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Hyperproduction and Application of α-Agarase to Enzymatic Enhancement of Antioxidant Activity of Porphyran

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The nucleotide sequence of the gene for the α-agarase, AgaA33, from *Thalassomonas* sp. strain JAMB-A33 was determined. The open reading frame for AgaA33 was revealed to encode 1463 amino acid residues. We succeeded in extracellular production of recombinant α-agarase (AgaA33) efficiently using *Bacillus subtilis* as a host. This is the first report of recombinant production of α-agarase. Furthermore, we demonstrated that hydrolysis of α-1,3 linkages in porphyran, a sulfated polysaccharide from marine red algae, by α-agarase is an important step for improvement of its antioxidant activity with regard to free-radical-scavenging capacity and superoxide radical anion scavenging activity, whereas the hydrolysis of β-1,4 linkages in porphyran by β-agarase did not increase on the antioxidant activity markedly.

KEYWORDS: α-Agarase; porphyran; antioxidant activity; recombinant enzyme

INTRODUCTION

Reactive oxygen species (ROS) can attack important biological molecules, such as DNA, proteins, or lipids, generating mutations and damaging membranes, leading to cell and tissue injuries (*1*). Those damages have been linked to the etiology of aging and many diseases, such as arteriosclerosis, arthritis, neurodegenerative disorders, and cancer. ROS can also affect food quality with the development of a rancid flavor and undesirable chemical compounds that lead, in part, to chronic diseases. These indicate a possible important role for antioxidants in disease prevention.

Photosynthesizing plants including seaweed are considered to be rich sources of antioxidants. They are able to survive without any serious photodynamic damage, although they usually are exposed to high solar irradiation and high oxygen concentrations in their habitat. Previously characterized antioxidants include chlorophylls, carotenoids, tocopherol derivatives, certain phenolic substances, and amino acids/peptides. Recently, the antioxidant activity of sulfated polysaccharides from marine algae such as *Laminaria japonica* (*2*), *Fucus vesiculosus* (*3*), and *Ulva pertusa* (*4*) has been reported. It is thought that the main function of sulfated polysaccharides is to protect algal cells from extreme environmental conditions, which lead to frequent ROS production (*5*).

Porphyran is a sulfated polysaccharide with antioxidant activity in vivo (*6*) and in vitro (*7*). It is a major component of the cell walls and intercellular matrixes of some red algae belonging *Porphyra*. *Porphyra yezoensis* and *Porphyra tenera* are traditionally and extensively consumed as popular food in East and Southeast Asia and are becoming popular worldwide as a component of the traditional Japanese sushi, for which it is used in the form of a dried vegetable sheet (nori) wrapped around rice. Porphyran is a galactan of the agaran group on the basis of its structure. The primary structure of porphyran is essentially the same as that of agarose, composed of alternating units of 1,4-linked 3,6-anhydro-L-galactose and 1,3-linked-D-galactose residues. It differs in terms of having partial modifications on sugar residues by O-sulfo and O-methyl groups (*8*, *9*).

Porphyran is a high molecular weight indigestible polysaccharide. It is likely that its partial degradation in stomach by gastric acid to lower molecular weight compounds is an indispensable step for activation in vivo and efficient translocation of orally ingested macromolecules from the gastrointestinal tract into the systemic circulation. In the primary structure of porphyran, α-1,3 linkages are the most susceptible to hydrolysis by acid in comparison with β-1,4 linkages and the other ester linkages. Several porphyran-degrading enzymes of bacterial origins have been reported (*9*−*11*). Most of them are able to act on both agarose and porphyran because they have the same backbone structures. Most enzymes can cleave only the β-1,4 linkages and produce agaran oligomers with D-galactose residues at their reducing ends (*9*). The degraded porphyran by the currently available enzymes, that is, β-agarases, might differ from the active compounds absorbed into the circulation after the intake of high molecular weight porphyran as an ingredient in food, especially with regard to the sugar residues at the termini of the saccharide chains.

