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Thermostable β-Agarase from a Deep-sea *Microbulbifer* Isolate*

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Abstract: *Microbulbifer* sp. strain JAMB-A3, isolated from the sediment in Sagami Bay, Japan, at a depth of 1174 m, was found to produce a novel β-agarase. The agarase gene was cloned and sequenced. It encodes a protein of 602 amino acids with a calculated molecular mass of 65,017 Da. The deduced amino acid sequence showed similarity to those of known β-agarases in glycoside hydrolase family 16, with 34–55% identity. Tandem sequences similar to a carbohydrate binding-like module were found in the C-terminal region of the enzyme. The recombinant agarase was hyper-produced extracellularly using *Bacillus subtilis* as the host, and the homogeneously purified enzyme had a high specific activity of 528 U/mg at pH 7.0 and 50°C. The optimal temperature and pH for activity were 54°C and around 7, respectively. The recombinant enzyme was thermostable with a half-life of 8.7 h at 50°C. It was very stable during incubation with EDTA, Na^+, K^+, Mg^{2+}, Ca^{2+} ions, and surfactants at high concentrations. *N*-Bromosuccinimide abolished the enzymatic activity, and agarose oligosaccharides protected the enzyme from inactivation by this chemical, suggesting that a tryptophan(s) residue is involved in the catalysis of the enzyme. The pattern of agarose hydrolysis showed that the enzyme is an endo-type β-agarase, and the final main product is neoagarotetraose.

Key words: *Microbulbifer*, β-agarase, glycoside hydrolase family 16, neoagaro-oligosaccharide

Agarose from the cell walls of some red algae consists of a linear chain of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactose.[1] Agaropectins have the same basic disaccharide-repeating units as agarose, although some hydroxyl groups of 3,6-anhydro-α-L-galactose residues are substituted for by sulfoxy or methoxy and pyruvate residues.[2] Agar-degrading bacteria that have been isolated so far from marine and other environments are strains of the genera *Pseudomonas*,[3] *Pseudoalteromonas*,[4] *Streptomyces*,[5] *Alteromonas*,[6] *Microscilla*,[7,8] *Vibrio*[9,10] and *Cytophaga*.[11] The agarolytic enzymes produced by these bacteria are α-agarase and β-agarase, which hydrolyze α-1,3 linkages and β-1,4 linkages in agarose, respectively. Most agarases that have been purified and characterized belong to the β-agarase group except for an α-agarase from *Alteromonas agarlyticus* GJ1B.[6]

Agarases have many applications in the food, cosmetic, and pharmaceutical industries for the production of oligosaccharides from agar.[12,13] Moreover, agarase can be used to degrade the cell walls of marine algae for extraction of labile substances with biological activities and for the preparation of protoplasts.[14] We have isolated a number of deep-sea, agar-degrading microorganisms, sequenced the genes encoding agarases and characterized the enzymes. In this report, we describe the cloning and sequencing of the gene for an agarase (*agaA3*) produced by a strain of deep-sea *Microbulbifer* isolated from Sagami Bay, Japan, at a depth of 1174 m. Moreover, we show that the recombinant enzyme expressed in *Bacillus subtilis* cells (RagaA3) is thermostable and resistant to various chemical reagents.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Microbulbifer* sp. strain JAMB-A3 was isolated from a sediment sample obtained from Sagami Bay, Japan, at a depth of 1174 m using the submersible Shinkai 2000. This strain has been deposited as a patented strain (FERM BP-8319) in the National Institute of Advanced Industrial Science and Technology of Japan. It was propagated at 25°C in marine agar 2216 (Difco). *Escherichia coli* HB101 (F′ *supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*) was used as the host for cloning and routinely grown at 37°C in Luria-Bertani (LB) broth (Difco) supplemented with 100 μg/mL ampicillin or 15 μg/mL tetracycline when required. Recombinant *B. subtilis* ISW1214 (*leuA8 metB5 hsrM1*) was cultured at 30°C, with shaking, in a medium composed of (w/v) 12% corn steep liquor (Nihon Syokuhin Kako), 0.2% Lab-Lemco powder (Oxoid), 0.1% yeast extract (Difco), 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2$, 6% maltose and 15 μg/mL tetracycline (CLT medium).

Isolation of DNA, transformation, and sequencing. Genomic DNA and plasmids were prepared as described by Saito and Miura[15] and Birnboim and Doly,[16] respectively. Restriction digestion and ligation were carried out using the methods of Sambrook *et al*.[17] Transformations

* Data deposition: The nucleotide sequence data reported in this study has been submitted to the DDBJ, EMBL and GenBank databases under accession number AB158516.

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Abbreviations: CBB, Coomasie Brilliant Blue R-250; LB, Luria-Bertani; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NBS, *N*-bromosuccinimide; TLC, thin-layer chromatography; aa, amino acid(s); NA6, neoagarohexaose; NA4, neoagarotetraose; NA2, neoagarobiose; MOPS, 3-morpholinopropanesulfonic acid-NaOH; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ORF, open reading frame; PCR, polymerase chain reaction.

of *E. coli* and *B. subtilis* with plasmids were performed by the methods of Hanahan[18] and Chang and Cohen,[19] respectively. Double-stranded DNA sequencing was performed with custom oligonucleotide primers using an ABI Prism Big Dye Terminator Cycle Sequencing kit and an ABI 377 Sequencer (Applied Biosystems). Computer sequence analysis was carried out using the GENETYX-MAC program ver. 10.1 (SDC Software Development).

