MOLECULAR CLONING, CHARACTERIZATION AND ENZYMATIC PROPERTIES OF A NOVEL BETA-AGARASE FROM A MARINE ISOLATE *PSUDOALTEROMONAS* SP. AG52

Chulhong Oh[1,3], Chamilani Nikapitiya[1], Youngdeuk Lee[1], Ilson Whang[2], Do-Hyung Kang[3], Soo-Jin Heo[3], Young-Ung Choi[3] and Jehee Lee[1,4]*

[1]Department of Marine Life Science, Jeju National University 66 Jejudaehakno, Ara-Dong, Jeju, 690-756, Republic of Korea; [2]Department of Life Science, Jeju National University 66 Jejudaehakno, Ara-Dong, Jeju, 690-756, Republic of Korea; [3]Korea Ocean Research & Development Institute, Ansan, 426-744, Republic of Korea; [4]Marine and Environmental Institute, Jeju National University, Jeju, 690-814, Republic of Korea.

ABSTRACT

An agar-degrading *Pseudoalteromonas* sp. AG52 bacterial strain was identified from the red seaweed *Gelidium amansii* collected from Jeju Island, Korea. A β-agarase gene which has 96.8% nucleotide identity to *Aeromonas* β-agarase was cloned from this strain, and was designated as a*ga*A. The coding region is 870 bp, encoding 290 amino acids and possesses characteristic features of the glycoside hydrolase family (GHF)-16. The predicted molecular mass of the mature protein was 32 kDa. The recombinant β-agarase (rAgaA) was overexpressed in *Escherichia coli* and purified as a fusion protein. The optimal temperature and pH for activity were 55 °C and 5.5, respectively. The enzyme had a specific activity of 105.1 and 79.5 unit/mg toward agar and agarose, respectively. The pattern of agar hydrolysis demonstrated that the enzyme is an endo-type β-agarase, producing neoagarohexaose and neoagarotetraose as the final main products.

Since, *Pseudoalteromonas* sp. AG52 encodes an a*ga*A gene, which has greater identity to *Aeromonas* β-agarase, the enzyme could be considered as novel, with its unique bio chemical characteristics. Altogether, the purified rAgaA has potential for use in industrial applications such as development of cosmetics and pharmaceuticals.

Key words: Agar; *Aeromonas sp.*; β-agarase; *Pseudoalteromonas* sp.; GHF-16; Neoagaro-oligosaccharides

INTRODUCTION

The main component of the cell wall of marine red algae (Rhodophyceaea) is agar, and it is composed of agarose (4) and agaropectin (12). Agarases are the hydrolytic enzymes mostly found in marine habitats (5, 39), which are responsible for the breakdown of agar and agar-derived compounds, and result in oligosaccharides that have various bioactivities (9, 25). Based on the pattern of hydrolysis of the substrates, agarases are grouped into α-agarases and β-agarases (3, 19). To date, most

of the agarolytic enzymes isolated, purified and characterized have been from microorganisms, with the majority of the genera being marine bacteria. Among these known agarases, most belong to the β-agarase group and few biochemical studies have been reported for α-agarases (39, 44). Several β-agarases have been purified and characterized from species including *Pseudoalteromonas* (43, 48), *Pseudomonas* (16, 17, 33), *Alteromonas* (19, 24, 29, 39, 49), *Agarivorans* (15, 20), *Vibrio* (2, 6, 14, 45), *Cytophage* (11, 47), *Bacillus* (46) and *Saccharophagus* (13). Cloning and expression of β-agarase encoding genes has been reported from *Pseudoalteromonas* (30), *Pseudomonas* (17, 27, 41), *Agarivorans* (28, 34), *Microbulbifer* (35-37), *Vibrio* (45, 50), *Zobellia* (22) and *Streptomyces* (23).

Our laboratory is currently conducting research on the characterization of a number of β-agarases and the biochemical properties of recombinant proteins, from various bacterial species isolated from the marine environment. In this study, *Pseudoalteromonas* sp. AG52 was isolated from the Jeju Island coastal environment, from which a β-agarase gene was subsequently isolated and designated as *aga*A. We cloned the gene, overexpressed the protein in *Escherichia coli* (*E. coli*) and purified the recombinant β-agarase (rAgaA) from *Pseudoalteromonas* sp. AG52. In addition, purified rAgaA was analyzed for biochemical properties such as specific activities and optimum reaction conditions. Until recently, the *Pseudoalteromonas* sp. β-agarase was an unknown entity. Herein we examine and describe in depth this enzyme, which upon analysis showed greater identity to *Aeromonas* β-agarase. The characterization of this novel enzyme could be beneficial to a variety of industries.

MATERIALS AND METHODS

Isolation of agarase-producing bacteria strain

Agarolytic bacteria were isolated from the red seaweed, *Gelidium amansii* from the south coast of Jeju Island, Republic of Korea. The crushed seaweeds were spread, on on SWT (0.3% Tryptone and 1.5% agar in seawater), SWY (0.3% yeast extract and 1.5% agar in seawater) and marine agar plates (Difco, Detroit, USA). Positive colonies showing clear zones or pits were picked out from the selection plates, and re-streaked The pure colonies were selected by repeat streaking under the same conditions and inoculated in their respective broths including 0.2% agar, then incubated at 30 °C. The stock was prepared from the bacteria culture using 20% glycerol, then samples were stored at -70 °C. Polymerase Chain Reaction (PCR) was performed for 16S rDNA sequence amplification from the extracted genomic DNA of isolated bacteria. The universal primers (16S-27F as forward and 16S-1492R as reverse) used for the PCR are shown in Table 1. The sequence was analyzed using the NCBI Blast N program and the DNAssist program.

Partial agaA gene amplification

All of the cloning experiments were carried out according to Sambrook et al. (42) with slight modifications. From analyzing the sequences of other agarase genes from NCBI database, three sets of forward and reverse primers were designed (Table 1) and those mix primers (Mix-AGA-F1 and R1, Mix-CY-F2 and R2, Mix-Sa-F3 and R3) were used for initial partial agarase amplification using genomic DNA as template and Ex Taq DNA polymerase (Takara, Japan).

Long and Accurate Polymerase Chain Reaction (LA PCR) for detection of full length agaA

The complete *agaA* was cloned using a LA PCR *in vitro* cloning kit (Takara, Korea), according to the manufacturer instructions. Genomic DNA was digested with separate restriction enzymes *BamH* I, *EcoR* I, *Hind* III and *Xho* I, and subsequently the digested products were ligated with a *BamH* I, *EcoR* I, *Hind* III and *Xho* I cassette, then used as a template for LA PCR. LA52-F1 and LA52-F2 primers and LA52-R1 and LA52-R2 primers were designed to identify the reverse and forward sequence from the known partial sequence of *agaA*, respectively (Table 1). The amplification of upstream or downstream of the known sequence was performed using LA52-R1 or LA52-F1 with C1 (from the cassette nucleotide

sequence), respectively. Taking the resultant product as the template, PCR was performed to obtain upstream or downstream of the known sequence using LA52-R2 or LA52-F2 with C2 (from the cassette nucleotide sequence), respectively. Product was sequenced, and the gene was analyzed by nucleotide BLAST and Protein BLAST of National Center for Biotechnology Information (NCBI)

database. The signal peptide sequence of AgaA was predicted through a SignalP program (http://www.cbs.dtu.dk/services/SignalP/). The LipoP 1.0 Server (http://www.cbs.dtu.dk/services/LipoP/) was used for a gram-negative bacteria lipoprotein site search analysis. Identity and percent similarity of full-length amino acid sequences were calculated using FASTA program (38).

