Gene Cloning, Expression, and Characterization of a β-Agarase, AgaB34, from *Agarivorans albus* YKW-34

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A β-agarase gene, *agaB34*, was functionally cloned from the genomic DNA of a marine bacterium, *Agarivorans albus* YKW-34. The open reading frame of *agaB34* consisted of 1,362 bp encoding 453 amino acids. The deduced amino acid sequence, consisting of a typical N-terminal signal peptide followed by a catalytic domain of glycoside hydrolase family 16 (GH-16) and a carbohydrate-binding module (CBM), showed 37−86% identity to those of agarases belonging to family GH-16. The recombinant enzyme (rAgaB34) with a molecular mass of 49 kDa was produced extracellularly using *Escherichia coli* DH5α as a host. The purified rAgaB34 was a β-agarase yielding neoagarotetraose (NA4) as the main product. It acted on neoagarohexaose to produce NA4 and neoagarobiose, but it could not further degrade NA4. The maximal activity of rAgaB34 was observed at 30°C and pH 7.0. It was stable over pH 5.0−9.0 and at temperatures up to 50°C. Its specific activity and k_{cat}/K_m value for agarose were 242 U/mg and 1.7×10^6/sM, respectively. The activity of rAgaB34 was not affected by metal ions commonly existing in seawater. It was resistant to chelating reagents (EDTA, EGTA), reducing reagents (DTT, β-mercaptoethanol), and denaturing reagents (SDS and urea). The *E. coli* cell harboring the pUC18-derived agarase expression vector was able to efficiently excrete agarase into the culture medium. Hence, this expression system might be used to express secretory proteins.

Keywords: β-Agarase, cloning, *Agarivorans albus*, neoagarooligosaccharide

Agar, the cell wall matrix of red algae, consists of two different components of agarose and agaropectin [2]. Agarose is a neutral linear polysaccharide composed of alternating residues of 3-*O*-linked β-D-galactopyranose (G-unit) and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose (A-unit) [11]. Agarases, catalyzing the hydrolysis of agar, were characterized as α-agarase (E.C. 3.2.1.158) that cleaves α-1,3 linkage to produce agarooligosaccharides [24] and β-agarase (E.C. 3.2.1.81) that cleaves β-1,4 linkage to produce neoagarooligosaccharides [14].

Neoagarooligosaccharides produced by β-agarase have potential applications in food, pharmaceutical, and cosmetic industries owing to their physiological and biological activities. They inhibit the growth of bacteria, slow down the degradation of starch, reduce the caloric value of food, and are used as low-calorie additives to improve food qualities [10]. Neoagarotetraose (NA4) and neoagarohexaose (NA6) showed high antioxidative properties [31] and exhibited as efficient moisturizers on skin [22]. Besides production of oligosaccharides, agarase also acted as excellent tools for preparation of protoplasts from red algae [3] and recovery of DNA from agarose gel [27].

Agarivorans is a recently described genus [15], which was named owning to its agar devouring ability. So far, several β-agarase genes from different genera of bacteria have been cloned and the agarases they encoded have been purified and characterized [5, 6, 13, 21]. There are two reports on agarases from *Agarivorans* strains [17, 20]. The two agarases from *Agarivorans* strains had molecular masses of more than 100 kDa, and their amino acid sequences indicated that they belong to glycoside hydrolase family 50 (GH-50).

Strain YKW-34 was isolated from the gut of turban shell, which was capable of degrading the cell walls of both brown algae *Laminaria japonica* and red algae *Gelidium amansii*. It was formerly classified as the genus *Vibrio* based on phenotypic features [33], but it was identified as *Agarivorans albus* based on its 16S rRNA gene sequence [8]. In our previous study, an alginate lyase [7] and two agarases (AgaA34 [8] and AgaB34) with excellent properties were found in the culture medium of this strain. In order to clone the genes encoding these enzymes, a functional cloning

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method was adopted to screen the chromosomal gene of this strain. In this study, gene cloning of a family GH-16 agarase (AgaB34), as well as expression, production, and purification of the recombinant enzyme (rAgaB34), is described. AgaB34 was different from other agarases produced by *Agarivorans* strains owing to its smaller molecular mass of 50 kDa and its catalytic domain of GH-16.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Agarivorans albus YKW-34 was isolated from the gut of turban shell, *Turbinidae batillus cornutus* [33]. The strain was grown at 20°C in marine broth medium (Difico, Detroit, MI, U.S.A.). *Escherichia coli* DH5α (iNtRON, Daejeon, Korea) [*endA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (Φ80d*lacZ*ΔM15)] was used as the host for the construction of the gene library and expression, and was grown at 37°C in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/ml) when required.

Cloning of Agarase Gene

Chromosomal DNA of *Agarivorans albus* YKW-34 was isolated as described by Wilson [30] and partially digested by Sau3AI. DNA fragments (3.0–8.0 kb) were ligated into the BamHI site of plasmid pUC18 using a T4 DNA ligase (iNtRON). The ligation mixture was transformed into *E. coli* DH5α. The clones showing agarase activity were selected on LB agar plates containing 100 μg/ml of ampicillin. The colonies that formed depressions on the agar plate were considered as harboring agarase activity. Plasmids, pUSS1 and pUSS2, purified from two agarase-positive colonies using a plasmid purification kit (DNA-Spin, iNtRON) were sequenced on an ABI Prism 3730 DNA analyzer using an ABI BigDye cycle sequencing kit with Solgent EF-*Taq* DNA polymerase (Solgent, Daejeon, Korea). The obtained sequences were compared with those in a nucleotide database, and the complete nucleotide sequence of *agaB34* was determined by using the BLAST algorithm at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/).

