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Characterization of a Glycoside Hydrolase Family 50 Thermostable β-agarase AgrA from Marine Bacteria *Agarivorans* sp. AG17

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An agar-degrading *Agarivorans* sp. AG17 strain was isolated from the red seaweed *Grateloupia filicina* collected from Jeju Island. A beta-agarase gene from *Agarivorans* sp. AG17 was cloned and designated as *agr*A. *agr*A has a 2,985 bp coding region encoding 995 amino acids and was classified into the glycoside hydrolase family (GHF)-50. Predicted molecular mass of the mature protein was 105 kDa. His-tagged *agr*A was overexpressed in *Escherichia coli* and purified as a fusion protein. The enzyme showed 158.8 unit/mg specific activity (optimum temperature at 65°C and pH 5.5 in acetate buffer) with unique biochemical properties (high thermal and pH stabilities). Enzyme produced neoagarohexaose, neoagarotetraose and neoagarobiose by degrading agar, and hydrolyzed neoagaro-oligosaccharides were biologically active. Hence the purified enzyme has potential for use in industrial applications such as the development of cosmetics and pharmaceuticals.

Key words: *Agarivorans* sp., Beta agarase, GHF-50, Neoagaro-oligosaccharides, Thermostable

Introduction

Agar is the most abundant polysaccharide found in the cell walls of many red seaweeds and is composed of agarose and agaropectin. Agarose is composed of alternating residues of 3-*O*-linked *β*-D-galacto-pyranose and 4-*O*-linked 3,6-anhydro-*α*-L-galactose (Duckworth and Yaphe, 1971). Even though agaro-pectin is composed of the same disaccharide-repeating units, sulfoxy or methoxy and pyruvate residues can replace some of the hydroxyl groups of 3,6-anhydro-*α*-L-galactose residues (Araki, 1966).

Agarase is the enzyme which hydrolyzes agarose. Agarase-producing organisms have been reported from marine and other environments. However, most of the known agarolytic organisms are bacteria (Zhang and Sun, 2007). Those include *Microbulbifer* sp. (Ohta et al., 2004a.; Ohta et al., 2004b), *Cytophaga* sp. (Van der Meulen and Harder, 1975) *Agarivorans* sp. (Fu et al., 2008b; Lee et al., 2006; Ohta et al., 2005), *Pseudomonas* sp. (Groleau and Yaphe, 1977; Ha et al., 1997; Lee et al., 2000; Morrice et al., 1983; Ryu et al., 2001) *Pseudo-alteromonas* sp. (Ma et al., 2007; Schroeder et al., 2003; Vera et al., 1998), *Alteromonas* sp. (Hassairi et al., 2001; Potin et al., 1993; Wang et al., 2006) *Streptomyces* sp. (Kendall and Cullum, 1984), *Vibrio* sp. (Aoki et al., 1990; Fu et al., 2008a; Sugano et al., 1993a; Sugano et al., 1993b), *Zobellia* sp. (Allouch et al., 2003; Jam et al., 2005), *Microscilla* sp. (Naganuma et al., 1993) and *Saccharophagus* sp. (Ekborg et al., 2006). To date, some of the agarases have been purified and characterized. These enzymes show a high degree of heterogeneity in terms of their amino acid sequence, molecular masses, catalytic properties and substrate specificities. However, the identified β-agarases are functionally similar by hydrolyzing β-1,4-linkages in the agar substrate (Ohta et al., 2004b).

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Based on the mode of action, agarases have been divided into α-agarase and β-agarase enzymes. α-agarase hydrolyzes α-1,3-linkages of agarose, resulting in agaro-oligosaccharides such as agarobiose (Young et al., 1978), while β-agarase hydrolyzes β-1,4-linkages in agarose (Duckworth and Turvey, 1969), resulting neoagaro-oligosaccharides such as neoagarohexaose, neoagarotetraose and neoagarobiose as the main products (Zhang and Sun, 2007). Most of the agarases that have been isolated and characterized belong to the β-agarases. Based on the amino acid sequence similarity, all of the β-agarases have been classified into three glycoside hydrolase families (GHFs) 16, 50 and 86 (http://afmb.cnrs-mrs.fr/CAZY/).

Many potential applications in the food, cosmetics, medical and other industries have been reported for the agarases, which produce oligosaccharides from agar or agarose (Kobayashi et al., 1997; Yoshizawa et al., 1995). Since agarase has ability to degrade the cell wall of seaweeds, it has potential to be used for the extraction of a variety of labile substances with bioactive compounds, and in addition, can be used for the preparation of protoplasts (Araki and Morishita, 1998). It has been reported that *AgaA* (GHF-50) from a *Vibrio* strain degrades agarose as well as agaro-oligosaccharides containing four sugars to yield neoagaro-biose (Sugano et al., 1993a) and possesses a whitening effect that can be applied in the cosmetic industry (Kobayashi et al., 1997). Some GHF-16 agarases promoted degradation of agarose and agaro-oligosaccharides containing six sugars to yield neoagaro-tetraose as main products (Schroeder et al., 2003).

Due to the diversification of β-agarase-producing bacteria and their agarase enzyme characteristics, further studies on biochemical and genetic variations of newly isolated strains producing these enzymes as well as on the enzymes of already identified bacterial strains are important. Ohta et al. reported agarases from *Agarivorans* sp. which belongs to GHF-50 with similar enzymatic properties (Ohta et al., 2004a; Ohta et al., 2004b). In the present study described herein, an agar-degrading bacteria *Agarivorans* sp. AG17 was isolated from a marine environment. The *agr*A was cloned, overexpressed in *Escherichia coli*, and the recombinant protein was purified. Finally, biochemical characterization of purified agarase (AgrA) and functional activity studies of neoagaro-oligosaccharides obtained from agar hydrolysates were conducted to assess the suitability of developing pharmaceutical and cosmetic products from this product.

Materials and Methods

Isolation and identification of agarase-producing bacteria strains

A strain of the *Agarivorans* sp. was isolated from the seaweed, *Grateloupia filicina* collected at the south coast of Jeju Island, Republic of Korea. The seaweeds were macerated and diluted with autoclaved sea water. Diluted samples were plated on 1.5% agar in seawater plates (SW) in order to screen agarase-producing bacteria. As another screening method, different seaweeds were maintained and grown on separate selection plates. The plates were incubated at 30°C for 3-5 days. Positive colonies having pits or clear zones were picked out and streaked on 0.3% tryptone and 1.5% agar in seawater (SWT), 0.3% yeast extract and 1.5% agar in seawater (SWY) and marine agar plates (Difco, Detroit, MI). Then, the plates were incubated at 30°C for 2-4 days. The pure colonies were selected under same conditions and inoculated in SWT, SWY and marine broth including 0.2% agar, then incubated at 30°C. The stock was prepared from the grown bacteria using 20% glycerol; samples were kept at -70°C.

