performed using either agy1-LA51 or agy1-LA31 with the C1 primer (corresponding to the cassette nucleotide

**Table 1.** Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Purpose</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S-27F</td>
<td>16S rDNA sequence amplification</td>
<td>AGAGTTGATCTMTGCTCAG</td>
</tr>
<tr>
<td>16S-1492R</td>
<td>16S rDNA sequence amplification</td>
<td>TACGGYTACCTTGTTACGACTT</td>
</tr>
<tr>
<td>agy1-F1</td>
<td>agy1 partial sequence amplification</td>
<td>ATGAAACACCAAAATGC</td>
</tr>
<tr>
<td>agy1-R1</td>
<td>agy1 partial sequence amplification</td>
<td>TTAGTTGCTAAGGCGTAAN</td>
</tr>
<tr>
<td>agy1-LA51</td>
<td>agy1 5’ region PCR</td>
<td>AATCCACCCAGCTCAACCGCATA</td>
</tr>
<tr>
<td>agy1-LA52</td>
<td>agy1 5’ region nested PCR</td>
<td>TGGCCGATTACACCATTACGGTGC</td>
</tr>
<tr>
<td>agy1-LA31</td>
<td>agy1 3’ region PCR</td>
<td>CAAACCACGTTCGTTGAGCGGT</td>
</tr>
<tr>
<td>agy1-LA32</td>
<td>agy1 3’ region nested PCR</td>
<td>CAGGGCCAAGCCAAGCATTGATAA</td>
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<tr>
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<td>Cassette primer</td>
<td>GTATATTTGCTTGATAGACCGTAATAGCCTCA</td>
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<tr>
<td>C2</td>
<td>Cassette primer</td>
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</tr>
<tr>
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<tr>
<td>agy1-ER</td>
<td>agy1 cloning to pET-16b</td>
<td>GAGAGAGATCCCTTAGTGTGCTAAGCGTGAACTTATAGG</td>
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</table>
Sequence Characterization

The sequenced agy1 gene was analyzed by the BLASTN and BLASTP programs. The signal peptide of Agy1 was predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/), and motif prediction was carried out using a motif scan prediction program (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The structural features were further analyzed with the conserved domains database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Multiple alignment of protein sequences from different species was performed by the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The phylogenetic tree was constructed by the neighbor-joining method using molecular evolutionary genetic analysis software (MEGA, version 5.05).

Construction of Recombinant Plasmid with agy1

The agy1-EF and agy1-ER primer set was designed to amplify agy1 without the signal sequence but with restriction enzyme sites for NdeI and BamHI at the 5'-end and 3'-end, respectively, for cloning into the pET-16b expression vector (Novagen, Germany). The PCR mixture (50 µl) consisted of 5 units of LA Taq polymerase, 5 µl of 10× LA PCR buffer II (Mg²⁺ free), 5 µl of 25 mM MgCl₂, 500 ng of gDNA, and 20 pmol of each primer. The thermal cycling conditions included an initial denaturation step (94°C, 5 min), 30 cycles of amplification (94°C, 30 sec; 45°C, 30 sec; 72°C, 180 sec), and a final extension step (72°C, 5 min). The PCR product and pET-16b vector were digested with the respective restriction enzymes and ligated together by overnight incubation at 16°C with T4 DNA ligase (Takara, Japan). The pET-16b-agy1 expression plasmid was then transformed into E. coli DH5α competent cells, and correct recombinants were selected after sequencing confirmation.

Overexpression and Purification of the Recombinant Protein, rAgy1

The sequence-confirmed rAgy1 (pET-16b-agy1) was transformed into E. coli BL21 (DE3) cells and grown at 37°C in 100 ml of Luria-Bertani (LB) broth (Difco, USA) supplemented with 100 µl of ampicillin (100 mg/ml). At the mid-exponential growth phase, rAgy1 overexpression was induced by the addition of 0.3 mM isopropyl-β-thiogalactopyranoside (IPTG) to the medium and further incubation at 10°C for 15 h. Cells harvested by centrifugation (3,500 rpm, 20 min, 4°C) were resuspended in 5 ml of binding buffer (His-Bind Kit; Novagen, Germany) and frozen at −20°C overnight. After thawing on ice, cells were disrupted by sonication and centrifuged (13,000 rpm, 20 min, 4°C) to collect the supernatant containing the crude enzyme. The rAgy1 was purified from the crude extract using the His-Bind Kit. The purified protein was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard [6].