Bioinformation Approaches

The nucleotide sequence of *agaB34* and its deduced protein product were analyzed by different programs. Similarity searches were conducted with BLAST at the NCBI Web site. The free energy of the palindrome structure sequence was calculated by M-Fold (http://frontend.bioinfo.rpi.edu/applications/mfold). The predicted signal sequence was identified with SignalP (http://www.cbs.dtu.dk/services/SignalP). The predicted molecular mass and pI value of the mature protein were calculated using Compute pI/Mw (http://us.expasy.org/tools/pi_tool.html). The predicted protein domains were identified with Conserved Domain BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Extracellular Expression of rAgaB34

The expression plasmid was constructed based on the plasmid pUSS1, which has a linear size of 5.1 kbp with an insert fragment of 2.4 kbp. There is a BamHI site in the insert fragment and a HindIII site in the multiple cloning site of the plasmid located 30 bp downstream of the 3' end of the insert fragment. The sequence (~500 bp) between

the site of BamHI and the C-terminal of the agarase gene was amplified by PCR from pUSS1 using *Pfu* DNA polymerase (Solgent). The F-primer used was 5'-AGATGTATGTAGATT<u>GGATCC</u>-3' containing a BamHI restriction site (underlined), and the R-primer used was 5'-GAA<u>AAGCTT</u>CTA*GTGGTGGTGGTGGTGGTG*CAAGAATTTGA AGCGCTGG-3' containing a HindIII restriction site (underlined), a stop codon (double underlined), and the nucleotide sequence encoding 6× His (italic). The amplified product was adenylated using *Taq* polymerase (Solgent) and ligated into plasmid pGEMT-Easy (Promega). The ligated plasmid, designated as pGBH1, was amplified in DH5á, isolated, and digested with BamHI and HindIII. The fragment (~ 510 bp) was purified and ligated with the 4.2 kbp fragment of pUSS1, which was digested previously by BamHI and HindIII. The constructed plasmid, designated as pUSH1, was sequenced to check any sequence change. pUSH1, which produced a derivative of rAgaB34 with a 6× His tag at its C-terminal, was then transformed into *E. coli* DH5α. The recombinant DH5α was cultured at 37°C in a LB broth supplemented with 100 μg/ml ampicillin. When the OD_{600} reached 0.5, IPTG was added to the culture medium to give a concentration of 1 mM. After incubation for an additional 6 h at 37°C, the culture fluid was centrifuged at 12,000 ×*g* for 15 min.

Purification of rAgaB34

The cell-free supernatant was concentrated by ultrafiltration (10-kDa cutoff membrane; Millipore, Bedford, MA, U.S.A.) and applied to a Ni$^+$ Sepharose 6 FF column (1×10 cm; Amersham, Uppsala, Sweden) pre-equilibrated with buffer A (50 mM Tris-HCl, 0.3 M NaCl, pH 8.0). After washing the column with buffer A supplemented with 10 mM histidine until the OD_{280} reached zero, the bound protein was eluted with buffer A supplemented with 250 mM histidine at a flow rate of 0.3 ml/min. Main active fractions were pooled and dialyzed against 50 mM Tris-HCl buffer (pH 8.0). The purified agarase was applied to SDS-PAGE on a 10% polyacrylamide gel by the method of Laemmli [16]. Protein bands were detected by staining the electrophorized gel with Coomassie Brilliant Blue R-250. The molecular mass markers (Sigma) consisted of albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20.1 kDa).

Enzyme Assay

The agarase activity was quantified by spectrophotometric determination of reducing sugars by the Nelson method [19]. After incubating 50 μl of enzyme solution with 200 μl of 0.1% agarose (Sigma, St. Louis, MO, U.S.A.) in 20 mM Tris-HCl (pH 8.0) at 40°C for 30 min, the amount of reducing sugar was determined by Nelson reagent using D-galactose as a standard. Enzyme activity (U/ml) was defined as the amount of enzyme required to liberate 1 μmol D-galactose per minute. Protein concentration was determined by the Bradford method [4] using bovine serum albumin as the calibration standard.

Characterization of rAgaB34

The effects of pH and temperature on the activity of rAgaB34 were assayed at each pH (4.0–12.0) at 40°C and each temperature (10–90°C) at pH 8.0. The pH and thermal stabilities of the agarase were determined by preincubating the enzyme solution at each pH (4.0–12.0) at 40°C and each temperature (10–90°C) at pH 8.0 for 1 h and then measuring the residual enzyme activity. The effects of various metal ions (100 mM) and several other reagents (10 mM) on enzyme activity were determined by adding 100 μl of each reagent to the reaction mixture, respectively. NA6

and NA4 (Dextra, Reading, U.K.) were used instead of agarose to identify the cleavage pattern of rAgaB34. Thin-layer chromatography was performed to determine the products [1]. K_m and V_{max} values for rAgaB34 acting on agarose (final concentration of 0.05–0.5 mg/ml) were calculated by linear regression analysis of Lineweaver-Burke plots [18]. Values of k_{cat} (turnover number) and k_{cat}/K_m (catalytic efficiency) were calculated based on K_m, V_{max}, and [E] (agarase concentration) values.