Identification of bacteria using 16S rDNA sequence analysis

Cultured bacteria cells were collected from growth medium by centrifugation at 15,000 rpm for 5 min. Genomic DNA was isolated using Accuprep genomic DNA extraction kit (Bioneer, Korea) following the manufacturer's instructions. Polymerase Chain Reaction (PCR) was carried out for 16S rDNA sequence amplification. Isolated genomic DNA was used as a template and universal primers (16S-27F as forward and 16S-1492R as reverse) were used for 16S rDNA sequence amplification, shown in Table 1. The sequences were analyzed using the software NCBI Blast N, and DNassist version 2.2.

PCR amplification of the *agr*A from *Agarivorans* sp. AG17

For the amplification of an agarase gene from the identified strain, AgF1 forward primer and AgR2 reverse primer were designed using other known *Agarivorans* sp. agarase sequences from the NCBI database (Table 1). The PCR mixture included 5 μL 10x Ex Taq polymerase buffer, 4 μL of 2.5 mM dNTP, 100 pmol of each primers, 400-600 ng of genomic DNA as template, and 3 units of Ex Taq DNA polymerase in 50 μL reaction volume. In the PCR reaction the initial denaturation step was carried out at 94°C for 5 min and amplification was carried out

Table 1. Primers used in this study

Name	Object	Sequence (5' to 3' direction)
16s-27F	16s rDNA sequence amplification	AGAGTTTGATCMTGGCTCAG
16s-1492R	16s rDNA sequence amplification	TACGGYTACCTTGTTACGACTT
AgF1	*agr*A gene amplification	ATGAAGATTAAATTTTTATCTGCAGC
AgR2	*agr*A gene amplification	TTACACTTTACGACGTCTTAG
AgF3	ORF amplification	$(GA)_3$ CATATGGCTACCTTAGTCACCTCTTTTG(NdeI)
AgR4	ORF amplification	$(GA)_3$GGATCCTTACACTTTACGACGTCTTAGTAAAAATAC (BamHI)

in 30 cycles (94°C, 45 s; 45°C, 45 s; 72°C, 180 s). The final extension step was carried out at 72°C for 5 min, and PCR product was purified using the Accuprep™ PCR purification kit (Bioneer Co., Korea).

Sequence characterization of *agr*A

Sequenced gene were analyzed by nucleotide BLAST and Protein BLAST of National Center for Biotechnology Information (NCBI) database. The signal peptide sequence of the *agr*A was predicted by utilizing a SignalP program (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). TMHMM Server v. 2.0 was used for the transmembrane site search (http://www.cbs.dtu.dk/services/TMHMM/).
DNassist version 2.2 was used for comparison of other sequences, and Pairwise and multiple alignment of the agarase gene was performed using the ClustalW multiple alignment 1.8 program (Thompson et al., 1994). MEGA 3.1 was used to produce the phylogenetic tree using the neighbor-joining (NJ) method (Kumar et al., 2004).

Cloning of *agr*A coding sequence into the pET16b expression vector

Primer set of AgF3 and AgR4 were designed with the corresponding restriction enzyme sites of *Nde*I and *BamH*I at the 5'-end and 3'-eend for *agr*A, respectively (Table 2) in order to clone the coding sequence into the pET16b expression vector (Novagen, USA) without including its signal sequence. Amplified PCR product was used as a template for coding sequence amplification. Briefly, a 50 μL PCR reaction was set up using 5 units of Herculase DNA polymerase, 5 μL of 10x Herculase buffer, 6 μL of 2.5 mM dNTP, 500 ng of template (amplified *agr*A) and 20 pmol of each primer.

After initial incubation at 94°C for 5 min, 30 cycles were carried out (94°C, 30 s; 50°C, 30 s; 72°C, 180 s), followed by a final extension at 72°C for 5 min. PCR products were analyzed on a 1% agarose gel and ethidium bromide staining. The PCR product was purified from the gel with the Accuprep™ gel purification kit (Bioneer Co., Korea) and digested with the respective restriction enzymes. The expression vector, pET16b was also digested with the same restriction enzymes as the PCR product and dephosphorylated with calf intestine phosphatase according to the vendor's protocol. The vector and the PCR product were purified on a 1% agarose gel using the Qiaex-II gel purification kit (QIAGEN Inc., USA). The ligation was carried out at 20°C for 2 h using a ligation mix (TaKaRa, Japan). The ligated product was transformed into *E. coli* DH5α cells, and correct recombinants (confirmed by restriction enzyme digestion and sequencing) were transformed into competent cells of *E. coli* BL21.

Overexpression and purification of recombinant AgrA

E. coli BL21(DE3) cells carrying the agarase gene (pET-16b-*agr*A) were overexpressed in the presence of isopropyl-β-thiogalactopyranoside (IP-TG). Briefly, 5 mL of an *E. coli* BL21(DE3) starter culture was inoculated into 100 mL Luria broth with 100 μL ampicillin (100 mg/mL). The culture was incubated at 37°C while shaken at 200 rpm until the cell count reached 0.5 at 600 nm optical density. The culture was shifted to 12°C for 30 min and induced by IPTG at 1 mM final concentration. After 24 h of induction, the cells were cooled on ice for 30 min, and harvested by centrifugation at 4,000×g for 20 min at 4°C, followed by removal of the supernatant. Then cells were resuspended in 5 mL ice cold 1x binding buffer (0.5 M NaCl, 20 mM Tris HCl, 5 mM imidazole, pH 7.9) and frozen at -20°C overnight. After thawing on ice, the bacterial cells were sonicated six times in short pulses of 10 s. The supernatant was taken as a crude enzyme after centrifugation at 9,000×g for 30 min at 4°C. Then, the recombinant AgrA fusion protein was purified using the His Bind Purification Kit (Novagen, USA). In the final purification step, bound fusion protein was eluted with 6 volumes of 1x elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Elutes were collected in 500 μL fractions and respective elutes were run on SDS-PAGE according to the standard procedure for

discontinuous SDS-PAGE. Gels were stained using 0.05% Coomassie Blue R-250, followed by a standard destaining procedure. The concentrations of purified proteins were determined by the method of Bradford using bovine serum albumin (BSA) as the standard (Bradford, M.M. 1976).