Cloning of the agaA3 gene. The genomic DNA of strain JAMB-A3 was digested with *Hin*dIII. The digests were purified using a High Pure PCR Product Purification kit (Roche Diagnostics) and ligated into pUC18, which had been digested with *Hin*dIII and then treated with a shrimp alkaline phosphatase (Roche Diagnostics) using a DNA ligation kit ver. 2 (Takara). The ligation mixture was transformed into competent *E. coli* HB101 cells, and transformants were selected on Luria-Bertani (LB) agar supplemented with 100 μg/mL ampicillin. Clones expressing agarolytic activity were detected as the colonies forming shallow craters around them on the agar. Then, to confirm the agarolytic activity expressed in them, 2% (w/v) iodine solution (Wako Pure Chemical) was poured onto the agar. The positive clones were visualized as having clear zones around the colonies with a dark brown background. The plasmid in one of the positive clones was designated pUA3 and its insert was sequenced in both directions.

Expression and purification of RagaA3. To produce recombinant agarase, the *agaA3* gene was amplified from genomic DNA of strain JAMB-A3 by PCR with LA *Taq* DNA polymerase. Two primers, 5′-CGTCGACTGTCATC CTCCGCGCTGGCTGCG-3′ and 5′-TTGGATCCCTGGT GATTAGTTACTCAGCAC-3′, were constructed incorporating *Sal*I and *Bam*HI restriction sites (underlined) into the 5′ and 3′ ends of the *agaA3* gene, respectively. The amplification product was digested with *Sal*I and *Bam*HI, and the digest was ligated into the *Sal*I-*Bam*HI site of the *E. coli-B. subtilis* shuttle vector pHSP64,[20] which was constructed for high-level expression of foreign genes in *B. subtilis*. The recombinant plasmid designated pA3AG was then introduced into both *E. coli* HB101 and *B. subtilis* ISW1214 cells. *E. coli* HB101 cells harboring pA 3AG were grown for 16 h on LB agar supplemented with 100 μg/mL ampicillin. The agarolytic activities of *E. coli* HB101 cells harboring pA3AG were confirmed by pit formation in the agar. The recombinant agarase produced by *B. subtilis* ISW1214 was designated RagaA3. *B. subtilis* ISW1214 cells harboring pA3AG were grown for 72 h in CLT medium. Cells and supernatants of the cultures were separated by centrifugation at 12,000×g for 10 min. The supernatant obtained was used for enzyme purification. All procedures for enzyme purification were carried out at temperatures below 4°C. The centrifugal supernatant (45 mL) was brought to 90% saturation with solid ammonium sulfate. The precipitates formed were collected by centrifugation (8000×g, 25 min) and resolved in a small volume of 20 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed against the same buffer overnight. After removal of insoluble materials by centrifugation at 8000×g for 15 min, the retentate was applied to a DEAE-Toyopearl 650M column (2.5× 15 cm, Tosoh) that had been equilibrated with 20 mM Tris-HCl buffer (pH 7.5). After washing the column with 200 mL of 20 mM Tris-HCl (pH 7.5) buffer containing 0.15 M NaCl, the enzyme was eluted over 500 mL with a linear 0.15–0.35 M NaCl gradient in the same buffer. The active fractions were combined and concentrated to 5.0 mL on a PM10 membrane (10,000-M_r cutoff, Millipore). The buffer of this enzyme preparation was replaced for 5 mM sodium phosphate buffer (pH 7.0) by 10-fold dilutions with 5 mM sodium phosphate buffer and concentration to 5 mL twice on the membrane. The retentate was loaded onto a hydroxyapatite column (2.5×15 cm, Seikagaku Kogyo) equilibrated with 5 mM sodium phosphate buffer (pH 7.0). After washing the column with 200 mL of 15 mM sodium phosphate buffer (pH 7.0), the enzyme was eluted over 500 mL with a linear 15–85 mM sodium phosphate gradient (pH 7.0). The active fractions were pooled and concentrated by ultrafiltration on a PM10 membrane. The concentrate was dialyzed overnight against 50 mM 3-morpholinopropanesulfonic acid-NaOH (MOPS) buffer (pH 7.0). The dialyzate (1.0 mL) was used as the final preparation of purified enzyme throughout the experiments.