Analysis of Enzymatic Product

Hydrolysis of 1.0% (w/v) agarose by rAgaB34 was carried out at 40°C with shaking at 120 rpm overnight. End products were determined by a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (QSTAR Pulsar I; Applied Biosystems, Foster City, CA, U.S.A.) and a carbon 13 nuclear magnetic resonance (^{13}C NMR) spectrometer (JNM-ECP 300M; Jeol, Tokyo, Japan).

Nucleotide Sequence Accession Number

The nucleotide sequence of *agaB34* was submitted to the GenBank nucleotide sequence database. The sequence is available in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number of EU200967.

RESULTS

Cloning of the Agarase Gene

About 5,000 transformants carrying recombinant plasmids were screened by plate assays, and two agarase-positive *E. coli* transformants were obtained. pUSS1 and pUSS2, the plasmids purified from these two agarase-positive colonies, had a 2.4-kb and a 3.0-kb Sau3AI fragment, respectively. Both fragments were sequenced, and the result indicated that they shared the same open reading frame (ORF) of 1,362 bp, which begins with the ATG codon and ends with the TAG codon. This ORF showed 77% nucleotide sequence homology to that of *agaA* from *Pseudoalteromonas* sp. CY24 (GenBank Accession No. AY150179) and *agaD* from *Vibrio* sp. PO-303 (AB221476). The nucleotide sequence of the agarase gene and its deduced amino acid sequence are shown in Fig. 1. The gene is orientated in the same direction as the *lac* promoter of pUC18. Putative promoter sequences, 5'-TAAGCA-3' for the –35 region and 5'-TTTAAC-3' for the –10 region, are located 39 bp and 15 bp upstream of the coding region, respectively, with an 18 bp spacing. A putative ribosome binding site (5'-AGGGAG-3') having homology to the Shine-Dalgarno (SD) sequence is present at 8 bp upstream of the coding region. A 41-bp palindrome sequence with a free energy value of –17.10 kcal/mol is located 46 bp downstream of the coding region. Thus, this sequence is a possible site for a ρ-independent transcriptional terminator [25].

Deduced Amino Acid Sequence of *agaB34* Gene

The predicted product of the agarase gene was a protein of 453 amino acids consisted of a possible N-terminal signal sequence of 23 amino acid residues, followed by a mature
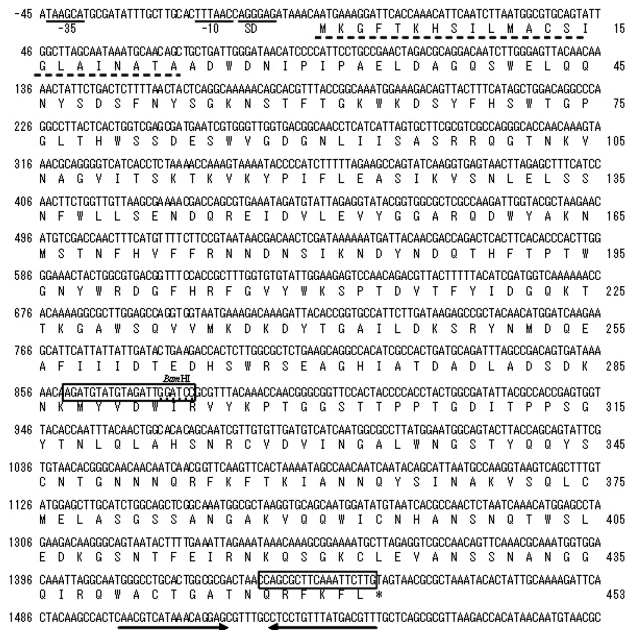

Fig. 1. Nucleotide sequence of *agaB34* gene and its deduced amino acid sequence.

The –35 and –10 regions of a possible promoter sequence and the potential Shine-Dalgarno (SD) sequence are underlined. The putative signal peptide is indicated by dashed underline. Gene sequences based on which the primers were designed are boxed and the BamHI site is indicated. The termination codon of the *agaB34* gene is indicated by an asterisk. The palindrome sequence is indicated by convergent arrows.

protein of 430 amino acid sequence with calculated molecular mass of 48,637 Da and pI value of 6.34. The possible cleavage site is between Ala23 and Ala24. Comparison of the N-terminal sequence of the deduced mature protein to those of the native agarases indicated that the gene product is AgaB34. The deduced amino acid sequence of *agaB34* showed a high homology to those of several agarases, such as 86% to AgaA from *Pseudoalteromonas* sp. CY24, 85% to AgaD from *Vibrio* sp. PO-303, 71% to AgaV from *Vibrio* sp. V134, and 61% to AgaY from *Janthinobacterium* sp. SY12.

Conserved domain search with the 453 amino acid sequence indicated that AgaB34 is a modular protein consisting of two domains (Fig. 2A); *i.e.*, a family GH-16 catalytic module (CM) consisting of 273 amino acid (Asp25-Lys297) in the N-terminus, and a carbohydrate-binding module (CBM) consisting of 137 amino acid (Tyr316-Phe452) in the C-terminus. The multiple alignments of amino acid sequences of AgaB34 and other putative proteins consisting of GH-16 CM (Fig. 2B) and CBM (Fig. 2C) indicated that most consensus sequences are conserved in AgaB34.