Biochemical characterization of recombinant AgrA

Agarase activity in the recombinant purified protein was determined using the modified method of Ohta et al., 2004a, by measuring the amount of reducing sugars released during incubation with 1% food grade agar, 1% agarose and 1% carrageenan separately as agarase substrates. A suitably diluted enzyme solution was added to different substrates in acetate buffer (pH 5.5) supplemented with 2 mM $MgSO_4$, and the mixture was incubated at 65°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 with D-galactose as the standard. One unit of the enzyme activity was defined as the amount of protein that produced 1 μmol of reducing sugar as D-galactose per min under the conditions of the assay.

Optimum temperature of AgrA was determined by monitoring the relative enzymatic activity at temperatures ranging from 40-70°C with 5°C in-tervals at pH 7.0. Optimum pH of AgrA was tested from pH 4.5-9.0 with pH 0.5 intervals at 45°C. Acetate buffer and phosphate buffer were used for pH 4.5-6.0 and pH 6.5-9.0, respectively. The thermo-stability of purified recombinant AgrA was evaluated by measuring the residual activity of the enzyme after incubation at the temperatures between 40-65°C for 30, 60 and 120 min. Effects of various metal ion salts and chelators on purified recombinant AgrA 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°C for 30 min. Control was the assay mixture without adding metal ion salts or chelators. In each experiment, 1% agar solution and purified agarase were mixed and incubated under the conditions of the assay. Agarase activity was determined by the DNS method.

Identification of AgrA hydrolyzed agar products

Thin layer chromatography (TLC) was used to identify the hydrolysis products of agar and neoagaro-oligosaccharides. 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 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°C for 30, 60, and 120 min. NA6 substrate was reacted separately with 20 μL of purified agarase at 45°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 consist of n-butanol: acetic acid: water (2:1:1, v/v). The resultant oligosaccharide spots after hydrolysis of substrates were visualized by spraying 10% H_2SO_4 to the plate and heating on a hot plate.

Preparation of neoagaro-oligosaccharides

One gram of agar was dissolved in 100 mL of triple-distilled water by boiling to make 1% agar solution. Purified AgrA was mixed into 1% agar solution and the reaction was performed at 45°C for 24 h. Degradation pattern of the products (neo-agaro-oligosaccharide) was tested using TLC to confirm the hydrolysis of agar. Finally, samples were dried in a freeze dryer and used to check for antioxidant activity *in vitro*.

Antioxidant activity

Antioxidant activity of neoagaro-oligosaccharides was determined by examining the results of the DPPH radical scavenging assay. A 100 μL aliquot of 0.01, 0.1 and 1 mg/mL neoagaro-oligosaccharides solution (NA2+NA4) was added into 100 μL of DPPH, and incubated at 25°C for 60 min in darkness. A concentration of 0.15 mM DPPH in ethanol was used as the colorimetric reagent, and absorbance was measured at 492 nm using a microplate reader ELISA.

Nucleotide sequence accession number

*Agr*A sequence data was submitted to the NCBI database under accession number FJ624295.

Results

Screening and identification of *Agarivorans* sp.

Initially, bacteria that showed agar-degrading ability were identified by a clearing zone formed on agar plates. Based on these results, different agarase-

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ATGAAGATTAAATTTTTATCTGCAGCAATCGCTGCAAGCTTAGCATTGCCATTAAGTGCTGCTACCTTAGTCACCTCTTTTGAGGAAGCC  90
M K I K F L S A A I A A S L A L P L S A A T L V T S F E E A  30
GACTACAGCAGCTCTGAAAACAATACTGAATTCTTGGAAGTGTCTGGAGATGCCACTTCTGAAGTTTCAACTGAACAAGCTACCGATGGT 180
D Y S S S E N N T E F L E V S G D A T S E V S T E Q A T D G  60
AATCAATCCATTAAAGCGTCTTTTGACGCGGCTTTCAAACCAATGGTTGTTTGGAACTGGGGAAGTTGGAACTGGGGCGCTGAAGATGTG 270
N Q S I K A S F D A A F K P M V V W N W G S W N W G A E D V  90
ATGTCAGTAGATGTTGTTAACCCTAACGACACTGACGTCACCTTTGCTATTAAGCTAATTGATAGTGATATTCTTCCTGATTGGGTAGAT 360
M S V D V V N P N D T D V T F A I K L I D S D I L P D W V D 120
GAGTCTCAAACCTCATTGGACTACTTTACGGTTTCAGCTAATACCACGCAGACCTTTAGCTTTAACTTAAATGGCGGCAACGAGTTCCAA 450
E S Q T S L D Y F T V S A N T T Q T F S F N L N G G N E F Q 150
ACTCATGGCGAAAACTTTAGTAAAGATAAAGTTATCGGTGTGCAGTTCATGCTCTCTGAAAACGATCCTCAAGTGTTGTACTTTGACAAC 540
T H G E N F S K D K V I G V Q F M L S E N D P Q V L Y F D N 180
ATTATGGTTGATGGCGAAACAGTCACTCCGCCACCAAGTGATGGTGCAGTGAATACACAAACCGCGCCTGTAGCCACCTTAGCGCAAATC 630
I M V D G E T V T P P P S D G A V N T Q T A P V A T L A Q I 210
GAAGACTTTGAAACCATTCCAGATTACTTACGACCTGATGGTGGGGTAAACGTTTCAACTACTACTGAGATTGTGACTAAAGGCGCTGCA 720
E D F E T I P D Y L R P D G G V N V S T T T E I V T K G A A 240
GCAATGGCTGCCGAGTTTACTGCAGGTTGGAACGGTTTAGTGTTTGCAGGTACTTGGAATTGGGCTGAACTAGGTGAACACACCGCAGTT 810
A M A A E F T A G W N G L V F A G T W N W A E L G E H T A V 270
GCCGTTGACGTTTCAAATACTAGCGATAGCAATATCTGGTTGTACTCACGTATCGAAGATGTAAATAGCCAAGGCGAAACAGCGACTCGC 900
A V D V S N T S D S N I W L Y S R I E D V N S Q G E T A T R 300
GGCGTATTGGTTAAAGCTGGCGAATCGAAAACCATCTACACCAGCTTAAATGACAACCCTTCATTGCTTACTCAAGATGAGCGCGTGTCA 990
G V L V K A G E S K T I Y T S L N D N P S L L T Q D E R V S 330
GCTTTAGGTTTACGTGATATTCCAGCTGACCCAATGAGCGCTCAAAATGGCTGGGGTGATTTTGTTGCTTTAGACAAATCTCAAATTACC 1080
A L G L R D I P A D P M S A Q N G W G D F V A L D K S Q I T 360
GCTATTCGTTACTTCATTGGCGAATTAGCCAGCGGTGAGACTAGCCAAACACTTGTGTTTGATAACATGCGTGTGATTAAAGACCTTAAC 1170
A I R Y F I G E L A S G E T S Q T L V F D N M R V I K D L N 390
CACGAATCAGCCTATGCAGAAATGACTGATGCTATGGGGCAAAACAACTTAGTCACTTATGCAGGTAAAGTTGCCAGCAAAGAAGAGTTA 1260
H E S A Y A E M T D A M G Q N N L V T Y A G K V A S K E E L 420
GCTAAGTTAAGTGATCCGGAAATGGCTGTTTTGGGTGAGTTAACCAATCGCAATATGTACGGTGGTAACCCAGATTCGTCGCCAACTACA 1350
A K L S D P E M A V L G E L T N R N M Y G G N P D S S P T T 450
GACTGTGTGCTCGCTACGCCTGCCTCGTTTAACGCTTGTAAAGACGCTGATGGTAACTGGCAATTGGTAGACCCTGCTGGTAATGCGTTC 1440
D C V L A T P A S F N A C K D A D G N W Q L V D P A G N A F 480
TTCTCAACCGGTGTTGATAACATTCGTTTGCAAGATACTTACACCATGACCGGCGTGTCGAGTGACGCCGAATCTGAGTCTGCACTTCGC 1530
F S T G V D N I R L Q D T Y T M T G V S S D A E S E S A L R 510
CAGTCAATGTTTACAGAAATTCCAAGTGATTATGTAAATGAAAACTATGGCCCTGTGCATAGTGGACCTGTTTCTCAAGGCCAAGCTGTA 1620
Q S M F T E I P S D Y V N E N Y G P V H S G P V S Q G Q A V 540
AGTTTTTACGCTAATAACTTAATTACCCGCCACGCTAGCGAAGACGTATGGCGAGACATTACTGTTAAGCGCATGAAAGACTGGGGCTTT 1710
S F Y A N N L I T R H A S E D V W R D I T V K R M K D W G F 570
AACACCTTAGGTAACTGGACCGATCCAGCGTTGTATGCAAACGGTGACGTTCCTTACGTGGCAAATGGTTGGTCAACCTCTGGTGCCGAT 1800
N T L G N W T D P A L Y A N G D V P Y V A N G W S T S G A D 600
CGTCTTCCCGTTAAACAAATTGGCAGCGGCTACTGGGGACCACTTCCTGATCCGTGGGATGCTAACTTTGCTACCAATGCCGCCACAATG 1890
R L P V K Q I G S G Y W G P L P D P W D A N F A T N A A T M 630
GCTGCAGAGATCAAAGCTCAGGTTGAAGGCAACGAAGAGTACTTAGTGGGTATTTTTGTTGATAACGAAATGAGCTGGGGCAATGTCACT 1980
A A E I K A Q V E G N E E Y L V G I F V D N E M S W G N V T 660
GATGTTGAAGGCTCTCGTTATGCGCAAACGCTAGCAGTGTTCAATACCGACGGCACTGATGCAACAACTAGCCCTGCTAAAAATAGCTTT 2070
D V E G S R Y A Q T L A V F N T D G T D A T T S P A K N S F 690
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Fig. 1. Nucleotide and deduced amino acid sequences of *agr*A of *Agarivorans* sp. AG17. Predicted signal peptide sequence is underlined and stop (TAA) codon is highlighted with an asterisk (*). C-terminal transmembrane domain (G^{968}-L^{990} aa) is indicated by the double underline.