Enzyme assay. A suitably diluted solution of enzyme preparation was incubated in 50 mM MOPS buffer (pH 7.0) containing 0.2% (w/v) of agar (Nacalai Tesque) at 50°C. Activity was expressed as the initial rate of agar hydrolysis by measuring the release of the reducing ends using the 3,5-dinitrosalicylic acid procedure[21] with D-galactose as the standard. One unit (U) of enzymatic activity was defined as the amount of protein that produced 1 μmol of reducing sugar as D-galactose per minute under the conditions of the assay. The kinetic parameters of K_m and k_{cat} for agar and neoagarohexaose (NA6) (Dextra Laboratories) were determined at 50°C in 50 mM MOPS buffer (pH 7.0). The initial rates of the hydrolysis of agar were determined at seven different substrate concentrations, ranging from 0.15- to 2.5-times the estimated K_m value. The hydrolysis of NA6 was monitored quantitatively by gel filtration chromatography on an Asahipak GS220 G7 column (6.7×500 mm, Asahi Kasei) using an LC-10Avp with CLASS-VP HPLC system (Shimadzu) equipped with a refractive index detector (RID-10A, Shimadzu). Reaction conditions were chosen so that $<$10% of the substrate was hydrolyzed, and the initial rates were determined based on the rates of substrate disappearance. Protein was determined using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard protein.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli[22] on slab gels [12.5% (w/v) acrylamide, 70×50 mm, 2.0-mm thick], and the gels were stained for protein with Coomasie Brilliant Blue R-250 (CBB, Bio-Rad). The molecular mass was estimated using SDS-PAGE 12% (w/v) acrylamide gels with low-range molecular mass standards (Bio-Rad), which included phosphorylase *b* (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Liquid chromatography-tandem mass spectrometry. The amino acid (aa) sequence of the C-terminal region,

and molecular mass of the purified enzyme were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after SDS-PAGE. The CBB-stained protein was manually excised from the gel and then digested *in situ* with 25 μg/mL *Staphylococcus aureus* V8 protease (Sigma) using established protocols.[23] The digests were extracted from the gel slices with four 15-min washes with 50% acetonitrile/1% formic acid (v/v). The peptides were dried and suspended in a solution of 2% acetonitrile/98% H_2O/0.1% trifluoroacetic acid (v/v). For on-line microcapillary LC-MS/MS analysis, the peptide mixtures were then chromatographed in a MAGIC 2002 HPLC system (Michrom Bioresources). All tandem mass spectra were recorded on a Thermo Finnigan LCQ Deca-plus ion-trap mass spectrometer (Thermo Finnigan) equipped with a nanospray ion source, using borosilicate PicoTips (Econo 10, New Objective). MS/MS spectra were acquired over the range of 450–2000 m/z. The MS/MS raw data were subsequently analyzed using TurboSEQUEST software (Thermo Finnigan)[24,25] that had been "indexed for speed" with carbamidomethylation as a static modification of cysteine (+57.0 Da), with and without oxidation of methionine (+16.0 Da). To identify the C-terminal aa of RagaA3, a database was created based on the deduced aa sequence of the *agaA3* gene and used for TurboSEQUEST searching.

Sequencing of amino-terminal regions of protein. The enzyme sample was blotted on a polyvinylidene difluoride membrane (Applied Biosystems) that had been wetted with methanol. The N-terminal aa sequence of the protein was determined directly using a protein sequencer (model 476A, Applied Biosystems).

Activity staining. SDS-PAGE was performed as described above. After electrophoresis, SDS in the gels was removed by soaking the gels in 50 mM MOPS buffer (pH 7.0) three times for a total of 30 min. The gels were then overlaid onto sheets containing 1.5% (w/v) agar and 50 mM MOPS buffer (pH 7.0) and incubated for 30 min at 37°C. Following incubation, the agar sheets were stained by flooding with 2% (w/v) iodine solution. Agarase activity was visualized as a clear zone on a brown background. Proteins were visualized by staining with CBB.

Treatments of the enzyme with chemicals. The purified enzyme (0.3 μM) was preincubated with chemicals at 25°C for 30 min. *p*-Chloromercuribenzoate (Nacalai Tesque) was used at 0.5 mM in 20 mM sodium acetate buffer (pH 5.5) containing 5% (v/v) dimethylsulfoxide (Nacalai Tesque). Iodoacetoamide (Kanto Chemical) was used at 0.5 mM in 20 mM MOPS buffer (pH 7.0). 2-Mercaptoethanol (Nacalai Tesque) and dithiothreitol (Amersham Biosciences) were used at 1 or 10 mM in the same buffer. EDTA (Bio-Rad) from 5 mM to 100 mM was used in the same buffer. Diethyl pyrocarbonate was used at 1 mM in the same buffer containing 5% (v/v) dimethylsulfoxide. 1-Ethyl-3-(3-dimethyl-aminopropyl)carbonate was used at 1 mM in 20 mM Tris-HCl buffer (pH 8.0). Nonidet P-40 (nonylphenylpolyethylene glycol, Nacalai Tesque), Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether, Nacalai Tesque), and Tween 20 (polyoxyethylene sorbitan monolaurate, Nacalai Tesque) were each used at 1% in 20 mM MOPS buffer (pH 7.0). Oxidation by *N*-bromosuccinimide (NBS) (Sigma) of the enzyme was carried out at various concentrations in 50 mM sodium acetate buffer (pH 5.0) at 25°C for 10 min essentially using the method of Spande and Witkop.[26] The oxidation was quenched with 5 volumes of 15 mM tryptophan. After preincubation of the enzyme with each of these chemicals, the enzyme was diluted (usually 1 : 50 dilutions) with 50 mM MOPS buffer (pH 7.0), and then the residual activity was determined under the standard conditions of enzyme assay.