We have extensively screened for various types of carbohydrate-degrading enzymes including agarases from deep-sea microorganisms with the aim of ecological and efficient production of physiologically functional compounds by the enzymes (*11*−*14*). We have recently reported a unique α-agarase, which hydrolyzes α-1,3 linkages not only in agarose but

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also in porphyran (*11*). This enzyme produces agarose oligomers with 3,6-anhydro-L-galactose residues at their reducing ends (agaro-oligosaccharides). In this report, we describe the sequence analysis, cloning of the gene, and recombinant production of this enzyme. To the best of our knowledge, this is the first report of recombinant production of α-agarase. Furthermore, we demonstrate that hydrolysis of α-1,3 linkages is important for improvement of the antioxidant activity of porphyran.

MATERIALS AND METHODS

Chemicals. Folin-Ciocalteu reagent was purchased from Nacalai Tesque (Kyoto, Japan). 2,2′-Bipyridyl, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from EMD Biosciences (La Jolla, CA). Superoxide dismutase (SOD) E.C. 1.15.1.1 from human (recombinant) and xanthine oxidase (XOD) E.C. 1.1.3.22 from buttermilk were purchased from Oriental Yeast (Tokyo, Japan). Nitro blue tetrazolium chloride (NBT) and xanthine were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Microorganism and Culture Conditions. *Thalassomonas* sp. strain JAMB-A33 was originally isolated from the sediment in Kagoshima Bay, Japan, at a depth of 230 m (*11*). It was propagated on marine agar (Difco) or in marine broth 2216 (Difco) supplemented with 0.1% agar at 30 °C. *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 was routinely grown at 37 °C in Luria−Bertani (LB) broth (Difco) supplemented with ampicillin (100 μg/mL) or tetracycline (15 μg/mL) when required.

Sequencing of the Amino Terminal and Internal Regions of AgaA33. AgaA33 was originally isolated from a culture of *Thalassomonas* sp. strain JAMB-A33 as described previously (*11*). The enzyme sample was blotted on a polyvinylidene difluoride membrane (Prosorb; Perkin-Elmer, Foster City, CA), which had been wetted with methanol. The N-terminal sequence of the protein was determined directly using a protein sequencer (model 476A; Perkin-Elmer). AgaA33 was digested with lysyl endopeptidase (Wako Pure Chemical Industries) and was applied to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). After blotting on a polyvinylidene difluoride membrane, 21-kDa and 27-kDa fragments were sequenced to determine the internal amino acid sequences of AgaA33.

Sequencing of the *agaA33* Gene. The complete α-agarase gene and its flanking region were sequenced using the cassette-ligation-mediated PCR method with a TaKaRa *LA* PCR in vitro Cloning kit, according to the manufacturer's instructions (TaKaRa Bio, Ohtsu, Japan). First, we designed primers A and B on the basis of the internal amino acid sequences TEGNINFD and TLVYFPYP of AgaA33 isolated from the culture of *Thalassomonas* sp. strain JAMB-A33, respectively. The nucleotide sequence of primer A was 5′-TCRAARTTNATRTTNC-CYTCNGT-3′ and that of primer B was 5′-ACNYTNGTNTAYTTY-CCNTAYCC-3′. PCR was carried out using genomic DNA (0.5 μg) of *Thalassomonas* sp. strain JAMB-A33 as a template and primers A and B. A 0.36-kb DNA fragment was amplified and subcloned into the *Sma*I site of plasmid DNA pUC18 (TaKaRa Bio) and then was sequenced. On the basis of the nucleotide sequence of the amplified fragment, primers for the amplification of its flanking regions were designed. The genomic DNA was digested with *Hind*III, *Eco*RI, or *Sau3A*I and was ligated to the cassette DNA fragments in the kit. PCR was conducted to amplify the fragment using the ligation mixture of *Hind*III digestion as the template and primers C (5′-AGATGATTA-CACCTCGTATTC-3′) and C1. The PCR-amplified DNA was used as a template for the second round of PCR with primers D (5′-CTAGTACATGCAATGTACTCTTC-3′) and C2. To determine the complete sequence of the agaA33 gene, primers were synthesized on the basis of the results of the successive sequencing of the PCR-amplified DNA fragments. Nucleotide sequencing was performed on an automated DNA sequencer (Model 377; Applied Biosystems, Foster City, CA) using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Both strands of DNA were sequenced, and computer analysis was performed with the GENETYX program (SDC Software Development, Tokyo, Japan). A database homology search was performed with the BLAST program provided by NCBI (http://www.ncbi.nlm.nih.gov/).