Expression and Purification of Recombinant AgaB34 (rAgaB34)

Time courses of cell growth of recombinant *E. coli* DH5α and production of rAgaB34 are shown in Fig. 3. The maximum agarase activity of 1.67 U/ml was detected in
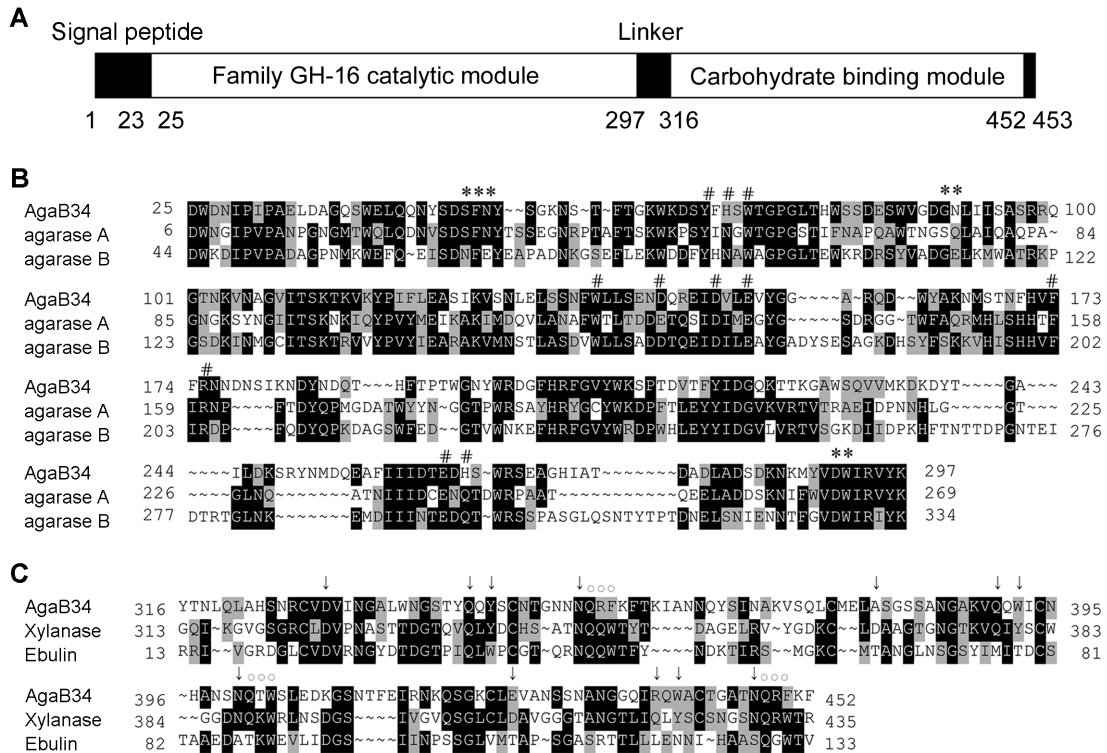
A

Signal peptide   Linker

| Family GH-16 catalytic module | | Carbohydrate binding module |

1 23 25   297 316   452 453

B

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                           ***                         # #  #                    **
AgaB34      25 DWDNIPIPAELDAGQSWELQQNYSDSFNY~~SGKNS~T~FTGKWKDSYFHSWTGPGLTHWSSDESWVGDGNLIISASRRQ 100
agarase A    6 DWNGIPVPANPGNGMTWQLQDNVSDSFNYTSSEGNRPTAFTSKWKPSYINGWTGPGSTIFNAPQAWTNGSQLAIQAQPA~  84
agarase B   44 DWKDIPVPADAGPNMKWBFQ~EISDNRBYEAPADNKGSELEKWDDFYHNAWAGPGLTEWKRDRSYVADGELKMWATRKP 122

                                              #     #   #  #  #  #                       #
AgaB34     101 GTNKVNAGVITSKTKVKYPIFLEASIKVSNLELSSNFWLLSENDQREIDVLEVYGG~~~~A~RQD~~WYAKNMSTNFHVF 173
agarase A   85 GNGKSYNGLITSKNKIQYPVYMEIKAKIMDQVLANAFWTLTDDETQSIDIMEGYG~~~~~SDRGG~TWFAQRMHLSHHTF 158
agarase B  123 GSDKINMGCITSKTRVVYPVYIEARAKVMNSTLASDVWLLSADDTQEIDILEAYGADYSESAGKDHSYFSKKVHISHHVF 202

            #
AgaB34     174 FRNNDNSIKNDYNDQT~~~HETPTWGNYWRDGFHRFGVYWKSPTDVTFYIDGQKTTKGAWSQVVMKDKDYT~~~~GA~~~ 243
agarase A  159 IRNB~~~~BTDYQPMGDATWYYN~GGTPWRSAYHRYGCYWKDPFTLEYYIDGVKVRTVTRAEIDPNNHLG~~~~~GT~~~ 225
agarase B  203 IRDB~~~~BQDYQPKDAGSWFED~~GTVWNKEFHRFGVYWRDPWHLEYYIDGVLVRTVSGKDIIDPKHFTNTTDPGNTEI 276

                          # #                                      **
AgaB34     244 ~~~~ILDKSRYNMDQEAFIIIDTEDHS~WRSEAGHIAT~~~~~~~~DADLADSDKNKMYVDWIRVYK 297
agarase A  226 ~~~~GLNQ~~~~~~~~ATNIIIDCENQTDWRPAAT~~~~~~~~~~~~QEELADDSKNIFWVDWIRVYK 269
agarase B  277 DTRTGLNK~~~~~~~~EMDIIINTEDQT~WRSSPASGLQSNTYTPTDNELSNIENNTFGVDWIRIYK 334
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C