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ATTTGGTTCTTAGAAAACCAGCGTTATACCGGTGGCATTGCTGACCTAAACGCAGCCTGGGGAACCGATTATGCGTCTTGGGATGCGATG 2160
 I  W  F  L  E  N  Q  R  Y  T  G  G  I  A  D  L  N  A  A  W  G  T  D  Y  A  S  W  D  A  M  720
CGCCCAGCGGCAAGAGTTAGCTTATGTGGCTGGCATGGAAGCTGATATGCAGTTCCTTGCTTGGCAGTTTGCGTTCCAATACTTCAACACC 2250
 R  P  A  Q  E  L  A  Y  V  A  G  M  E  A  D  M  Q  F  L  A  W  Q  F  A  F  Q  Y  F  N  T  750
GTAAACACGGCATTAAAAGCTGAGTTACCAAACCACTTGTACTTGGGCTCTCGCTTTGCAGATTGGGGACGTACTCCTGATGTAGTAAGT 2340
 V  N  T  A  L  K  A  E  L  P  N  H  L  Y  L  G  S  R  F  A  D  W  G  R  T  P  D  V  V  S  780
GCTGCTGCGGCTGTTGTTGATGTGATGAGTTACAACATCTACAAAGACAGTATTGCAGCTGCCGATTGGGATGCTGATGCCTTAAATCAA 2430
 A  A  A  A  V  V  D  V  M  S  Y  N  I  Y  K  D  S  I  A  A  A  D  W  D  A  D  A  L  N  Q  810
ATTGAAGCCATTGATAAGCCAGTAATTATTGGTGAGTTCCACTTCGGTGCGCTTGATAGCGGTTCGTTTGCAGAAGGTGTAGTAAATGCC 2520
 I  E  A  I  D  K  P  V  I  I  G  E  F  H  F  G  A  L  D  S  G  S  F  A  E  G  V  V  N  A  840
ACTTCGCAACAAGATCGTGCAGACAAAATGGTTAGCTTCTACGAATCAGTAAATGCCCATAAAAACTTTGTAGGTGCGCATTGGTTCCAA 2610
 T  S  Q  Q  D  R  A  D  K  M  V  S  F  Y  E  S  V  N  A  H  K  N  F  V  G  A  H  W  F  Q  870
TACATCGATTCACCATTAACGGGTCGTGCATGGGATGGCGAGAACTACAACGTTGGTTTTGTTAGCAATACTGACACGCCATATACATTG 2700
 Y  I  D  S  P  L  T  G  R  A  W  D  G  E  N  Y  N  V  G  F  V  S  N  T  D  T  P  Y  T  L  900
ATGACAGATGCTGCGCGTGAGTTTAACTGTGGTATGTACGGCACTGACTGCTCTAGCTTAAGCAATGCTACTGAAGCTGCTTCGAGAGCC 2790
 M  T  D  A  A  R  E  F  N  C  G  M  Y  G  T  D  C  S  S  L  S  N  A  T  E  A  A  S  R  A  930
GGTGAGTTGTATACCGGTACCAATATTGGTGTTAGCCACTCTGGCCCAGAAGCGCCAGATCCAGGTGAGCCAGTTGATCCTCCAATTGAT 2880
 G  E  L  Y  T  G  T  N  I  G  V  S  H  S  G  P  E  A  P  D  P  G  E  P  V  D  P  P  I  D  960
CCGCCAACACCACCAACAGGTGGCGTAACTGGCGGTGGCGGTAGCGCAGGTTGGTTATCGCTACTAGGTTTGGCCGGCGTATTTTTACTA 2970
 P  P  T  P  P  T  G  G  V  T  G  G  G  G  S  A  G  W  L  S  L  L  G  L  A  G  V  F  L  L  990
AGACGTCGTAAAGTGTAA                                                                        2988
 R  R  R  K  V  *                                                                         995
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Fig. 1. Continued.