Gene Expression and Purification of Recombinant AgaA33. First, we constructed an expression vector that performed well in *Bacillus subtilis*. A 1.0-kb DNA fragment including the part between the upstream region and coding region of the signal peptide of the cellulase from *Bacillus* sp. strain 1139 (*15*) was amplified by PCR using primers E (5′-GAGGAATTCACTCATCCGTTTATGCGTAAGTACAGCTTGC-3′)andF(5′-CCGGATCCTGCAAGAGCTGTCGGAAATAAAGATAGAAG-3′) and was inserted into plasmid pHY300PLK (TaKaRa) between the *Eco*RI and *Bam*HI sites. The expression vector constructed was designated as pEXBS. The structural gene for AgaA33 was amplified by PCR using primers G (5′-TTGGATCCGCTCGAGCCTCAGTAG-GTGAAGAAACTGGTAAC-3′) and H (5′- TTGTCGACCTAGT-GCGCTAGTTCTAAAATACCCC-3′) and was inserted into the *Bam*HI and *Sal*I sites of pEXBS. This recombinant plasmid, designated pEXA33, was used for the expression of the *agaA33* gene in *B. subtilis* ISW1214 (*leuA8 metB5 hsrM1*). Transformation of *B. subtilis* with plasmid DNA was carried out by the method of Chang and Cohen (*16*). Transformed *B. subtilis* ISW1214 cells were cultured at 30 °C, with shaking, in a medium composed of (w/v) 12% corn steep liquor (Nihon Syokuhin Kako, Shizuoka, Japan), 0.2% Lab-Lemco powder (Oxoid, Hampshire, United Kingdom), 0.1% yeast extract (Difco), 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2$, 4% maltose, and tetracycline (15 μg/mL). The supernatant obtained was used for enzyme purification. The purification was carried out following the procedure described previously (*11*). SDS-PAGE of the purified enzyme was performed essentially as described by Laemmli (*17*). Activity staining was carried out essentially as described previously (*14*). The molecular mass of the enzyme was estimated by SDS-PAGE with Precision-plus dual standards (Bio-Rad, Hercules, CA) as molecular mass standards.

Enzyme Assay. A suitably diluted solution of enzyme preparation was incubated at 45 °C in 50 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid-NaOH buffer (TAPS, pH 8.5), containing 1 mM $CaCl_2$ (standard condition) and 0.2% agar (Nacalai Tesque). The enzyme assay and quantification of reaction products followed the methods described previously (*11*). One unit (U) of enzymatic activity was defined as the amount of protein that produced 1 μmol of reducing sugar as D-galactose (Sigma Chemical, St. Louis, MO) per minute under the assay conditions.

Nucleotide Sequence Accession Numbers. The nucleotide sequence data of the gene for AgaA33 reported here has been submitted to the nucleotide sequence databases DDBJ, EMBL, and GenBank under accession number AB211981.