Chromatographic analysis of the products of agar and neoagaro-oligosaccharides hydrolysis. Thin-layer chromatography (TLC) was used to identify products. Enzymatic hydrolysis of agarose (Takara), NA6 and neoagarotetraose (NA4) (Dextra Laboratories) was carried out at 40°C in 50 mM MOPS buffer (pH 7.0) containing a 1.0% (w/v) substrate. The reaction mixtures were applied to silica gel 60 TLC plates (Merck) according to the method of Aoki *et al*.[27] The plates were developed using a solvent system composed of 1-butanol-acetic acid-H_2O (2 : 1 : 1, v/v). The spots, which were oligosaccharides resulting from the hydrolysis of agarose, were visualized by spraying with 10% (v/v) H_2SO_4 and heating. D-Galactose (Sigma), NA4 and NA6 were used as standards and for substrates. Quantification of reaction products was carried out by gel filtration chromatography using the HPLC system as described above.

RESULTS

Cloning of the agarase gene.

Because *Microbulbifer* sp. strain JAMB-A3 produced very low agarase activity, we could not isolate and purify the enzyme from the organism. Thus, the strategy was changed to cloning the *agaA3* gene and expressing it in *B. subtilis* cells as the host. First, the recombinant plasmid pUA3 was isolated from one of the positive clones of *E. coli*, and the insert (approximately 3.9 kb) was sequenced in both directions. The insert contained the incomplete open reading frame (ORF) lacking a downstream region, which had similarity to known agarase genes using the National Library of Medicine Retrieval System (http://www.ncbi.nlm.nih.gov) and BLAST algorithm to scan GenBank and other databases. To determine the entire sequence of the ORF, the downstream region of the insert was amplified using the inverse PCR method and sequenced directly. The ORF was composed of 1809 bp with a G+C content of 57.9 mol%, and code for a protein of 602 aa with a calculated molecular mass of 65,017 Da (Fig. 1). A potential ribosome-binding site of 5′-AAGGAG-3′ was located 6 bp upstream from the initiation codon ATG. A putative promoter sequence, 5′-TTGTTA-3′ for the −35 region and 5′-TATTAT-3′ for the −10 region, was located 136 bp upstream from the initiation codon with 19-bp spacing. An inverted-repeat sequence was found 15 bp downstream of the TAA stop codon. A possible signal sequence of 19 aa was present with a potential cleavage site between Ala19 and Ala20, suggesting that AgaA3 is localized on the outer membrane or in the periplasmic space as in the case of Gram-negative bacteria using the program PSORT (http://psort.