producing bacteria strains were isolated from the samples collected from the Jeju coastal environment. Of the tested bacteria isolated and screened from the red seaweed on SWT, SWY and marine agar, a particular bacteria was identified as *Agarivorans* sp. according to 16S rDNA sequence analysis. NCBI Blast N results showed that the identified bacterial strain has 99% identity to *Agarivorans* sp. JAMB-A11 and *A. albus*. We named the identified bacteria strain as *Agarivorans* sp. AG17.

Cloning and sequence characterization of *agr*A

*agr*A was identified from *Agarivorans* sp. AG17 after PCR amplification using specific primers as described in Materials and Methods. Nucleotide and amino acid sequence of the *Agarivorans* sp. AG17 agarase is shown in Fig. 1. It has a 2,985 bp ORF, encoding 995 amino acids. The theoretical molecular mass of *agr*A putative protein and mature protein was 107 and 105 kDa, respectively, with an isoelectric point of (p*I*) of 4.1. The N-terminal signal peptide (20 aa) and the C-terminal helical trans-membrane sequence were located at the region of amino acids 1-20 and 968-990 in *agr*A, respectively. ClustalW pairwise identity results revealed that *agr*A has the highest nucleotide (98.6%) and amino acid (99.7%) sequence identity to a β-agarase sequence of *Agarivorans* sp. JAMB-A11 (agaA11) (Accession number AB178483), which belongs to the GHF-50. In addition, the protein showed 99.5%, 99.2%, 99.0% and 98.2% amino acid identity for *Agarivorans* sp. QM38 agarase (agaD01) (Accession number EF051475), β-agarase (AgaA) from *Vibrio* sp. JT0107 (Accession number BAA-03541), *Agarivorans* sp. JA-β-agarase (Accession number EF100136) and *Vibrio* sp. PO-303 agaB gene (Accession number AB232056), which belongs to GHF-50, respectively.

Phylogenetic analysis of the *agr*A

To determine the relationship between isolated *agr*A with known agarase sequences, a phylogenetic tree was constructed based on their primary amino acid sequences using the Neighbor-joining method in MEGA3.1 (Fig. 2). Phylogenetic analysis results showed that agarase amino acid sequences, which belong to the GHFs 16, 50 and 86 positioned in separate clusters and within that, clusters were sub-divided into many sub-clusters. Moreover, *agr*A was related to a member of GHF-50 and formed a monophyletic clade with other agarase sequences in

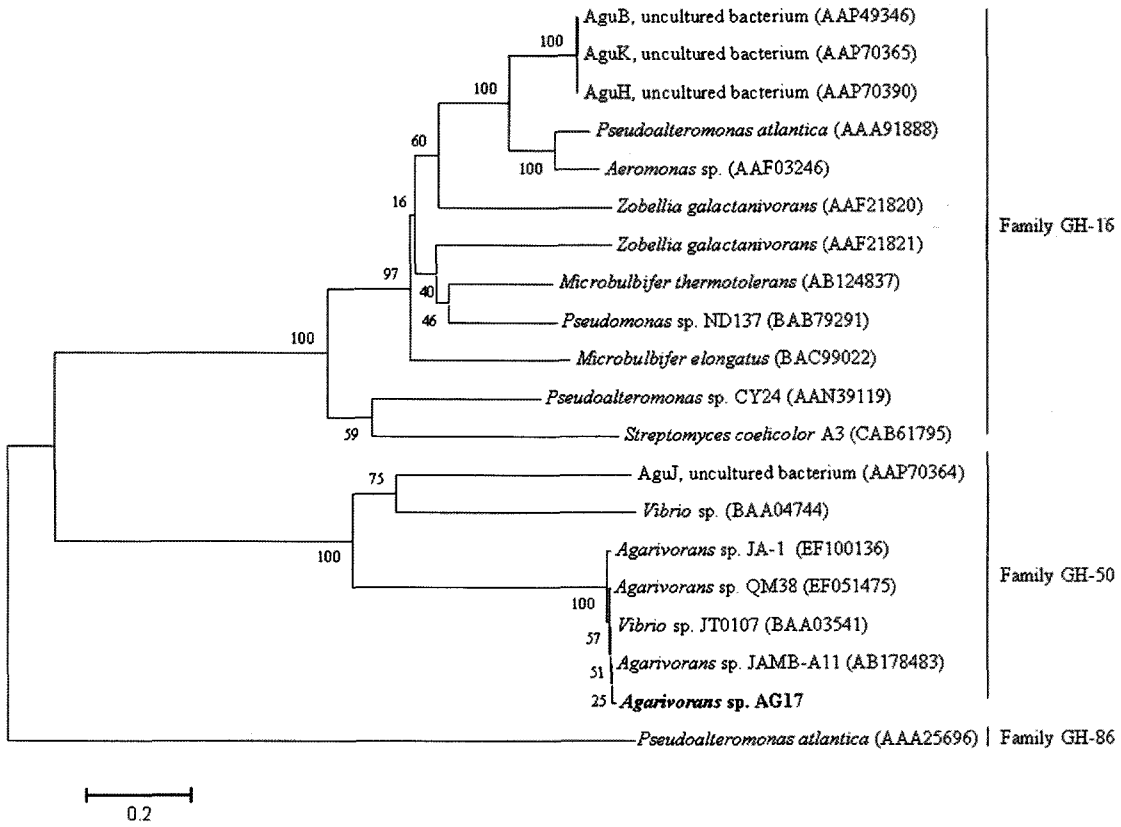

Fig. 2. Phylogenetic analysis of *agr*A with known agarases based on amino acid sequence. Phylogenetic analysis was done by NJ method using MEGA3.1 based on sequence alignment using ClustalW (1.81). Numbers indicate the bootstrap confidence values of 1000 replicates. The accession numbers of the selected 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 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).

the GHF-50.