Preparation and Analyses of Porphyran. Porphyran was prepared from dried nori, which was obtained by processing of *P. yezoensis* cultivated in the Ariake Sea off Kyushu, Japan, in 2005. Dried nori (20 g) was extracted with 1 L of distilled water at 120 °C for 30 min. Then, the extract was filtered through Whatman no. 1 paper (Middlesex, United Kingdom) and was centrifuged at 8300 rpm for 30 min, and 2 volumes of ethanol was added to the supernatants. The resulting precipitate was collected as crude porphyran after centrifugation (3000 rpm, 10 min). The precipitate was dissolved in 300 mL of 10 mM Tris/HCl, pH 7.4, at 4 °C. Nucleic acids included in the crude porphyran preparation were degraded by 250 U/mL deoxyribonuclease (Benzonase Nuclease HC; Novagen, Madison, WI) and 30 U/mL ribonucleasease (Nuclease P1; Roche Applied Science, Penzberg, Germany) at 20 °C for 16 h. Then, the solution was applied to an activated charcoal column (32−60 mesh, 2.5 × 15 cm; Nacalai Tesque). Two volumes of ethanol was added to the flow-through fraction. The resulting precipitate was collected as purified porphyran after centrifugation (3000 rpm, 10 min) and was disolved in 100 mL of distilled water at 4 °C. The total saccharide content, anhydrosaccharide content, and sulfate content of purified porhyran were measured using the phenol-sulfuric acid method (*18*) with D-galactose as the standard, the resorcinol method (*19*) with D-fructose as the standard, and the rhodizonate method (*20*) with sulfuric acid as the standard. Reducing sugar content was determined following the 3,5-dinitrosalicylic acid method (*21*). Reducing capacity was measured using the Folin-Ciocalteu (FC) method (*22*) with gallic acid as the standard.

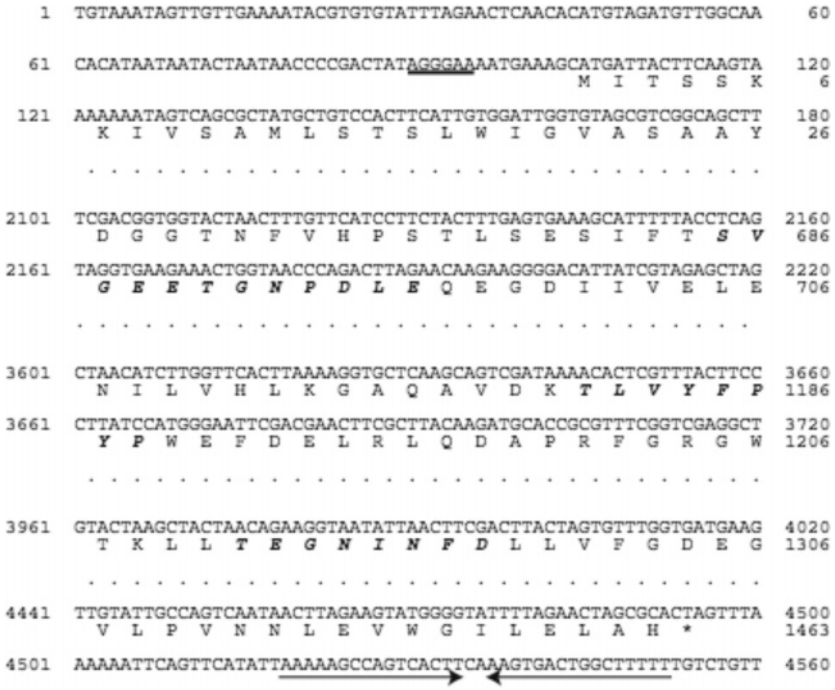

Figure 1. Partial nucleotide sequence and deduced amino acid sequence of the *agaA33* gene from *Thalassomonas* sp. strain JAMB-A33. The proposed ribosomal binding site is underlined. The stop codon is marked by an asterisk. The inverted repeat sequence downstream from the stop codon of the ORF is shown by convergent arrows. N-terminal and internal amino acid sequences of the mature AgaA33 isolated from the culture of *Thalassomonas* sp. JAMB-A33 are shown in bold-oblique type face.

α-Agarase (recombinant AgaA33) was prepared as described above. *β*-Agarase (recombinant AgaA), which produces agarose oligomers with D-galactose at their reducing ends (neoagaro-oligosaccharides), was prepared according to the method of Ohta et al. (23). The main product of agarose hydrolysis by the latter enzyme is neoagaro-oligosaccharide consisting of four sugar residues (neoagarotetraose), corresponding to 78% of total products. Each agarase (0.1 U/mL) was used for enzymatic cleavage of either α-1,3 or *β*-1,4 linkages in porphyran at a concentration of 2.0% in 50 mM Tris/HCl buffer, pH 7.4, for 3 h at 40 °C.