Table 1. Oligonucleotide primers

Name	Object	Sequence (5' to 3' direction)
16S-27F	16S rDNA sequence amplification	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence amplification	TACGGYTACCTTGTTACGACTT
Mix-AGA-F1	*aga*A partial sequence amplification	CWTCKTATATWAATGCTTGGC
Mix-AGA-R1	*aga*A partial sequence amplification	TGGYTGRTAATCTTGAAATGG
Mix-CY-F2	*aga*A partial sequence amplification	YTNGARTAYTAYATHGAYGG
Mix-CY-R2	*aga*A partial sequence amplification	TTRTANACNCKDATCCARTC
Mix-Sa-F3	*aga*A partial sequence amplification	TCNATHCAYYTNTAYGAYTTYCC
Mix-Sa-R3	*aga*A partial sequence amplification	CCAYTCNGCYTTNACNGG
52LA-F1	LA PCR Forward 1	TCGTCGCTACGGTGTTCATTGGAA
52LA-F2	LA PCR Forward 2	TAGTTCGCAGCGTTTCAGGTCCTA
52LA-R1	LA PCR Reverse 1	ACGTGCATACGTTGGTCAAACCAC
52LA-R2	LA PCR Reverse 2	TGCCTCCATCGCATCAATTTCCTG
C1	Cassette primers	GTACATATTGTCGTTAGAACGCGTAATACGACTCA
C2	Cassette primers	CGTTAGAACGCGTAATACGACTCACTATAGGGAGA
Ag52-F1	Cloning to pET-16b	(GA)$_3$ CATATGGCAGATTGGGACGCATATAGTA (*Nde* I)
Ag52-R2	Cloning to pET-16b	(GA)$_3$ GGATCCTTAGTTTGCTTTGTAGACACGTATC (*BamH* I)

Cloning of AgaA coding sequence into the expression vectors

Primer set Ag52-F1 and Ag52-R2 were designed with its corresponding restriction enzyme sites (Table 1) to clone the coding sequence into the pET16b expression vector (Novagen, USA), without including its signal sequence. The vector and PCR products were digested with restriction enzymes, ligated and transformed into *E. coli* DH5α cells, and correct recombinants (confirmed by restriction enzyme digestion and sequencing) were transformed into *E. coli* BL21(DE3). Recombinant cells were overexpressed in the presence of

isopropyl-ß-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Briefly, 5 mL of an overnight grown BL21(DE3) starter culture was inoculated into 100 mL Luria broth with 100 μL ampicillin (100 mg/mL) and 10 mM glucose (0.2% final concentration) and incubated at 37 °C. Recombinant pET-16b- AgaA cells were induced for 24 h at 12 °C, followed by cell harvesting (centrifugation at 4000 x g for 20 min at 4 °C). The cells carrying the pET-16b- AgaA were resuspended in 5 mL ice-cold 1x binding buffer (8x = 4M NaCl, 160 mM Tris HCl, 40 mM imidazole, pH 7.9) and frozen at -20 °C overnight. After thawing on ice, the bacterial cells

were sonicated and supernatant was taken as a crude enzyme after centrifugation. The crude rAgaA fusion protein fused with His tag was purified using the His Bind purification Kit (Novagen, USA). Elutes were collected in 500 μL fractions and respective elutes were run on 12% SDS-PAGE. The concentrations of purified proteins were determined by the method of Bradford using bovine serum albumin (BSA) as the standard.

Agarase enzyme assay

Specific activity of purified rAgaA was determined according to a modified method of Ohta et al. (35) using different substrates including 1% food-grade agar, 1% agarose and 1% carrageenan. Appropriately diluted enzyme solution was added to different substrates in phosphate buffer (pH 7.0) at 45 ^{o}C, and was incubated at 45 ^{o}C for 30 min. Activity was expressed as the initial rate of agar hydrolysis by measuring the release of reducing ends using the 3,5-dinitrosalicylic acid (DNS) procedure (32) with D-galactose as the standard. One unit of the enzyme activity was defined as the amount of protein per min produced 1 μmol of reducing sugar as D-galactose under condition of the assay.

Biochemical characterization of rAgaA

In each experiment, 1% agar solution and purified agarase were mixed and incubated at various times and temperatures as described. The relative agarase activity was determined by the DNS method. The optimum temperature of *r*AgaA activity was determined by monitoring the relative enzymatic activity at temperatures ranging from 40-65 ^{o}C with 5 ^{o}C intervals at pH 7.0. Optimum pH was tested from pH 4.5-9.0 with pH 0.5 intervals at 45 ^{o}C. Acetate buffer and phosphate buffer were used for pH 4.5-6.0 and pH 6.5-9.0, respectively. The thermostability of *r*AgaA was evaluated by measuring the residual activity of the enzyme after incubation at the temperatures between 40-55 ^{o}C for 30, 60 and 120 min. The effects of various metal ion salts and chelators on purified *r*AgaA activity were tested by determining the activity in the presence of 2 mM of various ions or chelators (CaCl$_2$, CuSO$_4$,

FeSO$_4$, KCl, MgSO$_4$, MnCl$_2$, NaCl and EDTA) in a final concentration and incubated at 45 ^{o}C for 30 min. The control was the assay mixture, with no addition of metal ion salts or chelators.

Identification of rAgaA hydrolyzed agar products

Thin layer chromatography (TLC) was used to identify the hydrolysis products of agar and neoagarooligosaccharides. Neoagarohexanitol (NA6) was purchased from Sigma (USA) and neoagarotetraose (NA4) and neoagarobiose (NA2) (NA4 + NA2) were prepared by digestion of neoagarohexanitol using commercial β-agarase (New England Biolab, USA). D-(+)-galactose was purchased from Sigma (USA), and all above mentioned oligosaccharides were used as standards. Moreover, food-grade agar and NA6 were used as substrates for the reactions. The reaction of purified agarase and agar was carried out in 200 μL reactions containing 20 μL of purified agarase and 180 μL of 1% agar at 45 ^{o}C for 30, 60, and 120 min. The NA6 substrate was incubated separately with 20 μL of purified agarase at 45 ^{o}C for 120 min. Subsequently, the reaction mixtures were applied to a silica gel 60 TLC plate (Merck, Germany). The TLC plates were developed using a solvent system consisting of n-butanol: acetic acid: water (2:1:1, v/v). After hydrolysis of substrates, the resultant oligosaccharide spots were visualized by spraying 10% H$_2$SO$_4$ on the plate, then heating it on a hot plate.

Nucleotide sequence accession number

The *Pseudoalteromonas* sp. AG52 β-agarase nucleotide sequence was submitted to the NCBI database with accession number FJ979637.

RESULTS

Identification of the agarase-producing bacteria

Initially, agarase-producing marine bacteria were isolated from selection plates showing clear zones. The 16S rRNA sequence analyses results showed that the isolated bacteria from seaweed was 99% similar to *Pseudoalteromonas* sp. and

is assigned to the genus *Pseudoalteromonas* and named *Pseudoalteromonas* sp. AG52. This strain was deposited in Korean Culture Collection Center of Microorganisms (KCCM), Rep. of Korea with accession number KCCM 42924.

Cloning of *β*-agarase from *Pseudoalteromonas* sp AG52

The partial *aga*A sequence (651 bp) followed by full length sequence was amplified as described in Materials and Methods. The nucleotide and deduced amino acid sequences of *Pseudoalteromonas sp.* AG52 β-agarase is shown in Figure 1. The *aga*A gene open reading frame (ORF) consists of a 870 bp encoding a protein of 290 amino acids. The *aga*A has a putative molecular mass of 32 kDa with an isoelectric point of 5.8. The signal peptide (1-21 aa) and lipoprotein signal peptide (signal peptidase II) were identified in the N-terminal sequence. Additionally, a characteristic GHF-16 β-agarase family domain (Cys^{22}-Lys^{287}), catalytically active site residues (Tyr^{69}, Asp^{71}, Trp^{72}, Trp^{139}, Ser^{145}, Asp^{150}, Glu^{153}, Phe^{176}, Arg^{178}, Glu^{257}, Glu^{259}), and calcium binding residues (Gln^{47}, Phe^{48}, Asn^{49}, Gly^{91}, Ala^{92}, Asp^{282}, Trp^{283}) were identified in the sequence. The *aga*A nucleotide sequence showed 96.4% and 74.9% nucleotide identity to β-agarase sequence of *Aeromonas* sp. (Accession number U61972) and *Pseudoalteromonas atlantica* (accession number M73783), respectively. Database searches using BLASTP yielded results showing homology to other known GHF-16 family agarases. Pairwise comparison of AgaA amino acid sequence to known agarases is shown in Table 2. Interestingly, AgaA amino acid sequence shares 96.9% identity and 99.3% similarity with *Aeromonas* sp. β-agarase (accession number AAF03246). The *P. atlantica* β-agarase coding sequence (accession number AAA91888) shares only 84.5% identity, and 92.4% similarity with AgaA. The multiple alignment shows conserved catalytic and calcium binding residues of AG52 when compared with other GHF-16 agarases (Figure 2). The phylogenetic analysis of the AgaA amino acid sequence including known β-agarase amino acid sequences of different members of the β-agarase family (GHF16, 52 and 82) was constructed using the neighbor-joining method as shown in Figure 3. The AgaA coding sequence was clustered with GHF-16 β-agarases, and formed a monophyletic clade with β-agarase of *Aeromonas* sp. and *P. atlantica* (AAA91888). However, it was also closely grouped with *Aeromonas* β-agarase.