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                  ↓              ↓           ↓ ○○○            ↓              ↓    ↓
AgaB34     316 YTNLQLAHSNRCVDVINGALWNGSTYQQYSCNTGNNNQRFKFTKIANNQYSINAKVSQLCMELASGSSANGAKVQQWICN 395
Xylanase   313 GQI~KGVGSGRCLDVPNASTTDGTQVQLYDCHS~ATNQQWTYT~~~~DAGELRV~YGDKC~~LDAAGTGNGTKVQIYSCW 383
Ebulin      13 RRI~~VGRDGLCVDVRNGYDTDGTPIQLWPCGT~QRNQQWTFY~~~~NDKTIRS~~MGKC~~MTANGLNSGSYIMITDCS  81

                  ↓ ○○○                              ↓  ↓           ↓ ○○○
AgaB34     396 ~HANSNQTWSLEDKGSNTFEIRNKQSGKCLEVANSSNANGGQIRQWACTGATNQRFKF 452
Xylanase   384 ~~GGDNQKWRLNSDGS~~~~IVGVQSGLCLDAVGGGTANGTLIQLYSCSNGSNQRWTR 435
Ebulin      82 TAAEDATKWEVLIDGS~~~~IINPSSGLVMTAP~SGASRTTLLLENNI~HAASQGWTV 133
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Fig. 2. General organization of AgaB34 **(A)**, and alignments of amino acid sequence of AgaB34 and other putative proteins consisting of the family GH-16 catalytic module **(B)** and carbohydrate-binding module **(C)**.
The amino acid numbers of each domain are indicated. Conserved amino acid residues are highlighted. Tildes indicate gaps to improve the alignment. Putative amino acid residues involved in active site (#), calcium binding site (*), Q-X-W(F) motif (○), and sugar binding site (↓) are marked. AgaB34 (ABW77762) is from *Agarivorans albus* YKW-34 (this study), agarase A (1URXA) and agarase B (1O4ZA) are from *Zobellia galactanivorans*, xylanase (1XYF_A) is from *Streptomyces olivaceoviridis*, and ebulin (1HWM_B) is from *Sambucus ebulus*.
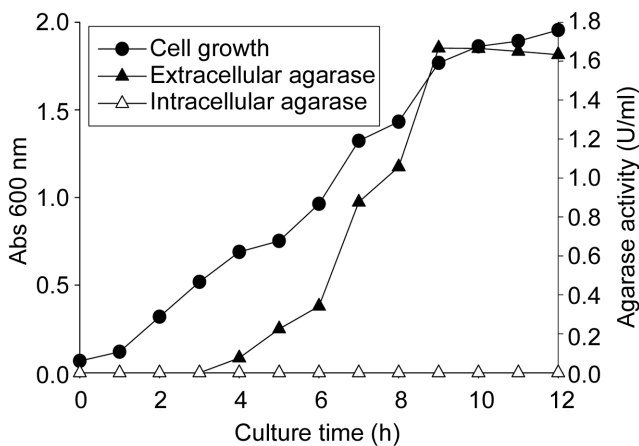

Fig. 3. Time courses of cell growth of recombinant *E. coli* DH5α and production of rAgaB34.

the culture fluid of DH5α after induction for 6 h with IPTG at 37°C. No agarase activity was determined in the intracellular fraction of the cells (Fig. 3), which indicated rAgaB34 is a secretory protein. The rAgaB34 was purified to homogeneity by affinity chromatography. The purification procedures from a 50-ml culture medium are summarized in Table 1. The specific activity of purified rAgaB34 for agarose at 40°C was 242.18 U/mg. Thus, the production of rAgaB34 was about 6.9 mg/l. The molecular mass of the rAgaB34 was estimated to be 49 kDa by SDS-PAGE analysis (Fig. 4), which was coincident with that of the native AgaB34 and that estimated from the gene sequence.

Characterization of rAgaB34

The pH and temperature profiles of rAgaB34 are shown in Fig. 5. The agarase exhibited the maximum activity and

Table 1. Purification of rAgaB34 expressed by *E. coli*.

Procedure	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Yield (%)	Fold
Culture fluid	5.20	83.73	16.09	100	1
Ni⁺ Sepharose 6	0.28	67.08	242.18	80	15