Free-Radical-Scavenging Capacity. The free-radical-scavenging capacity of porphyran preparations was evaluated against $ABTS^{\bullet+}$ generated according to previously reported protocols (*24*). $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate and by keeping the mixture in the dark at room temperature for 12−16 h before use. The product $ABTS^{\bullet+}$ was diluted to 100 μM in 50 mM Tris/HCl buffer, pH 7.4. Samples were suitably diluted with the same buffer prior to analysis and were mixed with the same volume of $ABTS^{\bullet+}$ solutions. The reduction of $ABTS^{\bullet+}$ (50 μM) by samples after 3-h incubation at 37 °C was measured spectrophotometrically with a microplate reader (Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan) by determining the decrease in absorbance at 734 nm. For each assay, Trolox in the same buffer was used to develop a 10−100 μM standard curve. All data were then expressed as Trolox equivalents (μmol). All determinations were carried out at least in triplicate.

Superoxide Radical Scavenging Activity. Superoxide radical scavenging activity was determined spectrophotometrically by monitoring the effect of the test samples on the reduction of NBT to blue chromogen formazan by superoxide radicals. Superoxide radicals were generated by the xanthine/XOD system as described previously (*25*). Briefly, 100 μL of aqueous SOD standard solutions (5, 10, 25, 50, and 100 U/mL) or suitably diluted sample solutions were separately added to a 100-μL mixture of 0.4 mM xanthine and 0.24 mM NBT in 50 mM Tris/HCl buffer, pH 7.4, containing 0.1 mM EDTA. Then, 100 μL of XOD (0.01 U/mL) in the same buffer was added, the resulting mixture was incubated at 37 °C for 20 min, and the absorbance was measured at 560 nm. The superoxide scavenging activity was calculated as SOD equivalents (U) from the SOD standard curve.

Statistical Analysis. Data of measurements of reducing capacity, free-radical-scavenging capacity, and superoxide radical scavenging activity of porphyran preparations were analyzed using the SIGMA STAT software for windows (version 3.5, HULINKS, Tokyo, JAPAN). Values were expressed as means ± standard deviations (SD). Analysis of variance (ANOVA) and Student−Newman−Keuls' test were used to assess significant differences ($p < 0.01$) between samples.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of the Gene for AgaA33. First, the two internal amino acid sequences of the purified AgaA33 (*11*) were determined to be TEGNINFD and TLVY-FPYP. Two primers designed from the amino acid sequences were used for PCR, and a 0.36-kb DNA fragment coding a part of AgaA33 was amplified and sequenced. On the basis of the results of the sequence analysis, the primers were designed and used to amplify the flanking regions that include the whole AgaA33 gene as described in Materials and Methods. Finally, the 4.7-kb DNA fragment containing the *agaA33* gene was sequenced. The G + C content of the gene was 43.1%. The fragment contains a single open reading frame (ORF), which begins with an ATG codon at nucleotide 105 and ends with a TAG codon at nucleotide 4496, as shown in **Figure 1**. An inverted-repeat sequence was found 23 bp downstream from the TAG stop codon. The free-energy value of this sequence for a stem-loop structure was calculated to be −126 kJ/mol, which would be sufficient for the termination of transcription. The ORF encodes 1463 amino acid residues. Upstream from this ORF, the putative ribosome-binding sequence AGGGAA was found, separated by 9 bp from the initiation codon ATG.

Amino Acid Sequence Analysis. As shown in **Figure 1**, the N-terminal amino acid sequence SVGEETGNPDLE and internal amino acid sequences TLVYFPYP and TEGNINFD of AgaA33 secreted by *Thalassomonas* sp. strain JAMB-A33 were all found in the deduced amino acid sequences 685−696, 1181−1188, and 1291−1298, respectively. The molecular mass of the mature enzyme secreted by *Thalassomonas* sp. strain JAMB-A33 was calculated on the basis of the deduced amino acid sequence