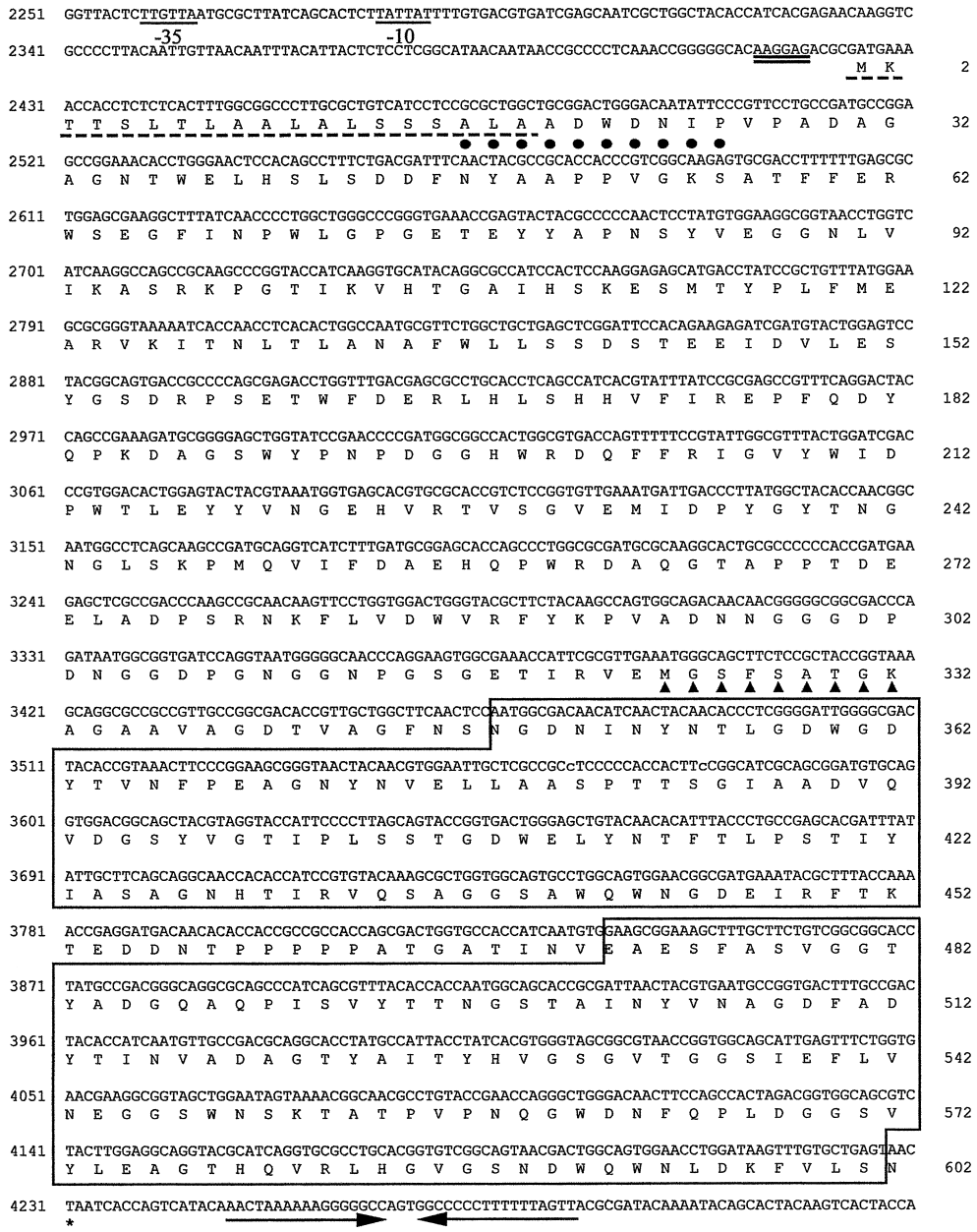

Fig. 1. Complete nucleotide and deduced aa sequences of AgaA3.

The nucleotide sequence of the *agaA3* gene and its flanking regions are shown. Sequences similar to the −35 and −10 consensus promoters of *E. coli* are underlined. A putative ribosome-binding site is double-underlined. The deduced aa sequence of the gene product is in single letter code under a nucleotide sequence. The putative signal peptide is shown by a dotted line. The aa residues from Ala17 to Pro 26 (●) refer to the N-terminal end of the recombinant enzyme secreted by *B. subtilis*. The aa residues (▲) from Met324 to Lys332 (the C-terminus) were determined using LC-MS/MS. Tandem repeats similar to carbohydrate binding-module family 6 are boxed. Inverted repeats downstream of the stop codon TAA (*) of the open reading frame are designated by convergent arrows.

nibb.ac.jp). The calculated molecular mass and isoelectric point of the mature enzyme deduced from its aa sequence were 63,185 Da (583 aa) and pH 4.22, respectively.

Comparison of the deduced aa sequence of the agaA3 gene product with those of other agarases.

Computer-assisted homology analysis using the BLAST algorithm with the full-length deduced aa sequence of AgaA3 showed homology to other known agarases, that are members of glycoside hydrolase family 16 (http://afmb.cnrs-mrs.fr/CAZY). The homologies between AgaA3 and the other agarases are as follows: 57% with β-agarase I (AAA9188) from *Pseudoalteromonas atlantica* ATCC19262; 56% with AagA (AAF03246) from *Aeromonas* sp. strain B9; 53% with AagA (BAB79291) from *Pseudomonas* sp. strain ND137; 51% each with AguB (AAP49346), AguD (AAP49316), AguH (AAP 70390), and AguK (AAP70365) from uncultured bacterium; 49 and 48% with AgaB (AAF21821) and AgaA (AAF21820), respectively, from *Zobellia galactanivorans* Dsij; 38% with AgaA (AAN39119) from *Pseudomonas* sp. strain CY24; and 34% with an DagA (CAA61795) from *Streptomyces coelicolor* A3(2). Multiple alignments showed that the homologous regions in all the agarases were located in the region that correspond to Ala20–Val293 in AgaA3.

Table 1. Summary of typical purification of RagaA3.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Culture supernatant	801	799	1.00	100	1.00
90% Ammonium sulfate	203	793	3.91	99.3	3.92
DEAE-Toyopearl 650M	10.0	592	59.0	74.0	59.2
Hydroxyapatite	0.98	519	528	65.0	529

Using the reverse position-specific BLAST algorithm to scan GenBank and other databases, AgaA3 was indicated to be a modular protein. It contains tandem domains similar to carbohydrate binding-module family 6 at Asn348–Lys452 and Glu472–Ser601 (Fig. 1).

Extracellular expression and purification of RagaA3.

High-level exoproduction of recombinant agarase, designated RagaA3, was examined using *B. subtilis* ISW1214 as the host. The supernatant from 83 mL of 72-h-cultured broth was obtained by centrifugation. The recombinant agarase RagaA3 was purified 529-fold after anion-exchange chromatography and hydroxyapatite chromatography, with a high specific activity of 528 U/mg and a final yield of 65% (Table 1). SDS-PAGE and activity staining of the purified enzyme gave a single band with an apparent molecular mass of 35 kDa (Fig. 2). This value is smaller than that deduced from the *agaA3* gene sequence (calculated molecular mass, 63,185 Da). The N-terminal aa sequence of RagaA3 was Ala-Leu-Ala-Ala-Asp-Trp-Asp-Asn-Ile-Pro from aa 17 to 26, as revealed by protein sequencing. LC-MS/MS showed that the C-terminal aa sequence was Met324 through Lys332 (the C-terminus) in RagaA3 (see Fig. 1). Thus, the aa sequence of the recombinant enzyme starts with Ala17 and ends with Lys332 (316 aa) with a calculated molecular mass of 35,167 Da, a value closely similar to that estimated by SDS-PAGE. These results also showed that the N-terminal part of AgaA3 that corresponds to Ala17–Lys332 contains the region responsible for its agarase activity.