[a] Agarase activity was determined by the Nelson method using agarose as the substrate.
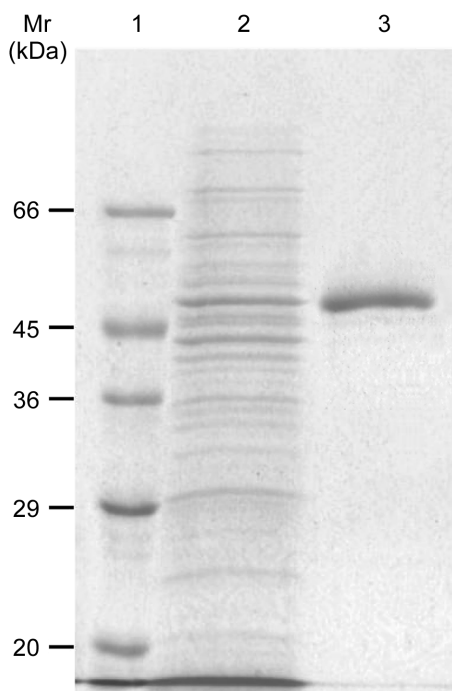
Fig. 4. SDS-PAGE analysis of rAgaB34 from *E. coli* harboring pUSH1.
Lane 1, molecular marker; lane 2, supernatant of culture fluid; lane 3, rAgaB34 purified from affinity chromatography.
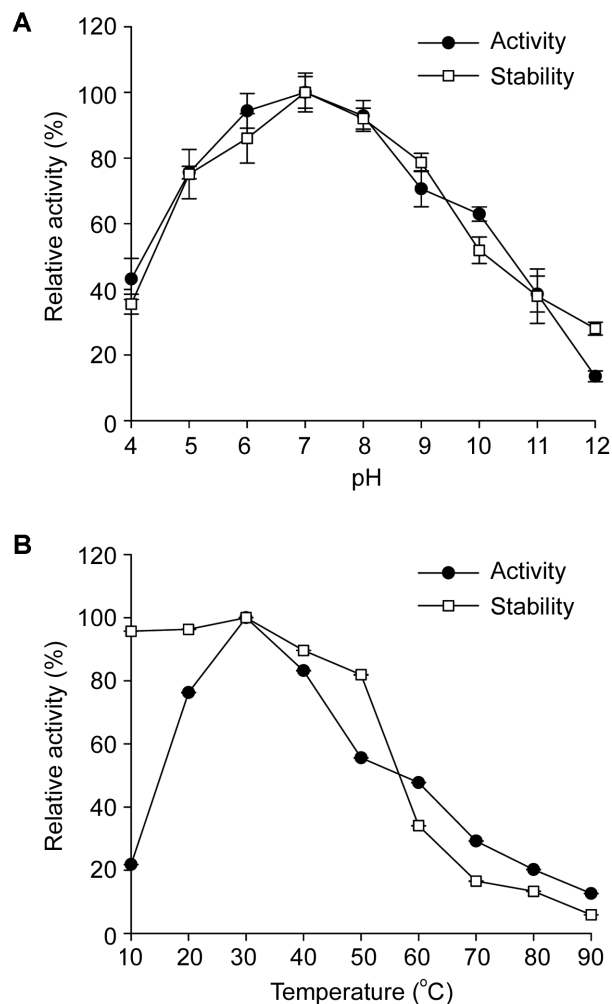

Fig. 5. Effects of pH **(A)** and temperature **(B)** on stability and activity of rAgaB34.
pH profiles were checked at 40°C in different buffers (pH 4.0–12.0). Temperature profiles were checked at different temperatures (10–90°C) in 0.1 M Tris-HCl (pH 8.0). Agarase activity was determined by the Nelson method using agarose as the substrate. The relative activity was defined as the percentage of activity determined with respect to the maximum agarase activity.

stability at pH 7.0. More than 70% activity was detected after incubation at a wide pH range of 5.0–9.0 for 1 h. The enzyme possessed maximum agarase activity at 30°C and was thermostable up to 50°C. Effects of various reagents on the activity of rAgaB34 were investigated (Fig. 6). Neither significant activation nor inhibition of rAgaB34 was observed by metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, and Ba^{2+}), chelators (EDTA and EGTA), reducing reagents (β-mercaptoethanol and DTT), and detergents (SDS and urea), whereas the activity of rAgaB34 was inhibited by heavy metal ions (Cu^{2+}, Mn^{2+}, Zn^{2+}, and Al^{3+}). For its cleavage pattern, rAgaB34 hydrolyzed NA6 into NA4 and NA2, but NA4 could not be further hydrolyzed into NA2 even after long incubation time (data not shown). The Lineweaver-Burk plot of rAgaB34 acting on agarose is shown in Fig. 7. K_m, V_{max}, k_{cat}, and k_{cat}/K_m values for agarose were 0.24 mg/ml (2.4×10^{-5} M), 50 U/mg, 41 /s, and 1.7×10^6 /sM, respectively.

Cleavage Pattern and Enzymatic Product
End products from agarose were determined by MALDI-TOF MS and ^{13}C NMR. As shown in Fig. 8, the dominant peaks with molecular masses of around 676 and 959 Da corresponded most probably to molecules of $[M+2Na]^+$ and $[M+Na]^+$, respectively. Thus, the enzymatic products were a tetramer (630 Da, $C_{24}H_{38}O_{19}$) and a hexamer (936 Da, $C_{36}H_{56}O_{28}$). The intensity of MALDI-TOF MS indicated that the tetramer was the dominant product (Fig. 8).

A typical pattern for neoagarooligosaccharides was observed according to the NMR spectrum (Fig. 9). The resonances at 96.960 and 92.979 ppm were assigned to be β and α anomeric carbons of the G-unit at the reducing end of the neoagarooligosaccharides, respectively. No resonance was observed at around 90.7 ppm, which is the characteristic signal of the A-unit at the reducing end of the agarooligosaccharides. These results indicated that the tetramer and hexamer produced by rAgaB34 were NA4 and NA6, respectively.