Agarolytic Activity Assay of rAgy1

The agarolytic potential of purified recombinant Agy1 was assayed by using the modified 3,5-dinitrosalicylic acid (DNS) method [33] with 1% agarose as the substrate. Briefly, the substrate was dissolved in phosphate buffer (pH 7.5), warmed to 45°C, mixed with rAgy1 enzyme solution, and incubated at 45°C for 30 min. Agarolytic activity (expressed as the initial rate of agar hydrolysis) was determined by measuring the release of reduced sugar ends. One unit of rAgy1 agarolytic activity was defined as 1 µmol of reducing sugar produced in 1 min as compared with the β-galactose standard under the same assay condition.

Biochemical Characterization of rAgy1

The optimum temperature for rAgy1 enzymatic activity was determined by monitoring the relative activity at temperatures ranging from 40–70°C with 5°C intervals at pH 7.0. The optimum pH for rAgy1 enzymatic activity was determined with a pH range of 5.0–9.0 with pH 0.5 intervals at 45°C. Acetate buffer and phosphate buffer were used for the lower (pH 4.5–6.0) and higher (pH 6.5–9.0) ranges, respectively. The protein’s thermostability was determined by incubation at temperatures of 45°C, 50°C, and 55°C for 30, 60, 90, 120, and 150 min. Additionally, the thermostability of rAgy1 in the presence of calcium ion was determined, where rAgy1 was incubated with 2 mM CaCl₂ for 30, 60, 90, 120, 150 min and then the agarolytic activity was measured. rAgy1 sensitivity to various metal ion salts and chelators was determined by DNS measurements of activity after 30 min of 45°C incubation in 1% agar solution supplemented with 2 mM of CaCl₂, CuSO₄, EDTA, FeSO₄, KCl, MgSO₄, MnCl₂, NaCl, or ZnSO₄.

Identification of Hydrolysis Products from Purified rAgy1

Thin layer chromatography (TLC) was used to identify the agarose degradation products by rAgy1. The reaction mixture (200 µl), containing 20 µl of purified rAgy1 and 180 µl of 1% agarose (USB, USA) or 180 µl of 1% neoagarohexanitol (Sigma, USA) substrate, was incubated at 45°C for 10, 20, 30, and 60 min. Subsequently, the reacted solutions were applied to a silica gel 60 TLC plate (Merck, Germany), which was developed using a solvent system consisting of n-butanol: acetic acid: water (2:1:1, v/v, v). The oligosaccharide spots that resulted from hydrolysis of the substrates were visualized by spraying 10% H₂SO₄ onto the plate and heating.

Results

Sequence Characterization of agy1

A novel agar-degrading bacterium, Saccharophagus sp. AG21, was identified by selection on a natural seawater
Fig. 1. Multiple sequence alignment of Agy1 with other previously reported agarases.

Identical residues among all orthologous sequences are denoted by uppercase letters. Similar residues (with single discrepancies among the orthologs) are denoted by lowercase letters or numbers. Gradations of similarity frequency are denoted by dark-to-pale shading (100%, 80%, and 60% respectively). Missing amino acids are denoted by dashes. The signal peptide and glycosyl hydrolase family 16 (GH16) domain are indicated by double-arrows. The carbohydrate binding modules family 6 (CBM6) are dashed-double-arrows. Down arrows indicate catalytic residues, and the calcium binding sites are indicated by solid triangles. The organisms used for alignment and their corresponding GenBank accession numbers are *Saccharophagus degradans* 2-40, YP_526649.1; *Microbulbifer agarolyticus*, BAE06228.1; *Alteromonas* sp. S89, ZP_09504881.1; *Simiduia agarivorans* SA1, AFV00509.1; *Pseudomonas* sp. ND137, BAD88713.1; and *Microbulbifer elongates*, BAC99022.1.
agar selection plate followed by 16S rDNA sequence analysis. The sequence (GenBank Accession No. JQ699229) showed 99.0% identity to *S. degradans* 2-40.

A novel agarase gene, *agy1* (GenBank Accession No. JQ743648), was identified from the newly identified strain by PCR amplification. The *agy1* had a 1,908 bp open reading frame (ORF), which encoded 636 amino acids. A signal peptide was located in the N-terminal region. In addition, a highly conserved GH16 domain and two carbohydrate binding modules of family 6 (CBM6) were identified in the polypeptide chain. Furthermore, catalytic residues and calcium binding residues were as predicted (Fig. 1).