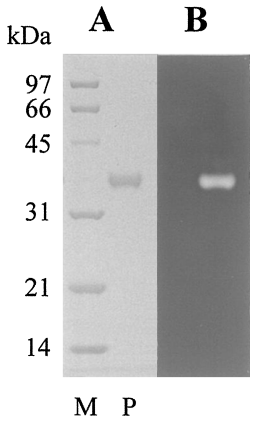

Fig. 2. SDS-PAGE and activity staining of purified RagaA3.

(A) SDS-PAGE of the purified enzyme (0.5 μg of protein) on a 12.5% (w/v) polyacrylamide gel. The proteins were stained with CBB (lane P). Protein mass markers (in kDa) are indicated on the left (lane M). (B) Activity staining of the purified enzyme. After SDS-PAGE, the slab gel was overlaid onto a sheet containing 1.5% (w/v) agar and 50 mM MOPS buffer (pH 7.0), followed by incubation for 30 min at 40°C. The activity was visualized as a clear zone by flooding the agar sheet with iodine solution.

Effects of pH on activity and stability.

The pH optimum for activity of RagaA3 was examined in various buffers. The buffers used were 50 mM Britton-Robinson universal buffers (pH 3.5–12.0), 50 mM 2-morpholinoethanesulfonic acid-NaOH buffers (pH 5.5–7.0), 50 mM MOPS buffers (pH 6.5–7.5), and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid-NaOH buffers (pH 7.0–8.2). Maximal activity was observed at around pH 7 in every buffer. To determine pH stability, RagaA3 was incubated at 40°C for 30 min at various pH values (3–12) in 50 mM Britton-Robinson universal buffers. The enzyme was the very stable at pH 5–8, retaining 90% of the original activity, and stable at pH 4.0–9.5, retaining 50% of the original activity.

Effects of temperature on activity and stability.

The optimal temperature for the activity of RagaA3 was calculated to be 54°C from Arrhenius plots (Fig. 3A), the value of which is the highest among those reported so far for agarases.[28] The activation energy was calculated from the plots to be 56.2 kJ/mol. The thermostability of RagaA3 was examined after incubation at 50 or 55°C in 50 mM MOPS buffer (pH 7.0) for various periods (Fig. 3B). RagaA3 was found to be stable up to 50°C. The first-order constant of irreversible thermoinactivation, k, at each temperature, was obtained by linear regression in semi-logarithmic coordinates. The enzyme half-life was calculated using the equation $t_{1/2}=\ln 2/k$. The half-lives of RagaA3 were 8.7 h and 2.2 h at 50 and 55°C, respectively.

Effects of cations and chemical reagents.

The enzyme was incubated with or without 10 mM EDTA at pH 7.0 in 50 mM MOPS buffer and at 0°C for 24 h and dialyzed extensively against distilled water. The specific activities in both samples were equal. The EDTA-treated enzyme was used to examine the effects of metal ions. The activities of some agarases such as β-agarase II from *P. atlantica*[29] and β-agarase PjaA from *Pseudomonas* sp. strain W7[3] are reported to be stimulated by NaCl. However, the major metal ions found in seawater, Na^+(added at 0.05–1.00 M), Mg^{2+}, K^+ and Ca^{2+} ions (each added at 5 mM and 0.1 M) did not essentially affect RagaA3 activity after incubation for 5 min at pH 7.0 in 50 mM MOPS buffer and at 50°C. Under the same conditions, Li^+, Co^{2+}, Cs^{2+}, Mn^{2+} and Fe^{3+} ions (each at 1 mM) were essentially no effect. Cu^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+} ions (each at 1 mM) inactivated the enzyme irreversibly within 5 min. The activity was not affected either by sulfhydryl inhibitors such as iodoacetoamide and *p*-chloromercuribenzoate or by thiol reagents such as dithiothreitol and 2-mercaptoethanol. RagaA3 had strong resistance to EDTA up to 100 mM. Diethyl pyrocarbonate and 1-ethyl-3-(3-

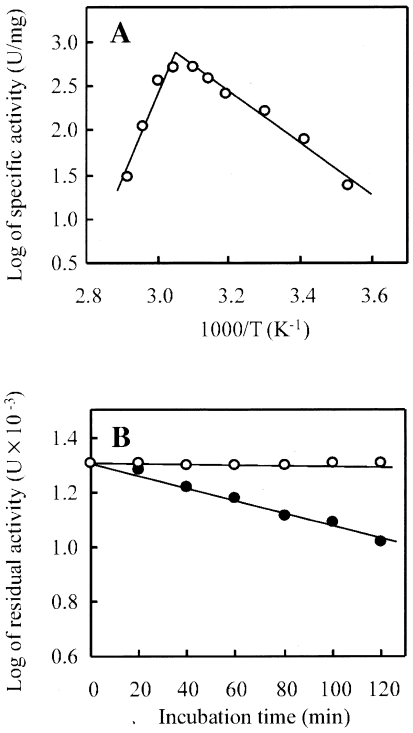

Fig. 3. Effects of temperature on the activity and stability of RagaA3.