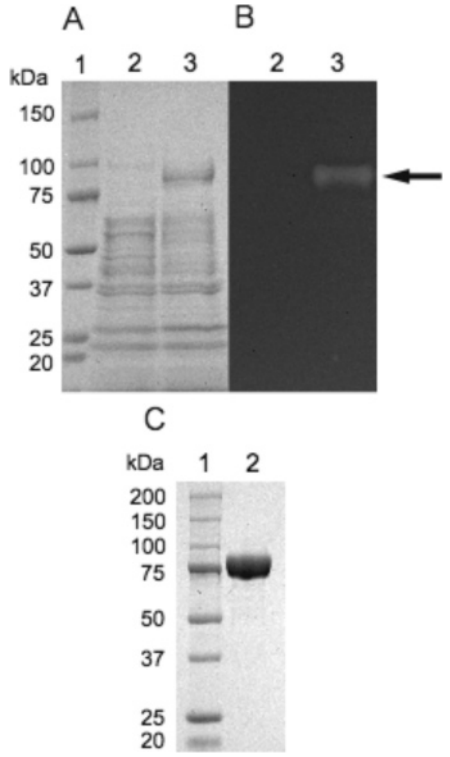

Figure 2. Recombinant AgaA33 produced by transformed *B. subtilis*. (**A**) SDS-PAGE analysis of proteins in the supernatant of culture broths of the transformants is shown. Proteins were separated on a 5–20% (w/v) polyacrylamide gel and were stained with Coomassie brilliant blue (CBB). Lane 1, protein mass markers (in kDa); lane 2, supernatant of the culture broth of the transformant with vector plasmid; lane 3, supernatant of the culture broth of the transformant with the plasmid containing the *agaA33* gene. **(B)** Activity staining of the enzyme after the SDS-PAGE. The activity was visualized as a clear zone by flooding the agar sheet with iodine solution. The arrow indicates the recombinant AgaA33. **(C)** SDS-PAGE of the purified recombinant AgaA33 on a 10% (w/v) polyacrylamide gel. Proteins were stained with CBB. Lane 1, protein mass markers (in kDa); lane 2, purified recombinant AgaA33.

(from 685 to 1463), to be 86 782 Da. Database searches using the BLAST program with the deduced amino acid sequence of AgaA33 indicated that the protein showed significant homology only to the α-agarase (accession number AAF26838) from *Alteromonas agarilytica* strain GJ1B (64% identity). That enzyme is the only reported α-agarase, except for AgaA33 (this study), to date.

Extracellular Production and Purification of Recombinant AgaA33. To obtain a sufficient amount of recombinant AgaA33, we used *B. subtilis* ISW1214 as a host and plasmid pEXBS as an expression vector. We amplified a 2.3-kb fragment of the *agaA33* gene corresponding to nucleotides 2157−4496, which encode the mature AgaA33 purified from *Thalassomonas* sp. strain JAMB-A33, using appropriate primers and cloned the fragment into pEXBS in frame. Exocellular production of the α-agarase was successful when *B. subtilis* was used as the host. SDS-PAGE and its activity staining of the supernatant of a culture broth gave a band of recombinant α-agarase with a molecular weight of 85 kDa (**Figure 2A** and **B**). This value corresponds to that of purified AgaA33 from *Thalassomonas* sp. strain JAMB-A33 (*11*). The total activity in the supernatant of the culture broth was 6950 U/L when measured under the standard assay conditions. The recombinant AgaA33 was purified (**Figure 2C**) using anion-exchange chromatography, hydroxyapatite chromatography, and gel-filtration chromatography, with specific activity of 44.7 U/mg and a final yield of 29.1%. The specific activity was almost the same as that of the native enzyme purified from *Thalassomonas* sp. strain JAMB-A33 (*11*).

To the best of our knowledge, this is the first report of recombinant production of α-agarase. When an enzyme is produced on a large scale and utilized commercially, the biological safety of the enzyme producer is strictly necessary. *B. subtilis* is one of the most thoroughly investigated bacteria and is considered to be a safe species. In addition, extracellular production of the enzyme allows easy purification procedures. We cloned the *agaA33* gene from *Thalassomonas* sp. strain JAMB-A33 and succeeded in efficient extracellular production of recombinant α-agarase (AgaA33) using the gene for AgaA33 and *B. subtilis* as a host. This achievement makes the enzyme widely available for the cleavage of α-1,3 linkages in agarose and porphyran.