To determine the relationship of Agy1 among the known β-agarase members from various species, a phylogenetic tree was constructed (Fig. 2). The tested agarases comprised three clades, which were represented by the GH16, GH50, and GH86 families, respectively. Agy1 grouped with the GH16 family and was most closely related to *S. degradans* 2-40 Aga16B (GenBank Accession No. YP_526649), with a similarity of 93.7%. The next closest relation was to agarase of *Microbulbifer agarilyticus* (GenBank Accession No. BAE06228), with a similarity of 84.9%.

![Fig. 2. Phylogenetic analysis of Agy1 with known agarases based on amino acid sequences. The numbers indicate the bootstrap confidence values of 1,000 replicates. The accession numbers of each agarase is indicated.](image1)

![Fig. 3. SDS-PAGE of rAgy1. Protein samples were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue. Lanes: M, molecular mass marker (Bio-Rad, USA); 1, total cellular extract from *E. coli* BL21 (DE3) before induction; 2, total cellular extract after induction; 3, purified rAgy1.](image2)
Expression and Purification of rAgy1

IPTG-induced overexpression of the his-tagged rAgy1 fusion protein was verified by SDS-PAGE (Fig. 3). A strong band at the predicted molecular mass of rAgy1 (69 kDa, including the 6-his tag) was detected.

Biochemical Characterization of Purified rAgy1

The rAgy1 enzyme showed specific activity towards agarose, with 85 U/mg. The optimum temperature for rAgy1 activity was 55°C (Fig. 4A). However, the activity was stable below 50°C for 30 min, after which the activity gradually decreased until 150 min (Fig. 4B). The maximum activity of rAgy1 occurred at pH 7.5. However, the activity was stable throughout a wide range of pH, with up to 60% activity being retained at pH 5.0–9.0 (Fig. 4C). The effects of metal ion, salts, and chelators on rAgy1 activity are shown in Fig. 4D. More than 50% of rAgy1 activity was inhibited by 2 mM of MnCl₂ and ZnSO₄. In contrast, the

Fig. 4. Biochemical properties of purified rAgy1.

(A) Effects of temperature on rAgy1 enzyme activity at temperatures in the range of 40–70°C. (B) Effects of thermostability on rAgy1 at different temperatures (45–55°C) and at different time points (30, 60, 90, 120, and 150 min). (C) Effects of pH on rAgy1 activity at pH ranging from pH 4.5–9.0. (D) Effects of metal ions and salts (all 2 mM) on Agy1 activity, measured after incubation at 45°C for 30 min. (E) Effect of CaCl₂ on the relative activity.
activity of rAgy1 was enhanced by FeSO$_4$ (40%), KCl (34%), and NaCl (34%). Only CuSO$_4$ had no observable effect on rAgy1 activity. In the presence of Ca$^{2+}$ ion in the reaction medium, the thermostability of Agy1 was increased (Fig. 4E) compared with the absence of Ca$^{2+}$ ion.

**Hydrolysis Products of rAgy1**

Hydrolysis patterns produced by purified rAgy1 against agarose and neoagarohexanitol are shown in Fig. 5. rAgy1 incubation with agarose generated NA4 and NA6 at the early stage of the reaction, and the amount of NA4 increased in a time-dependent manner. In contrast, rAgy1 incubation with neoagarohexanitol produced no products that were different from the control (NA6).

**Discussion**

In this study, we isolated a novel agarolytic bacterium from seaweed using natural seawater minerals and agar as the only carbon source as the first screening step. The identified bacterial strain was named *Saccharophagus* sp. AG21, based upon its high (99.0%) identity to *S. degradans* 2-40. Subsequently, the *Saccharophagus* sp. AG21 agarase gene, *agy*1, was isolated, cloned, expressed, and characterized. This agarase was found to be distinct from, but highly similar to (93% similarity), the β-agarase of *S. degradans* 2-40.