(A) The temperature profile of RagaA3 at pH 7.0 in 50 mM MOPS buffer is shown. The logarithm of specific activities were plotted against 1000/T (K^{-1}) (Arrhenius plots). (B) To assess the thermostability of RagaA3, the enzyme (0.2 U/mL) was heated at the indicated temperatures in 50 mM MOPS buffer (pH 7.0). Aliquots (0.1 mL) were removed at different times and used for the determination of the residual activity under the standard conditions of enzyme assay. The residual activities at 50°C (○) and at 55°C (●) are shown.

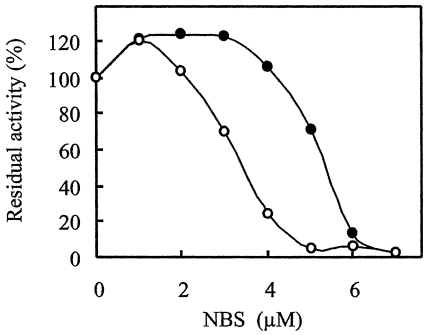

Fig. 4. Effect of NBS on RagaA3 activity.

Enzyme (0.3 μM of protein) was preincubated in the presence (●) and absence (○) of neoagaro-oligosaccharides at 40°C in 50 mM sodium acetate buffer (pH 5.0) for 20 min before addition of NBS at the indicated concentrations. After the oxidation by NBS was quenched by the addition of excess tryptophan, the residual activity was measured as described in MATERIALS AND METHODS.

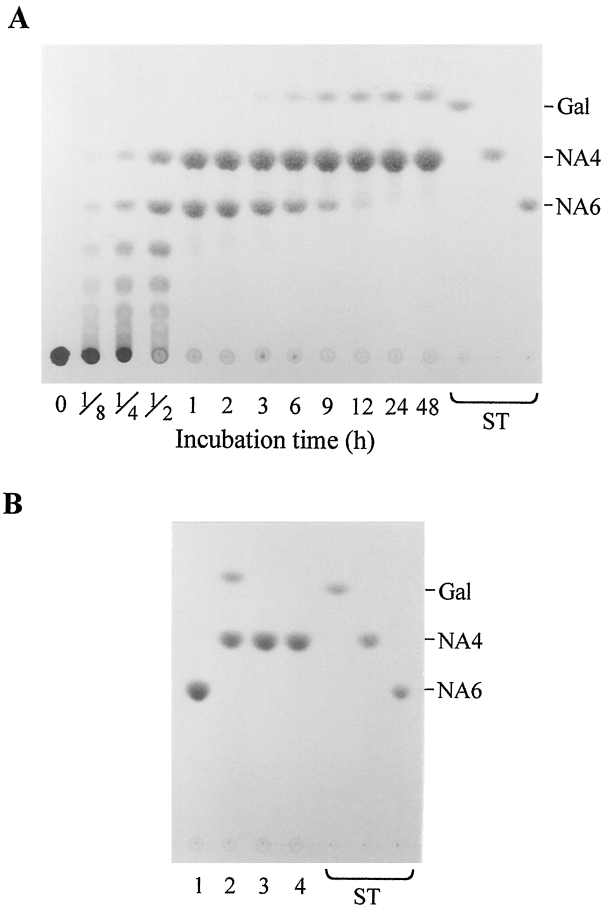

Fig. 5. TLC of the products of reaction by RagaA3.

(A) The reactions were carried out at 40°C at pH 7.0 in 50 mM MOPS buffer with 0.04 U/mL of enzyme and 1.0% (w/v) agarose. At intervals, aliquots from the reaction mixture were sampled and developed by TLC. (B) The reactions were carried out with NA6 and NA4 as substrates (each at 1.0%, w/v) and 1.0 U/mL of enzyme for 24 h. Lane 1, reaction with NA6 (0 h); lane 2, reaction with NA6 (24 h); lane 3, reaction with NA4 (0 h); lane 4, reaction with NA4 (24 h). ST shows standard sugars, including D-galactose (Gal).

dimethyl-aminopropyl)carbonate exhibited almost no inhibition. Moreover, the enzyme retained full activity even after treatment with 1.0% (w/v) each of Tween 20, Triton X-100 and Nonidet P-40.

The inhibition of some enzymes by NBS is caused by oxidation of tryptophan residues in the molecules.[26] The enzyme activity of RagaA3 was strongly inhibited by NBS at low concentrations (<10 μM). To assess whether tryptophan residues are actively involved in manifestation of the enzymatic activity, NBS oxidations were carried out in the presence or absence of neoagaro-oligosaccharides, which were prepared by digestion of 1% (w/v) agarose using RagaA3. Preincubation of RagaA3 with the substrates protected against NBS inhibition, as shown in Fig. 4. Therefore, tryptophan residue(s) may participate in the catalysis or maintain the structure of the enzyme.

Analysis of hydrolysis products and substrate specificity.