Purification and Biochemical characterization of rAgaA

Successfully purified rAgaA agarase in pET-16b as a fusion protein was approximately 33 kDa, which was in agreement with the predicted molecular mass of AgaA (Figure 4), hence enzyme characterization was carried out with the fusion protein.

The specific activity of purified rAgaA towards agar and agarose were 105.1 and 79.5 unit/mg, respectively. The optimum reaction temperature of the purified rAG52 was 55 $^{\circ}$C (Figure 5A), however, more than 50% of the relative activity was observed between 40 and 60 $^{\circ}$C. The effect of pH on purified enzyme is shown in Figure 5B. The maximal activity was observed at pH 5.5 and the enzyme was stable (more than 60% relative activity) in the range of buffers from pH 4.5-9 under the conditions of the assay. The temperature dependence of the rAgaA activity on agar was determined by measuring the activity at various temperatures for 120 min. The effect of temperature on the stability of rAG52 is shown in Figure 5C. The thermostability of rAgaA was retained upto 80% at 40 $^{\circ}$C for 30 min. When temperature was increased to 45, 50 and 55 $^{\circ}$C for 30 min, the enzyme relative activity was decreased to 20-30%. Nevertheless, the enzyme was fairly stable (50% relative activity) at 40 $^{\circ}$C for 60 min of incubation. Moreover, addition of 2 mM CaCl$_2$ to the reaction mixture at 40 $^{\circ}$C was enhanced the thermostability by 20 and 15% at 60 and 120 min, respectively (Figure 5D). The effect of metal ion salts and chelators on the activity of purified rAgaA enzyme is shown in Figure 6. rAgaA relative activity was totally inhibited by divalent metal salts such as CuSO$_4$ and ZnSO$_4$ (each at 2 mM final concentration). Moreover, 2 mM EDTA inhibited the enzyme activity by 60%. In contrast, 2 mM FeSO$_4$ and 2 mM KCl enhanced the activity of rAgaA more than the other metal ion salts present in seawater.

```
                                   GGTATTTTCATAAGCTTGAGTTTGAATA        28
TGGATACAAATAATAGAAGGTACACACAAAAGAGATTGTTTCATCTAGGGCCTGTTTATCTTTCGATGA        97
TTAAATTCACAAAAGTCACTCGCACTAGTTAAAGAAGCATATCTACATTAATTTGCATGGAGATTTTAT       166
ATGAATATATTAAAAACTACTATCCTGTTCTACTTGCGCAATACTCTGCACAGCAACACATGCTGCAGAT       235
 M--N--I--L--K--L--L--S--C--S--T--C--A--I--L--C--T--A--T--H--A--A--D--     23
TGGGACGCATATAGTATTCCGGCTTCTGCTGGATCAGGTAAAACATGGCAATTACAAACTGTTTCCGAC       304
 W--D--A--Y--S--I--P--A--S--A--G--S--G--K--T--W--Q--L--Q--T--V--S--D--     46
CAATTTAACTACCAAGCCGGTACTTCAAATAAACCGGCAGCATTTACCAATCGTTGGAATGCTTCGTAT       373
 Q--H--N--Y--Q--A--G--T--S--N--K--P--A--A--F--T--N--R--W--N--A--S--Y--     69
ATTAATGCTTGGCTTGGGCCTGGTGATACTGAATTCAGTTCAGGTCATTCCTACACTACTGGTGGTGCG       442
 I--N--A--W--L--G--P--G--D--T--E--F--S--S--G--H--S--Y--T--T--G--G--A--     92
TTAGGCCTTCAGGCAACTGAAAAAGCAGGAACAAATAAAGTGCTTGCAGGAATTGTTTCTTCAAAAGCA       511
 L--G--L--Q--A--T--E--K--A--G--T--N--K--V--L--A--G--I--V--S--S--K--A--    115
ACTTTTACATACCCACTTTATCTTGAGGCAATGGTAAAACCGAGTAATAACACTATGGCTAATGGTGTA       580
 T--F--T--Y--P--L--Y--L--E--A--M--V--K--P--S--N--N--T--M--A--N--G--V--    138
TGGATGTTGAGCTCTGATTCAACTCAGGAAATTGATGCGATGGAGGCATACGGCAGTGATCGTGTAGGG       649
 W--M--L--S--S--D--S--T--Q--E--I--D--A--M--E--A--Y--G--S--D--R--V--G--    161
CAAGAGTGGTTTGACCAACGTATGCACGTAAGTCACCATGTTTTTATACGTGAGCCATTTCAAGATTAC       718
 Q--E--W--F--D--Q--R--M--H--V--S--H--H--V--F--I--R--E--P--F--Q--D--Y--    184
CAACCAAAAGATGCAGGCTCTTGGGTATACAATAACGGCGAAACATACCGAAATAAATTTCGTCGCTAC       787
 Q--P--K--D--A--G--S--W--V--Y--N--N--G--E--T--Y--R--N--K--F--R--R--Y--    207
GGTGTTCATTGGAAGGACGCGTGGAACCTAGATTACTATATTGATGGTGTATTAGTTCGCAGCGTTTCA       856
 G--V--H--W--K--D--A--W--N--L--D--Y--Y--I--D--G--V--L--V--R--S--V--S--    230
GGTCCTAATATAATTGATCCTGAAGGCTATACCGGTGGCACAGGGCTAAGTAAACCAATGCACATCCTT       925
 G--P--N--I--I--D--P--E--G--Y--T--G--G--T--G--L--S--K--P--M--H--I--L--    253
TTAGATATGGAACATCAACCTTGGCGTGATGTAAAACCAAATTCAGCCGAGCTAGCTGATTCAAACAAA       994
 L--D--M--E--H--Q--P--W--R--D--V--K--P--N--S--A--E--L--A--D--S--N--K--    276
AGTATATTTTGGATTGACTGGATACGTGTCTACAAAGCAAACTAAGTCATTCTAAAATATTTGTAATAT      1063
 S--I--F--W--I--D--W--I--R--V--Y--K--A--N--*                              290
TAGGTTTTATTGCTTCTCGTTATACGACACGGAGCAATAAACTTTAAGGTCCCCAAAACTACTTAATGC      1132
GGCTATTACAGCCGCATTAAGTATAATTAACCTGAACTCTGGATAGTAAATCTATCTCGAGCAGCTATT      1201
GACGCGTGAATTCTCTCCCTATAGTGAGTCGTA                                        1234
```