Characterization of Recombinant AgaA33. The effects of the pH and temperature on the catalytic activity and stability of recombinant AgaA33 were examined. The recombinant AgaA33 showed activity in a wide pH range (4.5−9.5), with an optimum at pH 8.5. The enzyme was also stable in a broad pH range (6.5−10.5), retaining more than 75% of the original activity. The optimal temperature for the activity of the enzyme was 45 °C in 50 mM TAPS containing 1 mM $CaCl_2$. Reaction products from agarose hydrolysis after 24-h incubation were quantified by gel-filtration chromatography. The proportion of the products was calculated from area ratios on the chromatogram. The main product was the agaro-oligosaccharide consisting of four sugar residues (agarotetraose) corresponding to 79% of total products, with concomitant production of agarohexaose (7%), agarobiose (9%), and other agaro-oligosaccharides with different degrees of polymerization (5%). These enzyme characteristics of the recombinant AgaA33 were almost the same as those of the native enzyme purified from *Thalassomonas* sp. strain JAMB-A33 (*11*).

Chemical Analyses of Porphyran Preparations. The chemical composition of porphyran was analyzed and expressed as a percentage of dried solid. Total sugar, 3,6-anhydrogalactose, and sulfate contents were 79.2, 20.6, and 7.2%, respectively. The reducing sugar content was 5.0% with D-galactose as the standard, which increased to 14.3% after the α-agarase (recombinant AgaA33) treatment and 17.3% after the *β*-agarase (recombinant AgaA) treatment.

Reducing Capacity. The reducing capacity was measured using the FC method (*22*), which has gained popularity and is commonly known as the total phenols (or phenolic) assay. The basic mechanism of the FC method is oxidation/reduction mechanism, in which the oxidation of phenols by molybdotungstate heteropolyanion reagent yields a colored product. The FC method actually measures a sample's reducing capacity, but this is not reflected in the name "total phenolic assay" (*26*). The reactive compound with FC reagent in the porphyran preparation was lower than the detection limit of this method. This implied that contamination of porphyran preparation with reducing compounds including phenolic compounds from the seaweed extract was negligible. However, reducing capacities after α-agarase treatment were increased ($p < 0.01$) to 0.98 ± 0.02 mg/g in the total sample dry solid when expressed as gallic acid equivalent per gram of sample, where no significant reducing capacity was detected both in the undigested and *β*-agarase-digested porphyran.

Free-Radical-Scavenging Capacity. Free-radical-scavenging capacity of the agarase-digested and undigested porphyran was

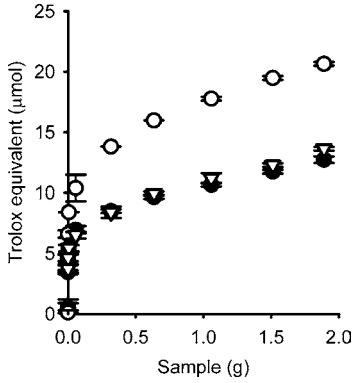

Figure 3. $ABTS^{\bullet+}$ scavenging capacity of the porphyran preparations. Activities of undigested (closed circles), α-agarase-digested (open circles), and β-agarase-digested (open triangles) porphyran were expressed as Trolox equivalents (μmol). Data points represent the mean of three replicate samples ± standard deviation.

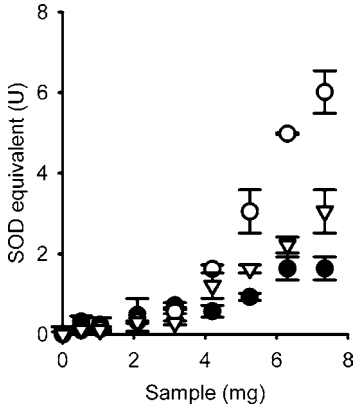

Figure 4. Superoxide radical anion scavenging activity of the porphyran preparations. Activities of undigested (closed circles), α-agarase-digested (open circles), and β-agarase-digested (open triangles) porphyran were expressed as SOD equivalents (U). Data points represent the mean of three replicate samples ± standard deviation.