The time course of hydrolysis products from agarose was examined using RagaA3 (0.04 U/mL) at 40°C for up to 48 h, as shown in Fig. 5A. In the initial stage, RagaA3 hydrolyzed agarose to generate many large oligosaccharides. After 1-h incubation, the amount of large oligosaccharides decreased, with a concomitant increase in tetramers and hexamers corresponding to NA4 and NA6 increased. This hydrolysis pattern indicates that RagaA3 is an endo-β-agarase. After incubation for a further 24 h, the main product was NA4 with concomitant production

of minor neoagarobiose (NA2), as judged by their R_f values on TLC.[28] The quantifications of reaction products after 24-h incubation were performed using gel-filtration chromatography. The composition (mol%) of the products was 1.5% NA6, 85.0% NA4 and 13.6% NA2. Excess RagaA3 (1.0 U) could not hydrolyze NA4, whereas it acted on NA6 to yield NA4 and NA2, as shown in Fig. 5B. The results show that RagaA3 hydrolyzes agarose, neoagaro-oligosacchrides larger than NA6, and NA6 to form NA4 as the major end product. The K_m values for agar and NA6 were 2.3 and 38 mg/mL, respectively. The catalytic efficiencies (k_{cat}/K_m) for agar and NA6 were 7.1 $\times 10^2$ and 2.3 mL/mg/s, respectively. The catalytic efficiency for agar is about 300-fold greater than that for NA6. RagaA3 did not degrade ι-, κ- or λ-carrageenans, which have the same backbones as agarose with substituted sulfoxy groups (data not shown).

DISCUSSION

Agarases reported to date have not been used widely for industrial applications due to their low activity, stability, and productivity. In this study, we cloned and sequenced the gene for β-agarase from *Microbulbifer* sp. strain JAMB-A3 isolated from a sample of deep-sea sediments in Sagami Bay, Japan, at a depth of 1174 m. The recombinant enzyme RagaA3 had a molecular mass of 35 kDa and a specific activity of 528 U/mg, with optimal temperature and pH at 54°C and around 7, respectively. The molecular mass is smaller than that deduced from its gene sequence (63,185 Da). The N-terminus and the C-terminus of RagaA3 were determined to be Ala17 and Lys332 by N-terminal protein sequencing and LC-MS/MS, respectively. Thus, the aa sequence of the recombinant enzyme is from Ala17 to Lys332 (316 aa) with a calculated molecular mass of 35,167 Da. This truncation may be caused by proteolytic activities in the culture broth of the host.

RagaA3 is an endo-type β-agarase, and the final main product is NA4. Based on the aa sequence similarity, known β-agarases are classified into the three glycoside hydrolase families 16, 50 and 86. For example, a β-agarase (DagA) from *S. coelicolor* A3(2),[5] two agarases from *Vibrio* sp. strain JT0107[9,10] and β-agarase from *P. atlantica* T6c[30] are members of families 16, 50 and 86, respectively. AgaA3 belongs to glycoside hydrolase family 16, based on its deduced aa similarity to those of enzymes in the same family. AgaA3 is a modular protein and contains tandem domains similar to carbohydrate binding-module family 6 at Asn348–Lys452 and Glu472–Ser601. We have no idea at present why the tandem domains are present in AgaA3. Some of the aa in them might be involved in binding to substrates. We are now attempting to express the entire *agaA3* gene using various hosts and plasmids.

Although several agarases have been reported so far,[3,4,9–11,27–29] the properties of RagaA3 are much more favorable for use in industrial applications. For example, the high activity and thermostability at temperatures higher than the gelling temperature of agar (around 40°C) are advantages for industrial agarose oligosaccharide production from agar or marine algae. Moreover, the strong resistance to salts at high concentrations shows that crude marine algae in high-salt seawater can be hydrolyzed by RagaA3 to produce oligosaccharides. Moreover, RagaA3 is resistant to excess EDTA and can also be used for extraction of DNA fragments from agarose gels after electrophoresis because gene manipulation buffers usually contain EDTA.

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深海から分離した *Microbulbifer* 属細菌の耐熱性β-アガラーゼ

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相模湾の深度 1174m の底泥サンプルから分離した海洋微生物 JAMB-A3 株由来の新規β-アガラーゼ遺伝子を単離し，その配列を解析した．本酵素遺伝子は 602 アミノ酸（65,017 Da）のタンパクをコードしており（Fig. 1），推定アミノ酸配列は既知のアガラーゼ（glycoside hydrolase family 16）と 34–55% の相同性を示した．また C 末端領域にはタンデムに並んだ carbohydrate-binding module 様の配列を有していた．枯草菌を宿主として組換え酵素を培地中に大量分泌生産させ，比活性 528 U/mg の精製酵素を得た（Table 1）．本酵素の最適 pH は 7，最適温度は 54℃ であり，50℃ における半減期は 8.7 h であった（Fig. 3）．本酵素は高濃度の EDTA，Na^+，K^+，Mg^{2+}，Ca^{2+}や界面活性剤共存下でも安定であった．また本酵素は*N*-Bromosuccinimide 処理によって失活するが，アガロース由来のオリゴ糖を共存させると，保護効果が認められた（Fig. 4）．このことから本酵素の触媒作用に Trp 残基が関与していることが示唆された．本酵素はネオアガロテトラオースを主生成物とするβ-アガラーゼであった（Fig. 5）．