Figure 1. The nucleotide and deduced amino acid sequences of the β-agarase of *Pseudoalteromonas* sp. AG52. The predicted lipoprotein signal peptide is underlined and signal peptide sequence is in bold face. The start (ATG) and stop (TAA) codons are in bold italics and stop codon is marked with an asterisk (*). The GHF-16 β-agarase domain is in italics. Active sites and calcium binding residues are in boxes and dotted boxes, respectively.
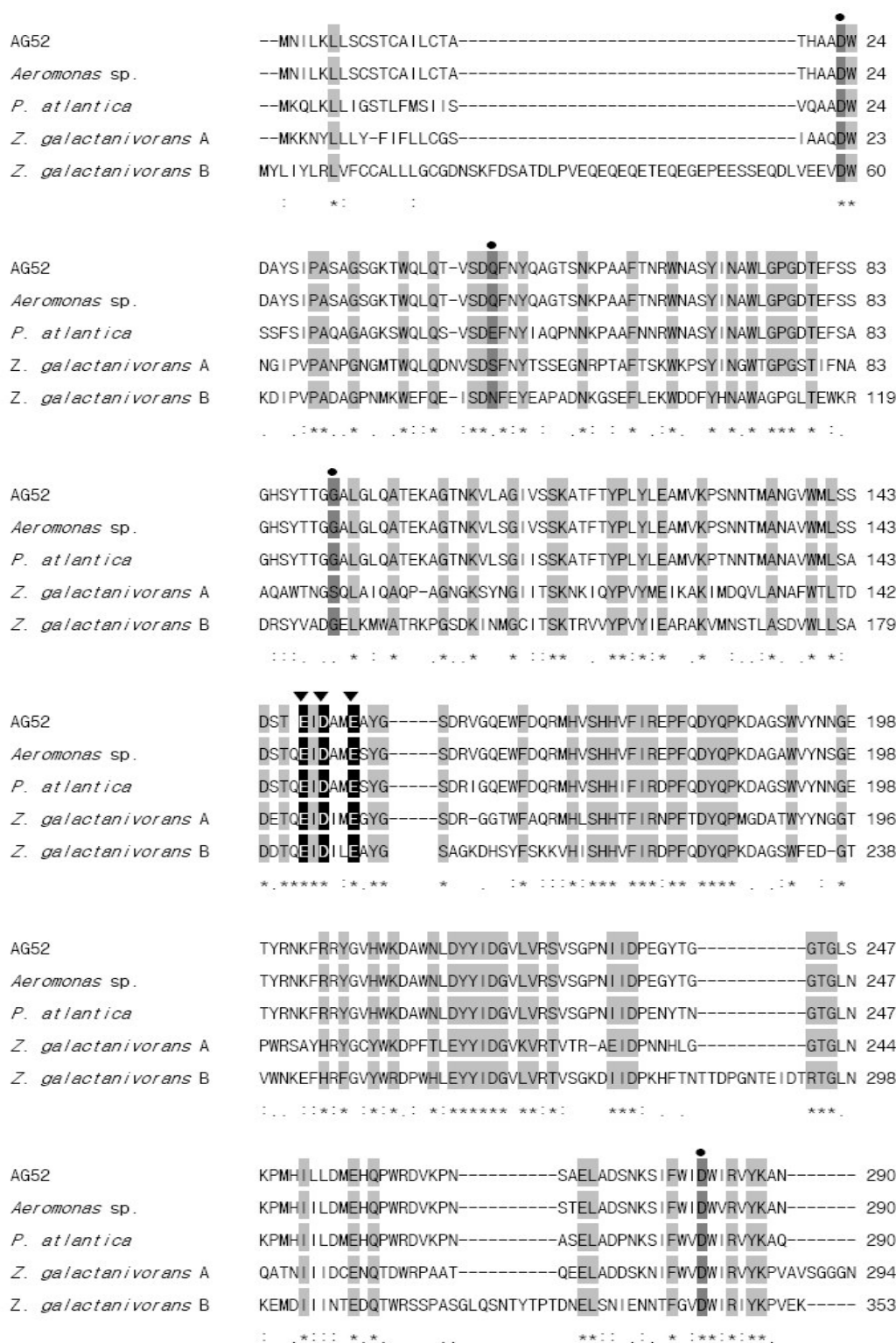
Figure 2. Multiple alignment of β-agarase amino acid sequences of *Pseudoalteromonas* sp. AG52 with known agarases. The inverted triangles (▼) highlight the conserved catalytic residues, and black circles (●) represent the conserved residues involved in calcium ion binding. Identical residues in all sequences are shaded in gray and indicated by (*) under the column, conserved substitutions are indicated by (:), and semi-conserved substitutions are indicated by (.). Deletions are indicated by dashes. Sequence sources: *Aeromonas* sp. β-agarase (AAF03246), *Pseudoalteromonas atlantica* β-agarase I (AAA91888), *Zobellia galactanivorans* β-agarase A (AAF21820), *Zobellia galactanivorans* β-agarase B (AAF21821).

Table 2. Pairwise analysis and comparisons of the deduced amino acid sequence of *Pseudoalteromonas* sp. AG52 β-agarase with other known β-agarases.