Furthermore, the Agy1 deduced amino acid sequence contained the GH16 β-agarase module and CBM6s domains, which are characteristic of β-agarases [13, 14]. Accordingly, phylogenetic analysis revealed that Agy1 was derived from the GH16 glycoside hydrolase family. Most of GH16 family recombinant agarases are reported to produce NA4 [13], similar to the novel rAgy1 described in this study. The CBM6 domains are found in a wide variety of glycosidases, such as cellulases, xylanases, mannanases, and agarases [1, 16, 29]. Proteins containing CBM6 have been described as binding to cellulose, xylan, mixed β-(1,3)(1,4)-glucan, and β-1,3-glucan [19, 36]. CBMs are believed to enhance the activity of the enzymes on insoluble substrates by increasing the effective enzyme concentration on the polysaccharide surface [16, 30]. Removal of CBM by proteolysis or genetic manipulation reduces the activity of the enzyme on insoluble polysaccharides, but not on soluble substrates [42]. Previous studies described that the presence of CaCl$_2$ did not show much effect on agarase activity [34, 36, 47]. In the present study, rAgy1 also showed no difference of agarolytic activity in the presence of Ca$^{2+}$ (Fig. 4D). Ca$^{2+}$ ions are reported to alter the bond cleavage preference of *Pseudomonas fluorescens* xylanase 10A, as well as protecting the enzyme from proteolytic attack [7, 39]. Glycoside hydrolases typically adopt a modular structure containing separate catalytic modules and noncatalytic modules. Carbohydrate binding modules (CBMs) are the most common noncatalytic modules, and probably enhance the activity of the catalytic modules by mediating a prolonged enzyme-substrate proximity [2, 5]. It has been reported that the thermostabilities were enhanced by binding of bivalent ions on the opposite site of the catalytic cleft in GH26 mannanase from *Bacillus subtilis* and GH5 cellulase from *B. subtilis* 168 [23, 37]. The thermal stability of rAgy1 was also involved in the calcium binding [26]. rAgy1 also showed a higher agarolytic activity after 90 min in the presence of, rather than the absence of, calcium ions.

In general, β-agarases can be divided into two types (type I and type II) according to their substrate specificity and product profile. The type I β-agarases degrade agarose into NA4, whereas the major product of the type II β-agarases is neoagarobiose [31]. rAgy1 produced neoagaro-oligosaccharides (mainly, NA4 and NA6), which is similar to the function of other β-agarase type I proteins. In previous reports, other bacterial β-agarase-derived neoagaro-oligosaccharides were used for their whitening and antioxidant activities [28, 35].

The gelling temperature of agar is ~38°C, and most of the reported β-agarases have shown optimal activity at
>40°C [15, 32]. The high activity and thermostability at temperatures higher than the gelling temperature of agar is an advantage for industrial oligosaccharide production from agar or marine algae [34].

The agarolytic activity of agarase is more efficient in liquid form than in solid form. However, rAgy1 showed optimum activity at the relatively high temperature of 55°C, indicating a potential application in the industrial production of neoagar-oligosaccharide directly from marine algae under cost-effective conditions [15]. The previously reported β-agarases have shown activity at a wide pH range. Vibrio sp. PO-303 (pH 4–8) [8], Vibrio sp. F-6 (pH 5–10) [13], Agarivorans sp. HZ105 (pH 6–10) [20], and Streptomyces coelicolor A3 (pH 4–9) [40] have also shown activity in a wide range of pH values.

The optimum pH of rAgy1 was 7.5, which corresponds to the weak alkaline pH of natural seawater and is similar to the optimum pH for the marine-derived agarases from Agarivorans albus (YKW-34) [14], Agarivorans sp. (LQ48) [27], Streptomyces coelicolor [41], and Saccharophagus degradans [24]. Moreover, the metal ions that are abundant in seawater, including Na⁺, K⁺, Ca²⁺, and Fe³⁺, also significantly affected the activity of rAgy1, similar to other previously reported agarases [4, 28, 44]. However, the activity of rAgy1 was found to be inhibited by Zn²⁺, Mn²⁺, and Cu²⁺. The underlying mechanism remains to be elucidated but may prove useful for manipulation of activity in industrial conditions.

In conclusion, an agarolytic bacterium was isolated from red seaweed, G. annansii, collected from Jeju Island and designated as Saccharophagus sp. AG21. The Saccharophagus sp. AG21 genome was found to encode a putative agarase, agy1. Subsequent cloning, sequencing, and TLC analysis of the overexpressed recombinant enzyme revealed that Agy1 could degrade agarose mainly into neoagarohexaose and neoagarotetraose. Thus, Agy1 was characterized as a β-agarase type I. Additionally, the biochemical activities of rAgy1 suggested several inherent features that may benefit the cosmetic, health, food, bioethanol, and chemical industries.

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**References**