DISCUSSION

A β-agarase gene, AgaB34, was cloned and sequenced from *A. albus* YKW-34. Upon BLAST analysis using the full-
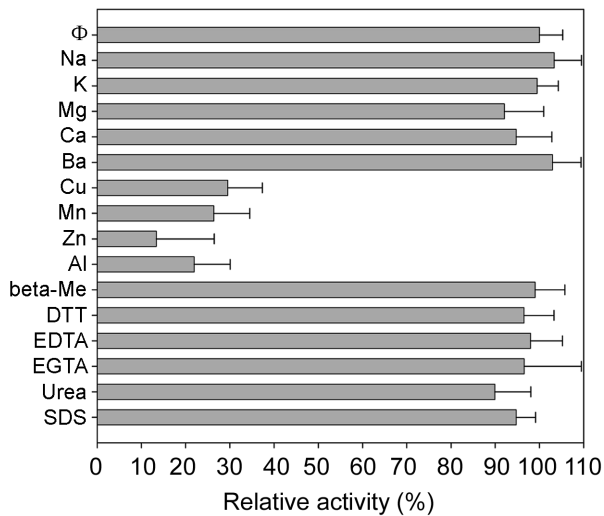
Fig. 6. Effects of various reagents on activity of rAgaB34.
Each kind of metal ion (final concentration of 100 mM) and other reagent (final concentration of 10 mM) was added, respectively, to the reaction mixture. Agarase activity was determined by the Nelson method using agarose as the substrate. The agarase activity determined under the standard conditions (Φ) was defined as 100%. Beta-Me, beta-mercaptoethanol.

length AgaB34 as a query, four proteins were found to have similarities of more than 50% homologies to AgaB34. These proteins were β-agarases belonging to family GH-16 (http://www.cazy.org/). Other enzymes belonging to this family, such as mannanase, glucosidase, and xylosidase, are also structurally related to AgaB34, but with lower similarity. Thus, AgaB34 belongs to the GH-16 β-agarase subfamily. The crystal structures of two agarases, agarases A and B
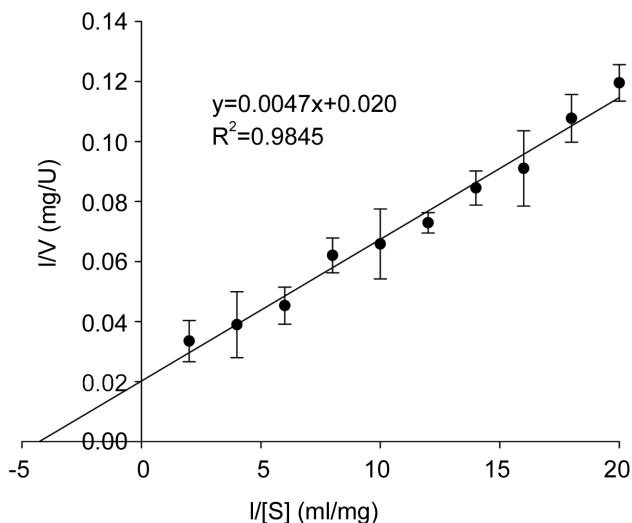
Fig. 7. Lineweaver-Burk plot to determine the kinetic parameters of rAgaB34 acting on agarose.
Agarase activity was assayed with agarose (final concentration of 0.05–0.5 mg/ml, molecular mass of 10 kDa) and rAgaB34 ([E] of 0.187 mg/ml) by the Nelson method.
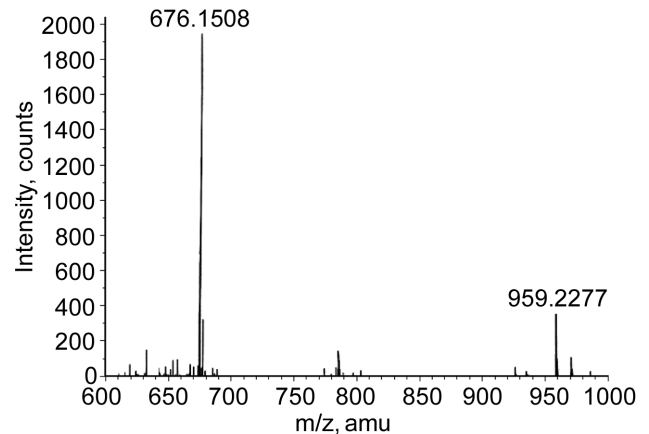

Fig. 8. MALDI-TOF mass spectrum of agarose hydrolysis fragments.
The sample was prepared by incubating rAgaB34 with agarose at 40°C with shaking at 120 rpm overnight.

from *Zobellia galactanivorans*, belonging to this subfamily were analyzed [13]. They are believed to have a family GH-16 catalytic module. Alignment of amino acid sequences of AgaB34 and the above two agarases indicated that several amino acid residues identified as active sites and calcium-binding sites in agarases A and B from *Zobellia galactanivorans* were also conserved in AgaB34 (Fig. 2B). These residues include aromatic amino acids (Phe51, Tyr68, Trp72, Trp138, Phe173, Try292), negative-charged amino acids (Asp144, Asp149, Glu152, Glu263, Asp291), and a positive-charged amino acid (Arg175). The alignment further identified that AgaB34 belongs to the GH-16 β-agarase subfamily. The products and cleavage pattern of rAgaB34 also agreed well with those of other agarases belonging to this subfamily [5, 6, 21].