| Species	Accession number	Identity (%)	Amino acids
Aeromonas sp. β-agarase	AAF03246	96.9	290
Pseudoalteromonas atlantica β-agarase	AAA91888	84.5	290
AguD uncultured bacterium β-agarase	AAP49316	45.2	449
Zobellia galactanivorans β-agarase B	AAF21821	39.3	353
Pseudomonas sp. ND137 agarase	BAB79291	38.7	441
Microbulbifer thermotolerans agarase	BAD29947	36.1	433
Microbulbifer elongatus agarase	BAC99022	35.6	441
Cellvibrio sp. OA-2007 putative agarase	BAH16616	32.3	596
Saccharophagus degradans β-agarase I	AAT67062	26.8	593
Zobellia galactanivorans β-agarase A	AAF21820	26.0	539
Pseudoalteromonas sp. CY24 agarase	AAN39119	24.1	453
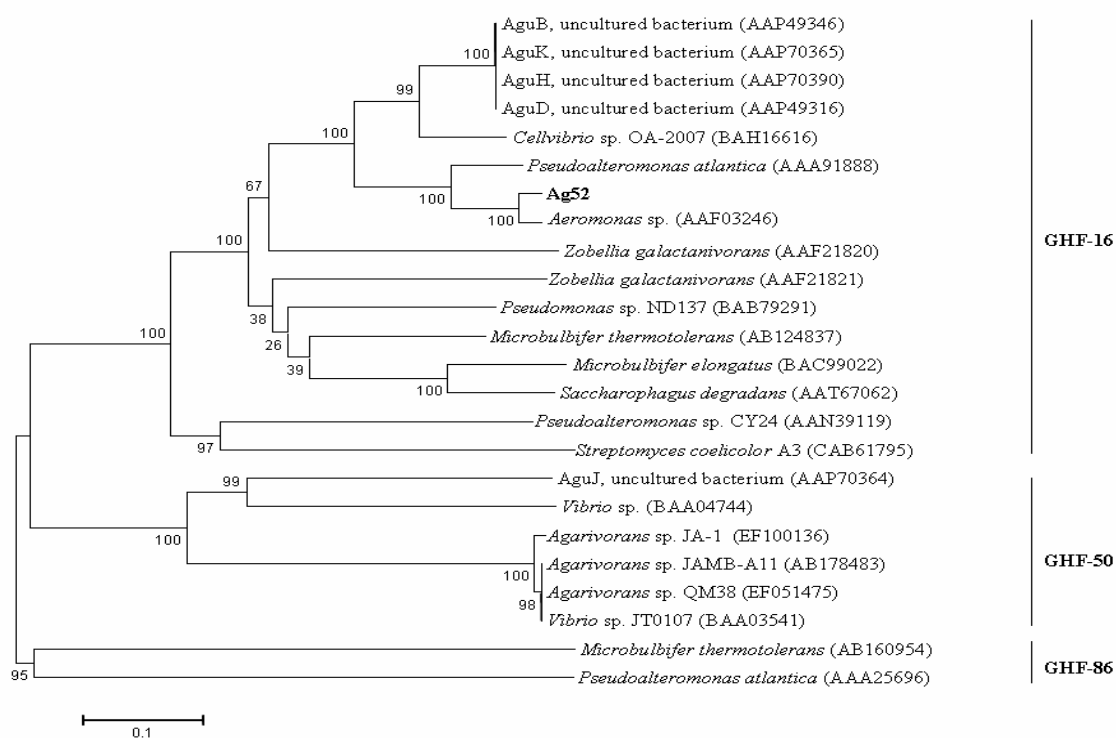
Figure 3. Phylogenetic analysis of AgaA with known agarases based on amino acid sequence. Phylogenetic analysis was done by the Neighbor Joining method using MEGA3.1, based on sequence alignment using ClustalW (1.81). Numbers indicate the bootstrap confidence values of 1000 replicates. The accession numbers of the selected agarase sequences are as follows: AB178483, agarase (*Agarivorans* sp. JAMB-A11); EF051475, QM38 agarase (*Agarivorans* sp. QM38); EF100136, β-agarase (*Agarivorans* sp. JA-1); AAA25696, β-agarase precursor (*Pseudoalteromonas atlantica*); AAP49346, AguB; AAP70390, AguH; AAP70365, AguK; AAP49316, AguD from uncultured bacterium; AAA91888, β-agarase I (*Pseudoalteromonas atlantica*); AAF03246, β-agarase (*Aeromonas* sp.); AB124837, agarase (*Microbulbifer thermotolerans*); BAC99022, agarase (*Microbulbifer elongatus*); BAB79291, agarase, (*Pseudomonas* sp. ND137; AAF21821, β-agarase B precursor (*Zobellia galactanivorans*); AAF21820, β-agarase A precursor (*Zobellia galactanivorans*); AAN39119, extracellular agarase precursor, (*Pseudoalteromonas* sp. CY24); CAB61795, extracellular agarase precursor (*Streptomyces coelicolor* A3); AAP70364, AguJ (uncultured bacterium); BAA04744, β-agarase (*Vibrio* sp.); BAA03541, β-agarase (*Vibrio* sp. JT0107); BAH16616, agarase (*Cellvibrio* sp. OA-2007); AAT67062, β-agarase I (*Saccharophagus degradans*); AB160954, β-agarase (*Microbulbifer thermotolerans*).
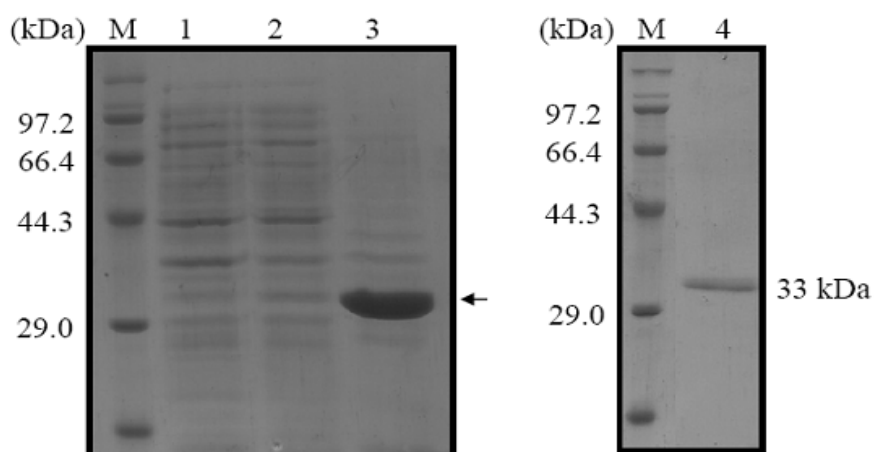
Figure 4. SDS-PAGE of the rAgaA. Samples of rAgaA were separated on 12% SDS-PAGE and stained with Coomassie brilliant blue. M: molecular mass marker (BioRad, USA). Lane 1: total cellular extract from *E. coli* BL21 (DE3) before induction; lane 2: total cellular soluble extract after induction; lane 3: total cellular insoluble extract after induction; lane 4: purified rAgaA.
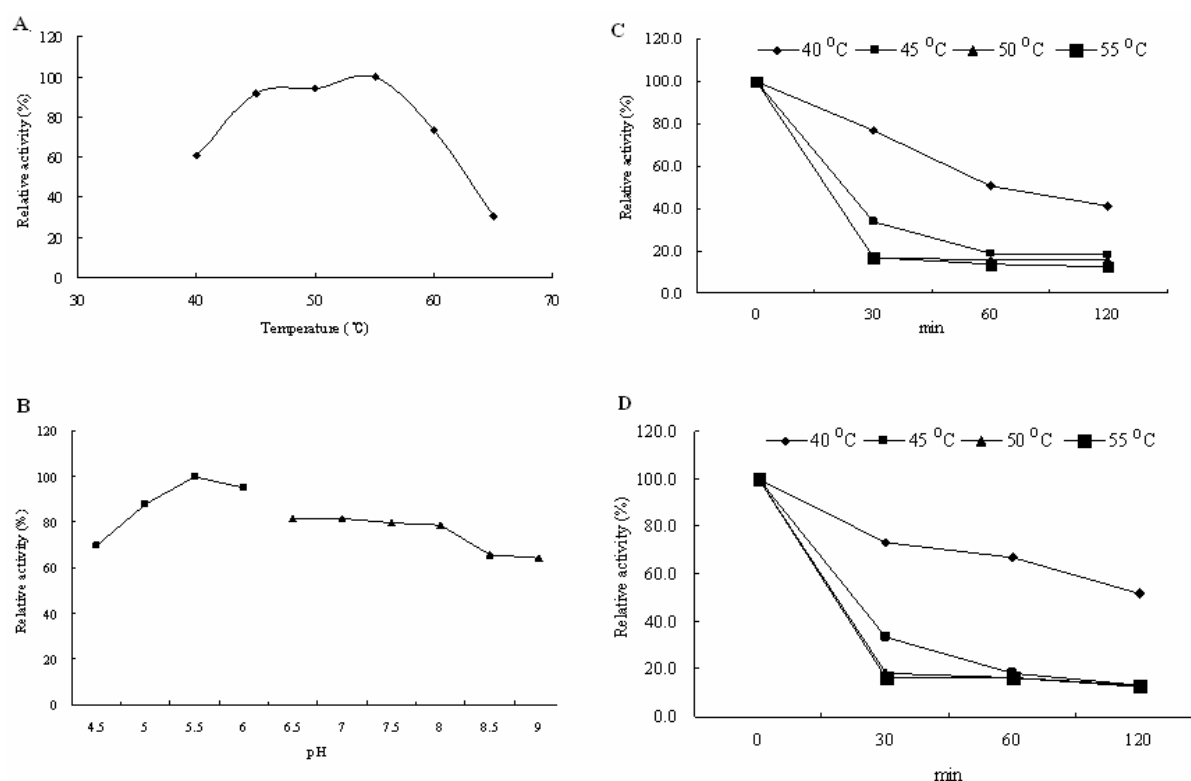