Expression and purification of recombinant AgrA

*agr*A mature sequence was cloned into the pET16b expression vector that fused with a his tag at the N-terminal. The recombinant AgrA was overexpressed in *E. coli* BL21 (DE3) cells by IPTG induction and purified as a his-tag fusion protein. The 12% SDS-PAGE results of different steps in the recombinant AgrA purification is shown in Fig. 3. It was very clear that AgrA was highly induced (lane 2) compare to un-induced cells (lane 1). Then, the recombinant AgrA fusion protein was purified from soluble crude protein extract by a Ni^{2+} binding column (lane 4). The size of the purified protein was identified as 108 kDa, which is identical to the mature protein size together with the his-tag protein.

Biochemical characterization of recombinant AgrA

Specific activities were determined using agar, agarose and carrageenan as substrates. Purified recombinant AgrA showed 158.8 and 155.3 unit/mg towards agar and agarose, respectively. However, no agarase activity was detected towards carrageenan. The effect of temperature on the activity of AgrA is shown in Fig. 4. Optimum temperature of purified recombinant AgrA activity was determined at

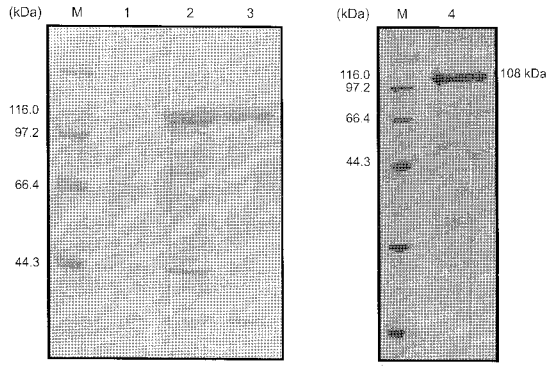

Fig. 3. SDS-PAGE of the recombinant protein AgrA. Protein samples were separated on 12% SDS-PAGE and stained with Coomassie brilliant blue. M: molecular mass marker (BioRad, USA). Lane 1: total cellular extract from *E. coli* BL21 (DE3) before induction; lane 2: total cellular soluble extract after induction; lane 3: total cellular insoluble extract after induction; lane 4: purified recombinant AgrA.

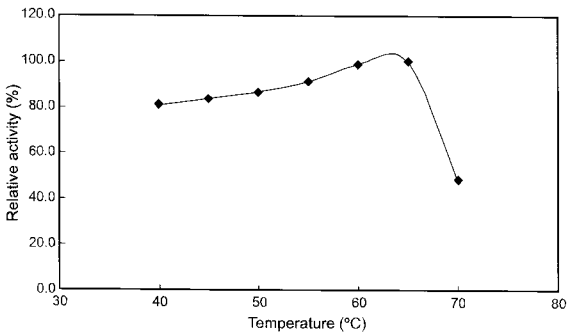

Fig. 4. Effect of temperature on the activity of purified recombinant AgrA. Effect of temperature on enzyme activity was determined under standard assay conditions as described in Materials and Methods, at temperatures ranging between 40-70°C.

different temperatures ranging from 40-70°C in 5°C intervals. Within the range of t he tested temperatures, the activity of the enzyme increased up to 65°C, and this was shown as the optimal temperature for the enzyme. Once the temperature rose above 65°C, the activity of the enzyme decreased drastically. However, relative activity was retained approximately 50% at the temperature of 70°C.

Effect of pH on the purified AgrA is shown in Fig. 5. Higher activity was obtained in the range of pH from 4.5-8.5 and in all this range, more than 90% of relative activity was retained. However, optimum pH was obtained at pH 5.5 in acetate buffer.

Fig. 6 shows the thermostability of AgrA. Six different temperatures (40, 45, 50, 55, 60 and 65°C) were used to determine the thermostability of AgrA

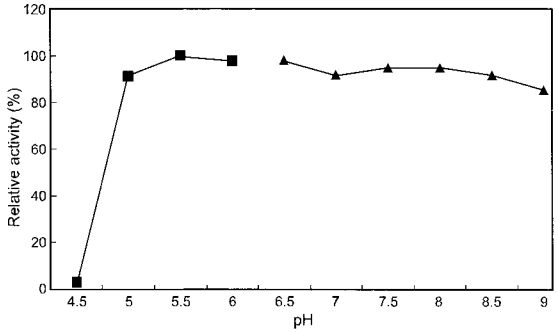

Fig. 5. Effect of pH on the activity of purified recombinant AgrA. Optimum pH for AgrA activity was tested from pH 4.5-9.0 at pH 0.5 intervals at 45°C under standard assay conditions as described in Materials and Methods using acetate (pH 4.5-6.0) and phosphate buffer (pH 6.5-9.0).

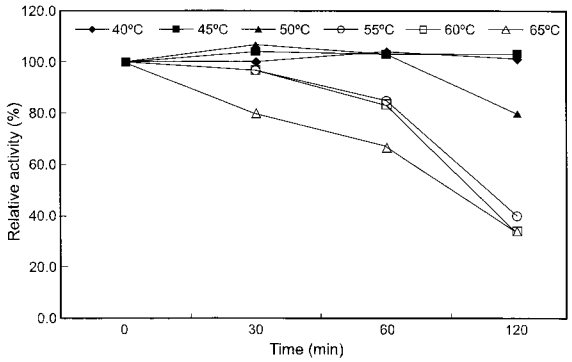

Fig. 6. Effect of thermostability on purified recombinant AgrA at different temperatures for different time points. Thermostability was determined by measurement of residual activity under standard assay conditions as described in Materials and Methods at temperatures between 40-65°C for 30, 60 and 120 min.

with respect to different incubation time points at 30, 60 and 120 min. Enzyme was thermostable (almost 100% of relative activity) at 40 and 45°C. Moreover, about 80% of the relative activity was retained at 50°C up to 120 min. Interestingly, AgrA showed 82% activity after incubation at 55 and 60°C for 1 h and activity was retained about 40% for 2 h at 55°C. Even though the thermostability decreased at 60 and 65°C at the time of 120 min (up to 40% relative activity), thermostability was retained more than 60% at 60 min.

Effects of metal ion salts and chelators on activity of purified AgrA is shown in Fig. 7. More than 40% of the relative activity was inhibited in the protein by 2 mM divalent metal salts such as $CuSO_4$ and $ZnSO_4$, and also by $FeSO_4$ and $MnCl_2$ (by 20%). In contrast, $MgSO_4$, NaCl, KCl enhanced the relative activity

when compared to the control. However, there was no difference observed in relative activity of AgrA, which had $CaCl_2$ and EDTA in the reaction mixtures, when compared to the control samples.