Another conserved domain existing in AgaB34 is CBM, which was found in xylanase from *Streptomyces olivaceoviridis* [9] and ebulin from *Sambucus ebulus* [23], whose crystal structures were also analyzed. Highly specific ligand binding
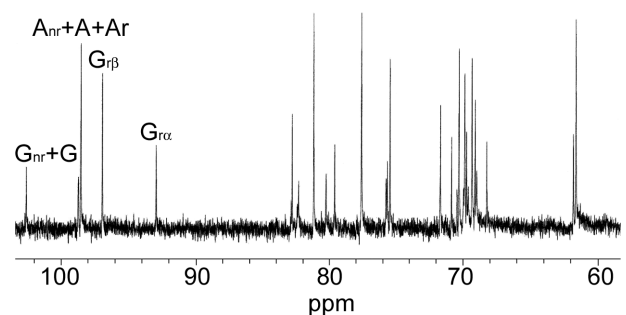

Fig. 9. ^{13}C NMR spectrum of the enzymatic products of agarose by rAgaB34.
The sample was prepared by incubating rAgaB34 with agarose at 40°C with shaking at 120 rpm overnight. G, β-D-galactopyranose; A, 3,6-anhydro-α-L-galactopyranose; r, reducing end; nr, nonreducing end; α, α anomer; β, β anomer.

was found to occur on the exposed surface of CBM. CBM includes three Q-X-W(F) motifs whose role was found to target the catalytic domain to the substrate [26], and involves some amino acid residues that serve as sugar binding sites [23]. Sequence alignment of AgaB34 and the above two proteins indicated that key amino acid residues of CBM, including those in Q-X-W(F) motifs and some of the sugar binding sites, were highly conserved in AgaB34 (Fig. 2C).

Based on the amino acid similarity, β-agarases were classified into three families of GH-16, GH-50, and GH-86 (http://www.cazy.org/), among which family GH-16 β-agarases are most abundant with members from different microorganisms. However, there is no report of GH-16 agarase from the strain belonging to the *Agarivorans* genus. Two agarases reported from *Agarivorans* strains belonged to family GH-50 [17, 20]. Thus, AgaB34 was the first family GH-16 agarase derived from the *Agarivorans* genus. Because AgaB34 is from an *Agarivorans* strain, a special agar devouring strain, it is reasonable to assume that AgaB34 might possess novel properties comparing with other family GH-16 agarases.

rAgaB34 had a molecular mass of 49 kDa and a specific activity of 242 U/mg. The specific activity was higher than those of many reported family GH-16 agarases; 63.6 U/mg of AgaA [5] and 16.4 U/mg of AgaD [6] from *Vibrio sp.* PO-303, 160 U/mg of AgaA and 100 U/mg of AgaB from *Zobellia galactanivorans* [13], but lower than 398 U/mg of RagaA7 from the deep-sea novel microorganism *Microbulbifer* sp. JAMB-A7 [21]. rAgaB34 was stable at pH 5–9 and had maximum activity at pH 7.0. Its pH properties were similar to those of reported agarases [5, 6, 21]. with regard to thermal stability, rAgaB34 was stable up to 50°C, whereas reported agarases were stable up to 40°C [20] or 37°C [6]. Only RagaA7 from *Microbulbifer* sp. JAMB-A7 [21] showed a similar thermal stability with rAgaB34. Reported agarases possessed optimal activity at temperature of 40°C [5, 6] or 50°C [21], which is above the gelling temperature of agar (around 38°C). It is novel that rAgaB34 could hydrolyze the gelated agarose and had the maximal activity at 30°C. Thus, it might be used for industrial production of neoagarooligosaccharide directly from marine algae under economic conditions. The resistance of rAgaB34 against commonly existed metal ions in marine environment and various other chemical reagents also benefits its industrial application. Moreover, the kinetic properties of rAgaB34 were superior to those of other reported agarases. rAgaB34 had a K_m value of 0.24 mg/ml against agarose, which was lower than the 1.1 mg/ml of AgaD from *Vibrio sp.* PO-303 [6], 2.33 mg/ml of AgaA from *Vibrio sp.* PO-303 [5], and 3.0 mg/ml of RagaA7 from *Microbulbifer* sp. JAMB-A7 [21].

The recombinant agarase rAgaB34 was produced extracellularly by *E. coli* DH5α with a satisfactory yield of 1,670 U/l. When *E. coli* was used as a host, most recombinant agarases were produced intracellularly with a relatively lower yield. AgaA and AgaB from *Zobellia galactanivorans* and AgaD from *Vibrio* sp. PO-303 were produced intracellularly by *E. coli* with yields of 160 U/l, 800 U/l [13], and 620 U/l [6], respectively. Excretion of rAgaB34 to the culture medium resulted in higher agarase yield and also greatly facilitated purification of this enzyme.

Intracellular production of a protein has certain disadvantages. High-level accumulation of the expressed protein often leads to the formation of inclusion body, which needs to be further solubilized and refolded to obtain the native protein conformation. On the other hand, the intracellular soluble protein may be destroyed as a result of proteolytic degradation. It was reported that the proteins transported outside were at least 10 times more stable than those that remained in the cytoplasm [28]. There have been various attempts to establish a secretory expression system [12, 29, 32] because excreting protein to the medium allows the protein to be obtained in its soluble and perfectly folded form, and to be more easily purified without cell lysis. However, because of the outer membrane barrier, the Gram-negative bacterium *E. coli* is generally not considered to be an appropriate host for the secretion of proteins into the culture medium. In this study, *E. coli* cells harboring the pUC18-derived agarase expression vector pUSH1 were able to efficiently excrete agarase into the culture medium. This expression system might be used to express secretory proteins.

The high-level expression of a recombinant agarase and its relatively excellent specific activity, thermal stability, and kinetic properties, as well as the novel thermal activity, enlighten the potential application of this agarase. Moreover, the achievements in secretion of agarase using *E. coli* provides an attractive approach to the construction of recombinant strains for the large-scale production of secretory proteins.

Acknowledgments

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