Figure 5. Characterization of biochemical properties of purified rAgaA. **A**. The effect of temperature on the rAgaA. The effect of temperature on enzyme activity was determined under standard assay conditions as described in Materials and Methods, at temperatures ranging between 40-65 °C. **B.** The effect of pH on the activity of rAgaA. Optimum pH for rAgaA activity was examined from pH 4.5-9.0 at pH 0.5 intervals at 45 °C under standard assay conditions (described in Materials and Methods) using acetate (pH 4.5-6.0) and phosphate buffer (pH 6.5-9.0). **C.** The effect of thermostability on rAgaA at different temperatures at different time points. Thermostability was determined by measurement of residual activity under standard assay conditions as described in Materials and Methods at temperatures between 40-55 °C for 30, 60 and 120 min. **D.** The effect of 2 mM $CaCl_2$ on thermostability of rAgaA.
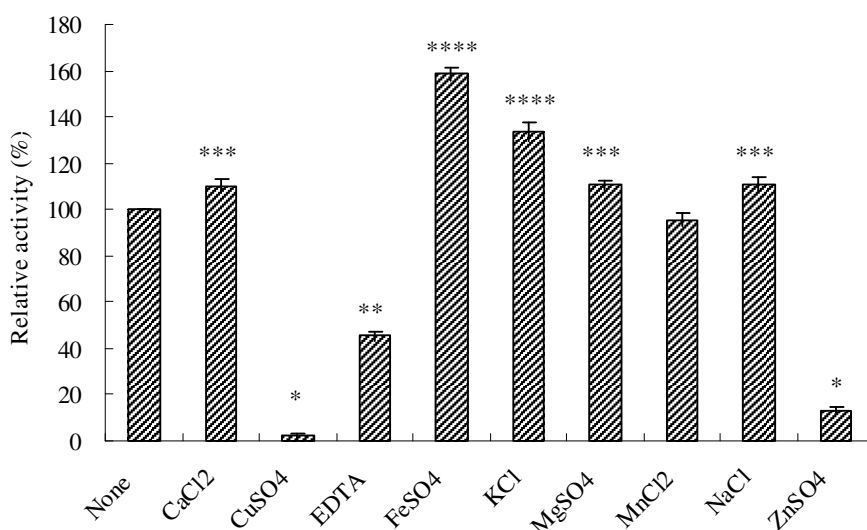
Figure 6. The effects of metal ions and metal salts on the activity of purified rAgaA. Various ions or chelators (CaCl$_2$, CuSO$_4$, FeSO$_4$, KCl, MgSO$_4$, MnCl$_2$, NaCl and EDTA) at a final concentration of 2 mM were included in the reaction buffer to test the activity of rAgaA at 45 ^{o}C for 30 min. The data presented are the average of three replicates. Means with the same number of stars are not significantly different at p<0.05, based on ANOVA. Error bars represent $\pm$ SD.

Identification of hydrolysis products of the rAgaA by TLC

Hydrolysis patterns of the purified rAgaA against food-grade agar and neoagarohexanitol (NA6) are shown in Figure 7. When rAgaA was incubated with agar, two distinct spots namely, NA6 and NA4 were observed on TLC plates at 30, 60 and 120 min after the reaction. Furthermore, the distinct spot of NA4 was observed when rAgaA was incubated with NA6.
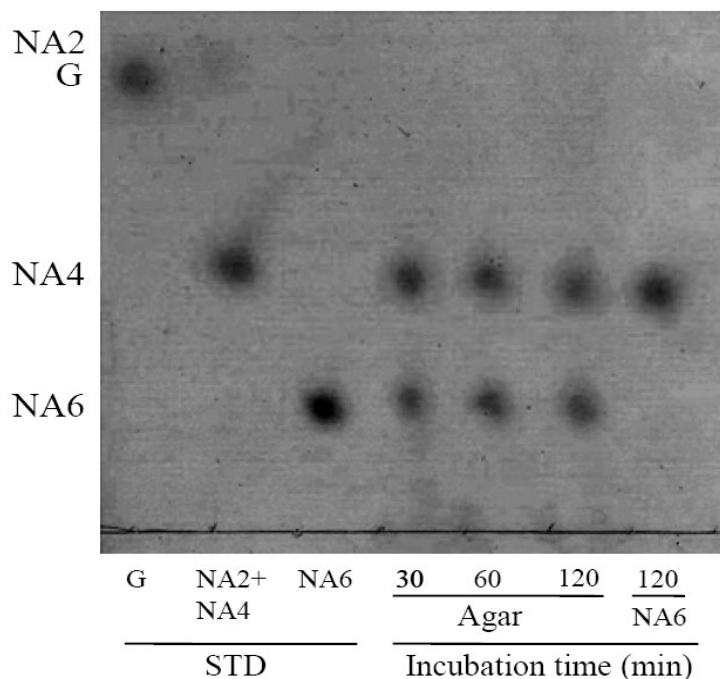

Figure 7. TLC of hydrolysis products of the purified rAgaA on food-grade agar and neoagaro-oligosaccharides. The assay of rAgaA and agar were performed in 200 μl reactions containing 20 μL of purified agarase and 180 μL of 1% agar at 45 ^{o}C for 30, 60, and 120 min. The NA6 substrate was incubated with 20 μl of rAgaA separately at 45 ^{o}C for 120 min. Neoagarohexaitol (NA6), neoagarotetraose (NA4), neoagarobiose (NA2) and D-(+)-galactose (G) were used as standards (STD).

DISCUSSION

In this study, a newly found marine bacterial isolate was assigned to the genus *Pseudoalteromonas* based on the 16S rDNA sequence analysis. We report herein the cloning and sequencing of the β-agarase gene with β-agarase activity from *Pseudoalteromonas* sp. AG52, which has greater identity to the *Aeromonas* β-agarase coding sequence than the *Pseudoalteromonas* sp. β-agarase coding sequence in the NCBI Genbank. It was shown in this study that *aga*A was genetically closely related to the GHF-16 β-agarases, and hence this gene should be classified into the GHF-16 family. The primary structure of the *Pseudoalteromonas* sp. AG52 β-agarase shows regions homologous to GHF-16 family members, which include catalytic domains belonging to GHF-16, which hydrolyze the internal β-1,4-linkage of agar, producing neoagarooligosaccharides. BLASTP and pairwise analysis of *aga*A full length coding sequence with other agarases which belong to the GHF-16 family showed overall sequence identities ranging from 24-97%. Some of the β-agarases encode for a modular protein consisting of a signal peptide, catalytic module and C-terminal domain of unknown function or a carbohydrate binding module (1, 35), and this may be the reason for having low identity, even within the same family. It has been reported that homologous regions in most of the reported GHF-16 agarases were present in catalytic module of the β-agarases (1). This further confirms the heterogeneity of the amino acid sequences in length, catalytic properties and substrate specificities in agarases. Furthermore, the results of the phylogenetic analysis support the idea that many β-agarases may have evolved from a common ancestral form, and, that domain shuffling may contribute significantly to the diversity of the agarases (7,10). Even though the protein is divergent from the primary sequences, AgaA features strictly conserved catalytic residues, which show conservation among the GHF-16 family members. Glutamic and aspartic acid are the highly conserved active site residues, which are responsible for catalytic activity in the GHF (30). According to previous reports (1, 31), the conserved Glu[148] and Glu[153] are responsible

for acting as the nucleophile and the acidic/basic residues, respectively, in AgaA. The third conserved acidic residue at Asp[150], is responsible for maintaining the charges in the environment of catalytic amino acids. The lipoprotein signal peptide is one of the secretion systems reported for gram negative bacteria (40) and such a signal was predicted in the AgaA sequence at the N-terminal where Cys[16] may be linked to the lipid moiety. However, in this study, the cleavage of N-terminal hydrophobic segment is by either signal peptidase I or signal peptidase II is not known clearly and remains to be elucidated. Using pET16b, intracellular AgaA was expressed efficiently and purified as a fusion protein. The purified agarase had a molecular mass of 33 kDa, which is close to those reported for β-agarases from *Pseudoalteromonas* sp. N-1 (33 kDa) (48) and *Pseudomonas atlantica* (32 kDa) (33); but, smaller than agarases reported for *Alteromonas* sp. (52 kDa) (29) and *Pseudomonas* sp. W7 (59 kDa) (17); and, larger than agarases reported for *Vibrio* sp. AP-2 (20kDa) (2). AgaA hydrolyzes agar to give NA6 and NA4 as the main products, suggesting that the enzyme is a β-agarase, in accordance with most of the reported GHF-16 β-agarases (1, 43).