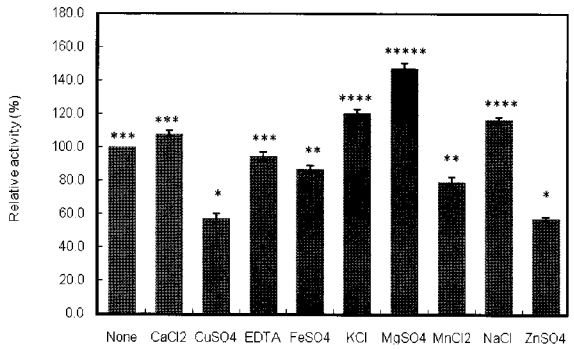

Fig. 7. Effect of metal ions and metal salts on the activity of purified recombinant AgrA. The effect of various metal ion salts and chelators on purified recombinant AgrA activity. Various ions or chelators ($CaCl_2$, $CuSO_4$, $FeSO_4$, KCl, $MgSO_4$, $MnCl_2$, NaCl and EDTA) at a final concentration of 2 mM were included in the reaction buffer to test the activity of AgrA at 45°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 ± SD.

Identification of hydrolysis products of the recombinant AgrA on TLC

Hydrolysis patterns of the purified AgrA against food grade agar and neoagarohexanitol (NA6) are shown in Fig. 8. When the AgrA is incubated with food grade agar, NA4 and NA2 were detected on TLC plate at early stage of the reaction. The amount of NA2 production was increased in a time-dependent manner. A higher amount of NA2 was observed at 60 and 120 min, compared to 30 min of hydrolysis. Furthermore, when the AgrA was incubated with NA6 at 120 min, two reaction products, NA4 and NA2 were observed on TLC.

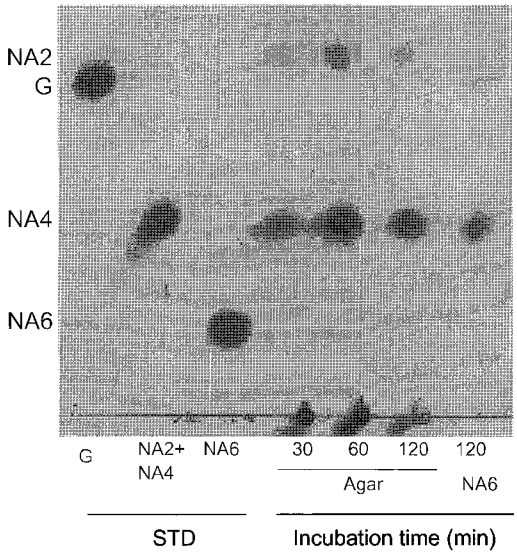

Fig. 8. TLC of hydrolysis products of the purified AgrA enzyme on food grade agar and neoagaro-oligosaccharides. The assay of purified AgrA and agar were performed in 200 μL reactions containing 20 μL of purified agarase and 180 μL of 1% agar at 45°C for 30, 60, and 120 min. NA6 substrate was incubated with 20 μL of purified AgrA separately at 45°C for 120 min. Neoagarohexaitol (NA6), neoagarotetraose (NA4), neoagarobiose (NA2) and D-(+)-galactose (G) were used as standards (STD).

Discussion

In the present study, the isolation, cloning, expression and characterization of a thermostable *agr*A from *Agarivorans* sp. AG17, isolated from red seaweed *G. filicina* at Jeju coastal area was described. Reported selection plates, which have been used for screening of agarase producing bacteria are rich medium containing agar Lee et al., 2006; Vera et al., 1998). However, in this study, SW agar plates were used, which contained only agar as a sole carbon source that allows for the growth of different kind of bacteria for the first step of screening of agarase-producing bacteria. An agarase-producing bacteria strain was identified by 16S rDNA sequencing and it showed the highest identity (99%) to *Agarivorans* sp. JAMB-A11 and *A. albus*. Therefore, the identified bacteria strain was named as *Agarivorans* sp. AG17 and was selected for isolation of the agarase encoding gene *agr*A. At present, many agarase-producing bacteria have been identified. However, there are a few bacteria that belong to genus *Agarivorans,* which produce agarases that have been reported such as *Agarivorans* sp. JAMB-A11 (Ohta et al., 2005), JA-1 (Lee et al., 2006), QM35 (Du et al., 2007) and *A. Albus* YKW-34 (Fu et al., 2008b). In some of these strains, the primary structure of the agarase gene has been characterized their biochemical properties of the enzyme were also characterized.

It has been reported that the molecular masses of reported *Agarivorans* sp. agarases ranged from 105-107 kDa except for the agarase AgaA34 (50 kDa) purified from *A. Albus* YKW-34 (Fu et al., 2008b). The *agr*A also showed a 107 kDa theoretical molecular mass with mature protein of 105 kDa. The first 20 residues of amino acids in *agr*A constitute a hydrophobic region (70%) that function as a signal

peptide for secretion of the agarase. When the *agr*A amino acid sequence was compared with other agarase sequences for their consensus signature motifs, no characteristic motifs and carbohydrate binding modules were found, suggesting that it does not belong to GHF-16 or 86. Phylogenetic analysis results of this study shows that *agr*A is deviated from the GHF16 clade and positioned separately in a separate monophyletic clade, suggesting that *agr*A belongs to the GHF-50. Agarase enzymes share a common ancestor (Allouch et al., 2003) and data indicate that *agr*A has diverged from the primary ancestral sequence. However, agarases feature common catalytic residues in their sequence. The glutamic and aspartic acid are the highly conserved active site residues, which responsible for catalytic activity in GHF (Ma et al., 2007). There were 7.8 and 5.6% aspartic and glutamic acids present in the *agr*A amino acid sequence, respectively, and some of those may responsible for the catalytic activity of the protein. *Pseudoalteromonas atlantica* T6c AgrA (AAA25696) which belongs to GHF-86 showed low amino acid identity (8%) to *agr*A. However, amino acid sequence from 202-354 of *P. atlantica* T6c and 301-480 of *agr*A showed 50% of conserved catalytic residues within that range. Conserved catalytic residues may differently position among agarases in different GHFs due to the diversification of their sequences. This confirms the heterogeneity of the amino acid sequences, catalytic properties and substrate specificities in agarases.

AgrA hydrolyzes agar to give neoagarohexaose, neoagarotetraose and agarobiose as the main products. After 30, 60 and 120 min incubation of 1% agar with AgrA, the main hydrolysis products observed were NA4 and NA2. This suggests that the purified recombinant enzyme is an endo-type β-agarase that catalyzed the hydrolysis of β-1,4-linkages in agar and belongs to GHF-50. Similar to this study, it has been reported that AgaA of GHF-50 from *Vibrio* sp. strain JT0107 degrades agarose as well as agarose oligosaccharides containing at least four sugars to yield neoagarobiose (Sugano et al., 1993a). In contrast, AgrA from *P. atlantica* T6c in GHF-86 degrades agarose into a more polymerized compound than hexamers to yield neoagarohexaose (Ohta et al., 2004a). Some agarases of GHF-16 degrade agarose and agarose oligosaccharides containing six sugars to yield neoagarotetraose (Schroeder et al., 2003; Allouch et al., 2003).