assessed using ABTS radical cation ($ABTS^{\bullet+}$) as a free radical. The ABTS method is based on single-electron-transfer reactions and is widely utilized to determine the antioxidant potential of food products (*27*). Free-radical-scavenging capacities expressed as Trolox equivalents (μmol) are shown in **Figure 3**. All porphyran preparations showed considerable radical-scavenging activity. Among the three preparations, α-agarase-digested porphyran showed the greatest scavenging capacity at the concentrations higher than 0.01 g/L ($p < 0.01$) in accordance with the reducing capacity determined by FC method. On the other hand, β-agarase-digested and undigested porphyran had almost the same capacity, although those two preparations have different molecular weights and amounts of reducing sugars. β-Agarase-digested porphyran has D-galactose residues at the reducing ends of the saccharide chains. In contrast, α-agarase digested-porphyran has 3,6-anhydro-L-galactose residues at the corresponding positions. D-Galactose residues exist mostly in the pyranose structure, unlike 3,6-anhydro-L-galactoses residues that exist as open-chain forms in equilibrium between aldehydic and hydrated forms in aqueous solutions (*28*). The sugars in the pyranose structure are more inert than those in the open-chain structure. The greater free-radical-scavenging capacity of α-agarase-digested porphyran is probably due to the contribution of 3,6-anhydro-L-galactose residues at the reducing ends.

Superoxide Radical Anion Scavenging Activity. Superoxide radical anion scavenging activity was examined in the xanthin/XOD system. Scavenging activities were expressed as equivalents of SOD activity. Superoxide radical anion is a reactive oxygen frequently generated in living cells. All of the porphyran preparations showed considerable activity against superoxide radicals. α-Agarase-digested porphyran showed greatest activity among all the samples (**Figure 4**), particularly at the high concentrations (>6.3 mg/mL, $p < 0.01$). The relationship between superoxide radical anion scavenging activity and the molecular weight of sulfated polysaccharides from a Chlorophyta (*Ulva pertusa Kjellm*) was investigated using saccharides of different molecular weights prepared by hydroxy peroxide degradation (*4*). They suggested that molecular weight has a significant effect on the antioxidant activity of the saccharide with low molecular weight saccharides having stronger antioxidant activity. In addition, the results presented herein suggest that the antioxidant activities of porphyran against $ABTS^{\bullet+}$ as a free radical and superoxide radical anion are related to the structure of the reducing ends.

In conclusion, our results showed that hydrolysis of α-1,3 linkages by α-agarase is an important step for improvement of the antioxidant activity of porphyran with regard to free-radical-scavenging capacity and superoxide radical anion scavenging activity. Moreover, enzymatic digestion also lowers the viscosity and improves solubility by breaking polysaccharide chains. This is an advantage for handling during large-scale processing in the food industry. On the contrary, the hydrolysis of β-1,4 linkages by β-agarase did not have a markedly favorable effect on their antioxidant activity, in spite of their lower molecular weight compared with undigested porphyran. In the processes of chemical or physical degradation of polysaccharides, harsh conditions—such as acidic solvent at high temperature, microwave radiation, or sonication—are often used. They lead to the deterioration of other chemically labile bioactive components such as vitamins, proteins, and many other compounds in red algae. Enzymatic degradation of polysaccharides can be carried out under mild conditions that avoid the deterioration of other compounds. The hyperproduction of α-agarase as an enzyme for the processing of porphyran presented in this report together with the antioxidant activity of the products will provide a rational step for developing physiologically functional materials for human health.

ABBREVIATIONS USED

ROS, reactive oxygen species; ABTS, 2,2′-bipyridyl, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; SOD, superoxide dismutase; XOD, xanthine oxidase; NBT, nitro blue tetrazolium chloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; NCBI, National Center for Biotechnology Information; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid-NaOH; FC, Folin-Ciocalteu; EDTA, ethylenediaminetetraacetic acid; ORF, open reading frame; bp, basepair.

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