Generally, most of the GHF-16 β-agarases that have been characterized so far, have optimal function at 40 $^\circ$C and pH optimums in the range of neutral to mild alkaline (50). rAgaA has an optimum temperature (55 $^\circ$C) that is higher than the gelling temperature of agar (40 $^\circ$C) with broad range of pH. These are properties which are useful for the production of industrially important oligosaccharides from marine algae or agar. These findings are in contrast with earlier reported pH and temperature optima at 7 and 30 $^\circ$C, respectively, for a purified β-agarase from *Pseudoalteromonas* sp. (48). A temperature optimum at 40 $^\circ$C and a pH optimum of 6.0 have been reported for recombinant β-agarase AgaB from *Pseudoalteromonas* sp. CY24 (30). In contrast to the experimentally determined optimum temperature of rAgaA activity, thermal stability profiles of this protein showed that the enzyme is stable under 45 $^\circ$C even after 30 min incubation, however, it becomes inactivated at higher temperatures. The enzyme at 40 $^\circ$C retained 50% activity even after 1 h of

incubation, indicating that this enzyme might be used under mild heating conditions. In addition, the enzyme stability was considerably enhanced in the presence of $CaCl_2$, and enzyme retained more than 15% of its initial activity after 1 and 2 h of incubation with $CaCl_2$. Several published studies support these results, where a major role is proposed for Ca^{2+} in stabilization of enzymes at higher temperatures (18, 21). It was reported that the reason for this may be due to the strengthening of interactions inside the protein molecule, and probably by the binding of Ca^{2+} to an autolysis site (8, 26). It has been reported that a catalytic module of β-agarase (β-AgaA_CM) of *Z. galactetanivorans* Dsij, had Ca^{2+} bound on the convex face of the protein in an octahedral geometry, coordinating with the backbone carbonyl oxygen atoms of Ser^{47}, Ser^{91}, and Asp^{279}, a carboxylate oxygen of Asp^{279}, Asn^{49}, and Asp^{22}, and one water molecule. In the β-agarase (β-AgaB) of *Z. galactetanivorans* Dsij, the Ca^{2+} was positioned in the same locations in a pentahedral manner to the backbone carbonyl oxygen atom of Asn^{83}, Gly^{127}, and Asp^{343}, a carboxylate oxygen of Asp^{343}, and one water molecule (1). Interestingly, in AG52, the Ca^{2+} binding residues were located at the same positions at Gln^{47}, Gly^{91} and Asp^{282}, however, the precise geometrical arrangement of Ca^{2+} binding needs to be investigated in future. In addition, analysis of the amino acid sequence for *aga*A confirms the evidence that the Asp, which is responsible for side-chain interaction with Ca^{2+}, is conserved among the GHF-16 family members (1). Moreover, among the metal ions, which are mainly present in seawater (Na^+, Mg^{2+}, K^+ and Ca^{2+}), K^+ shows considerable effect on rAgaA activity, suggesting that the red algae is originating from the marine environment. It is possible that the reason that Zn^{2+} has an inhibitory effect on rAgaA might be the structural alteration of the enzyme due to the affinity of the heavy metals for the SH, CO and NH moieties of the amino acids in the protein.

In conclusion, a marine bacterium *Pseudoalteromonas* sp. was isolated from red algae in the Jeju island (Korea) coastal environment. AgaA features homologous catalytic domains belonging to the GHF-16 family, and has characteristic properties indicating neoagarooligosaccharide production. Due to the reported functional properties of the neoagarooligosaccharides (25) when obtained from agar, the purified r rAgaA enzyme has potential for usage in industries for the production of pharmaceuticals and cosmetics. To further advance the research, the crystal structure and three-dimensional structure analyses need to be carried out in order to understand the mechanism of the catalysis of rAgaA in detail, and thereby investigate the structure-function relationships of the protein. Since no primary structural and functional characteristics have been reported for *Aeromonas* β-agarases to date, the current investigation will be useful for studying the structure, function and evolution of *Aeromonas* β-agarase as well.

ACKNOWLEDGEMENTS

REFERENCES

1. Allouch, J.; Jam, M.; Helbert, W.; Barbeyron ,T.; Kloareg, B.; Henrissat, B.; Czjzek, M. (2003). The three-dimensional structures of two beta agarases. *J. Biol. Chem.* 278, 47171-47180.

2. Aoki, T.; Araki, T.; Kitamikado, M. (1990). Purification and characterization of a novel beta-agarase from *Vibrio* sp. AP-2. *Eur. J. Biochem.* 187, 461-465.

3. Araki, C. (1959). Seaweed polysaccharides. In: Wolfrom ML (ed.), Carbohydrate chemistry of substances of biological interests. Pergamon Press, London, pp 15-30.

4. Araki, C. (1966). Some recent studies on the polysaccharide of agarophytes. In: Young EG, Maclachan JL (ed.), Proceedings International seaweed symposium. 5, 1965. Pergamon Press, London, pp 3-17.

5. Araki, C.; Arai, K. (1967). Studies on the chemical constitution of Agar-agar. XXIV. Isolation of a new disaccharide as a reversion product from acidic hydrolysate. *Bull. Chem. Societ. Japan.* 40, 1452-1456.

6. Araki, T.; Lu, Z.; Morishita, T. (1998). Optimization of parameters for isolation of protoplasts from *Gracilaria verrucosa* (Rhodophyta). *J. Mar.*

Biotechnol. 6, 193-197.

7. Barbeyron, T.; Gerard, A., Potin, P.; Henrissat, B.; Kloareg, B. (1998). The kappa-carrageenase of the marine bacterium *Cytophaga drobachiensis.* Structural and phylogenetic relationships within family 16 glycoside hydrolases. *Mol. Biol. Evol.* 15, 528-537.

8. Beg, Q.K.; Gupta, R. (2003). Purification and characterization of an oxidation-stable. Thiol depedent serine alkaline protease from Bacillus mojavensis. *Enzyme Microb. Technol.* 32, 294-304.

9. Chen, H.M.; Zheng, L.; Yan, X. J. (2005). The preparation and bioactivity research of agaro-oligosaccharides. *Food Technol. Biotechnol.* 43, 29-36.

10. Davies, G.; Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure.* 15; 3(9), 853–859.

11. Duckworth, M.; Turvey, J. R. (1969). The action of a bacterial agarase on agarose, porphyran and alkali treated porphyran. *Biochem. J.* 113, 687-692.

12. Duckworth, M.; Yaphe, W. (1971). Structure of agar. I. Fractionation of complex mixture of polysaccharides. *Carbohydr. Res.* 16,189-197.

13. Ekborg, N.A.; Taylor, L.E.; Longmire, A.G.; Henrissat, B.; Weiner, R. M.; Hutcheson, S.W. (2006). Genomic and proteomic analyses of the agarolytic system expressed by *Saccharophagus degradans* 2-40. *Appl. Environ. Microbiol.* 72, 3396-3405.

14. Fu, W.; Han, B.; Duan, D., Liu, W., Wang, C. (2008a). Purification and characterization of agarases from a marine bacterium *Vibrio* sp. F-6. *J. Indust. Microbiol. Biotechnol.* 35, 915-922.

15. Fu, X.T.; Lin, H.; Kim, S.M. (2008b). Purification and characterization of a novel beta-agarase, AgaA34, from *Agarivorans albus* YKW-34. *Appl. Microbiol. Biotechnol.* 78, 265-273.

16. Groleau, D.; Yaphe, W. (1977). Enzymatic hydrolysis of agar: purification and characterization of beta-neoagarotetraose hydrolase from *Pseudomonas atlantica. Can. J. Microbiol.* 23, 672-679.

17. Ha, J.C.; Kim, J.T.; Kim, S.K.; Oh, T.K.; Yu, J.H; Kong, I.S. (1997). Beta agarase from *Pseudomonas* sp. W7: purification of the recombinant enzyme from *Escherichia coli* and the effects of salt on its activity. *Biotechnol. Appl. Biochem.* 26,1-6.

18. Haddar, A.; Bougatef, A.; Agrebi, R.; Sellami-Kamoun, A.; Nasri, M. (2009). A novel surfactant-stable alkaline serine protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. *Process. Biochem.* 44, 29-35.

19. Hassairi, I.; Ben, A.R.; Nonus, M.; Gupta, B.B. (2001). Production and separation of alpha-agarase from *Altermonas agarlyticus* strain GJ1B. *Biores. Technol.* 79, 47-51.

20. Hu, Z.; Lin, B.K.; Xu, Y.; Zhong, M.Q.; Liu, G.M. (2009). Production and purification of agarase from a marine agarolytic bacterium *Agarivorans* sp. HZ105. *J. Appl. Microb.* 106, 181-190.

21. Jacobs, M.; Elkiasson, M.; Uhlen, M.; Flock, J.I. (1985). Cloning, sequencing and expression of subtilisin carlsberg from *Bacillus licheniformis. Nucleic Acids Res.* 13, 8913-8927.