The known β-agarases are grouped into the GHF-16, 50 and 86, and GHF-16 is the most abundant among them. Although the sequence-based GHFs contain enzymes of similar folding and identical molecular mechanisms, there are varying substrate specificities within the same families. Therefore, different sub-strates such as agar, agarose and carrageenan were selected to identify the substrate specificity of AgrA and it was confirmed that the recombinant enzyme selectively hydrolyzed agar-derivative products.

Generally most of the β-agarases show a temperature optimum around 40°C (higher than the gelling temperature of agarose) and an optimum pH around neutral to mild alkaline (Zhang and Sun 2007; Lee et al., 2006). The high thermostability and activity at temperatures higher than the gelling temperatures of agar is useful for industrial oligosaccharide production from marine agar or algae (Ohta et al., 2004c) which could degrade its cell wall for extraction of labile substances with many biological activities (Yoshizawa et al., 1995; Araki and Morishita, 1998). Interestingly, the recombinant AgrA showed optimum activity at 65°C and pH 5.5. It was reported that the optimum temperature of the enzyme below the gelling temperature (38°C) of agarase reduces the enzyme action due to compact bundles of gel structure in agarose (Van der Meulen and Harder, 1975). Therefore, we could suggest that the higher optimum temperature at 65°C would help to enhance the AgrA activity. So far, the highest thermostability reported for agarases isolated from *Agarivorans* sps. were between 35-50°C (Van der Meulen and Harder, 1975; Kirimura et al., 1999; Suzuki et al., 2003). Interestingly, the thermostability of AgrA was observed at 65°C, which retained its activity more than 60% for 60 min, as reported in the present study herein. With agreement to this, the thermostable β-agarase (rAgaA) from novel marine bacterium JAMB-A94 was stable up to 60°C, and more than 80% of the activity was retained at 65°C for 15 min of heating (Ohta et al., 2004c). Nevertheless, AgrA in the present study represents the novel biochemical properties compared to previously reported agarases from *Agarivorans* sp. Therefore, AgrA could be much more useful in the industries for preparation of oligosaccharides than the reported enzymes due to the unique characteristic features of the enzyme.

In the preliminary AgrA overexpression studies, a higher amount of insoluble protein was detected at higher induction temperatures such as 25°C and 37°C. Therefore, low temperature (12°C) overexpression was used, and it may be useful to increase the solubility of the protein and reduce the inclusion body formation. Furthermore, it suggests that the

conditions utilized here favored to produce the correct folding conformation of AgrA to give the thermostable and pH stable properties of the protein, which would be beneficial for industry. To understand the exact catalysis mechanism of AgrA, the crystal structure of the enzyme needs to be examined in future. Similar to previous reports of the beta-agarases, $MgSO_4$, NaCl and KCl positively affected the activity of the recombinant AgrA. The maximal activity (specific activity 167 U/mg) of recombinant *Agarivorans* sp. JA-1 was at 40°C and pH 8.0 in the presence of 1 mM NaCl and 1 mM $CaCl_2$ (Lee et al., 2006). As reported herein, the $CaCl_2$ did not show significant effect on the activity of the recombinant AgrA. However, $CaCl_2$ could stabilize the activity of GHF-86 β-agarase AgaO and enhanced the activity of AgaB of the *Pseudoalteromonas* sp. (Ohta et al., 2004a; Ma et al., 2007). Similar results have been reported by Zhang and Sun (Zhang and Sun, 2007) with no measurable effect on the activity of the recombinant β-agarase AgaV enzyme (isolated from V134, a marine isolate of the *Vibrio* genus) at concentrations of up to 10 mM $CaCl_2$, and exerted an apparently inhibitory effect at higher concentrations (Zhang and Sun, 2007). This further confirms the high degree of heterogeneity among β-agarases.

It has been reported that kojic acid and arbutin are natural tyrosinase inhibitors, which have been used for the tyrosinase inhibition and whitening effects. However, the major drawback of using these is their cytotoxicity (Briganti et al., 2003). The whitening effect of neoagaro-oligosaccharides on the B16F10 murine melanoma cell line was studied by Lee et al. (Lee et al., 2006) and a similar whitening effect was observed at 10 μg/mL in the present study. Similar to their study, the AgrA hydrolyzed neoagaro-oligosaccharides were not cytotoxic to B16F10 murine melanoma and normal human fibroblast cells at concentrations 0.1-100 μg/mL (data not shown in the present study, to avoid repetition). Moreover, in this study, neoagaro-oligosaccharides prepared by purified AgrA showed antioxidant activities (50% at 1.0 mg/mL) by scavenging production of free radicals, however, at a lower level than commercial vitamin C and E (90%). Several explanations on the *in vitro* antioxidation mechanism of polysaccharide have been reported (Jimenez-Escrig et al., 2001; Xue et al., 2001). DPPH scavenging activity is widely accepted and an easy-to-use *in vitro* assay to measure the activity of various antioxidants through a proton donation mechanism (Chen et al., 2005; Hu et al., 2004). Inhibiting the production of lipid peroxidation and inhibiting the production of NO were some of the antioxidant effects of agaro-oligosaccharides (Chen et al., 2005). This confirms that neoagaro-oligosaccharides produced by AgrA are biologically active as reported by Kobayashi et al. (Kobayashi et al., 1997) and Lee et al. (Lee et al., 2006).

In conclusion, an agar-degrading bacteria was isolated from red seaweed, *G. filicina* samples collected from Jeju Island and was named as *Agarivorans* sp. AG17 on the basis of 16S rDNA sequence analysis. The *agr*A was cloned and sequenced from *Agarivorans* sp. AG17. Recombinant enzyme was overexpressed and purified using an *E. coli* bacterial expression system and it showed distinguishable biochemical characteristics compared to reported agarases. TLC analysis revealed that AgrA could degrade agar mainly into neoagarohexaose, neoagarotetraose and neoagarobiose functionally confirming that AgrA is β-agarase. Together with the biochemical and functional characteristics, purified recombinant AgrA could be used for the production of neoagarohexaose, neoagarotetrose and neoagarobiose from agar. Since generated simple neo-oligosaccharides from complex polysaccharides have whitening and antioxidant activity, the hydrolysis products identified here could be useful for industrial applications. Furthermore, the reaction mechanisms of agar oligosaccharide functional activities need to be studied more in detail in the future.

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