22. Jam, M.; Flament, D.; Allouch, J.; Potin, P.; Thion, L.; Kloareg, B.;

Czjzek, M.; Helbert, W.; Michel, G.; Barbeyron. T. (2005). The endo-beta-agarases AgaA and AgaB from the marine bacterium *Zobellia galactanivorans:* two paralogue enzymes with different molecular organizations and catalytic behaviours. *Biochem. J.* 385, 703-713.

23. Kendall, K.; Cullum, J. (1984). Cloning and expression of an extracellular-agarase from *Streptomyces coelicolor* A3(2) in *Streptomyces lividans* 66, *Gene* 29, 315-321.

24. Kirimura, K.; Masuda, N.; Iwasaki, Y.; Nakagawa, H.; Kobayashi, R.; Usami. S. (1999). Purification and characterization of a novel beta agarase from an alkalophilic bacterium, *Alteromonas* sp. E-1. *J. Biosci. Bioeng.* 87, 436-441.

25. Kobayashi, R., Takisada, M., Suzuki, T., Kirimura, K.; Usami, S. (1997). Neoagarobiose as a novel moisturizer with whitening effect. *Biosci. Biotechnol. Biochem.* 61, 162-163.

26. Lee, S.; Jang, D. J. (2001). Progressive rearrangement of subtilisin Carlsberg into orderly and inflexible conformation with Ca^{2+} binding. *Byophys. J.* 81, 2972-2978.

27. Lee, S.; Park, J.; Yoon, S.; Kim, J.; Kong, I. (2000). Sequence analysis of a beta-agarase gene (pjaA) from *Pseudomonas* sp. isolated from marine environment. *J. Biosci. Bioeng.* 89, 485-488.

28. Lee, D.G.; Park, G.T.; Kim, N.Y.; Lee, E.J.; Jang, M.K.; Shin, Y.G.; Park, G.S.; Kim, T.M.; Lee, J.W.; Lee, J.H.; Kim, S.J.; Lee, S.H. (2006). Cloning, expression, and characterization of a glycoside hydrolase family 50 beta-agarase from a marine *Agarivorans* isolate. B*iotechnol. Lett.* 28,1925-1932.

29. Leon, O.; Quintana, L.; Peruzzo, G.; Slebe, J.C. (1992). Purification and properties of an extracellular agarase from *Alteromonas* sp. strain C-1. *Appl. Environ. Microb.* 58, 4060-4063.

30. Ma, C.; Lu, X.; Shi, C.; Shi, C.; Li, J.; Gu, Y.; Ma, Y.; Chu, Y.; Han, F.; Gong, Q. (2007). Molecular cloning and characterization of a novel beta-agarase, AgaB, from marine *Pseudoalteromonas* sp. CY24. *J. Biol. Chem.* 282, 3747-3754.

31. Michel, G.; Chantalat, L.; Duee, E.; Barbeyron, T.; Henrissat, B.; Kloareg, B.; Dideberg, O. (2001). The kappa-carrageenase of *Pseudoalteromonas carrageenovora* features a tunnel-shaped active site: a novel insight in the evolution of clan-B glycoside hydrolases *Structure.* 9, 513-525.

32. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31,426-428.

33. Morrice, L. M.; McLean, M.W.; Williamson, F.B.; Long, W.F. (1983). Beta-agarases I and II from *Pseudomonas atlantica.* Purifications and some properties. *Eur. J. Biochem.* 135, 553-558.

34. Ohta, Y.; Hatada, Y.; Ito, S.; Horikoshi, K. (2005). High-level expression of a neoagarobiose-producing beta-agarase gene from *Agarivorans* sp. JAMB-A11 in *Bacillus subtilis* and enzymic properties of the recombinant enzyme. *Biotechnol. Appl. Biochem.* 41,183-191.

35. Ohta, Y.; Hatada, Y.; Nogi, Y.; Li, Z.; Ito, S.; Horikoshi, K. (2004a). Cloning, expression, and characterization of a glycoside hydrolase family 86 beta agarase from a deep-sea *Microbulbifer*-like isolate. *Appl. Microb.*

Biotechnol. 66, 266-275.

36. Ohta, Y.; Hatada, Y.; Nogi, Y.; Miyazaki, M.; Li, Z.; Akita, M.; Hidaka, Y.; Goda, S.; Ito, S.; Horikoshi, K. (2004b). Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from a novel species of deep-sea *Microbulbifer. Appl. Microb. Biotechnol.* 64, 505-514.

37. Ohta, Y.; Hatada, Y.; Nogi, Y.; Miyazaki, M.; Li, Z.; Hatada, Y.; Ito, S.; Horikoshi, K. (2004c). Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta agarase from the novel marine isolate, JAMB-A94. *Biosci. Biotechnol. Biochem.* 68 (5), 1073-1081.

38. Pearson, W.R. (1990). Rapid and Sensitive Sequence Comparison with FASTP and FASTA. *Methods Enzymol.* 183, 63-98.

39. Potin, P.; Richard, C.; Rochas, C.; Kloareg, B. (1993). Purification and characterization of the alpha-agarase from *Alteromonas agarlyticus* (Cataldi) comb. nov., strain GJ1B. *Eur. J. Biochem.* 214, 599-607.

40. Pugsley, A.P. (1993). The complete general secretory pathway in gram negative bacteria. *Microb. Rev.* 57, 50-108.

41. Ryu, S.K.; Cho, S.J.; Park, S.R.; Lim, W.J.; Kim, M.K.; Hong, S.Y.; Baed, W.; Park, Y.W.; Kim, B.K.; Kim, H.; Yun, H.D. (2001). Cloning of the cel9A gene and characterization of its gene product from marine bacterium *Pseudomonas* sp. SK38. *Appl. Microb. Biotechnol.* 57, 138-145.

42. Sambrook, J.; Fritsch, E.F.; Maniatis. T. (1989). Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

43. Schroeder, D.C.; Jaffer, M.A.; Coyne, V.E. (2003). Investigation of the role of a beta (1-4) agarase produced by *Pseudoalteromonas gracilis* B9 in eliciting disease symptoms in the red alga *Gracilaria gracilis*. *Microbiol.* 149, 2919-2929.

44. Sugano, Y.; Kodama, H.; Terada, I.; Yamazaki, Y.; Noma, M. (1994). Purification and characterization of a novel enzyme, α-neoagarooligosaccharide hydrolase (α-NAOS hydrolase), from a marine bacterium, *Vibrio* sp. strain JT0107. *J. Bacteriol.* 176, 6812-6818.

45. Sugano, Y.; Terada, I.; Arita, M.; Noma, M.; Matsumoto, T. (1993). Purification and characterization of a new agarase from a marine bacterium, *Vibrio* sp. strain JT0107. *Appl. Environ. Microbiol.* 59, 1549-1554.

46. Suzuki, H.; Sawai, Y.; Suzuki, T.; Kawai, K. (2003). Purification and characterization of an extracellular beta agarase from *Bacillus* sp. MK03. *J. Biosci. Bioeng.* 93, 456-463.

47. Van der Meulen, H.J.; Harder, W. (1975). Production and characterization of the agarase of *Cytoplaga flevensis. Antonie van Leeuwenhoek.* 41, 431-447.

48. Vera, J.; Alvarez, R.; Murano, E.; Slebe, J.C.; Leon, O. (1998). Identification of a marine agarolytic *Pseudoalteromonas* isolate and characterization of its extra cellular agarase. *Appl. Environ. Microbiol.* 64, 4378-4383.

49. Wang, J.; Mou, H.; Jiang, X.; Guan, H. (2006). Characterization of a novel beta-agarase from marine *Alteromonas* sp. SY37-12 and its degrading products. *Appl. Microbiol. Biotechnol.* 71, 833-839.

50. Zhang, W.W.; Sun, L. (2007). Cloning, characterization, and molecular application of a beta-agarase gene from *Vibrio* sp. strain V134. *Appl. Environ. Microbiol.* 73, 2825-2831.