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A Novel Agarolytic β-Galactosidase Acts on Agarooligosaccharides for Complete Hydrolysis of Agarose into Monomers

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Marine red macroalgae have emerged to be renewable biomass for the production of chemicals and biofuels, because carbohydrates that form the major component of red macroalgae can be hydrolyzed into fermentable sugars. The main carbohydrate in red algae is agarose, and it is composed of D-galactose and 3,6-anhydro-L-galactose (AHG), which are alternately bonded by β1-4 and α1-3 linkages. In this study, a novel β-galactosidase that can act on agarooligosaccharides (AOSs) to release galactose was discovered in a marine bacterium (*Vibrio* sp. strain EJY3); the enzyme is annotated as *Vibrio* sp. EJY3 agarolytic β-galactosidase (*Vej*ABG). Unlike the *lacZ*-encoded β-galactosidase from *Escherichia coli*, *Vej*ABG does not hydrolyze common substrates like lactose and can act only on the galactose moiety at the nonreducing end of AOS. The optimum pH and temperature of *Vej*ABG on an agarotriose substrate were 7 and 35°C, respectively. Its catalytic efficiency with agarotriose was also similar to that with agaropentaose or agaroheptaose. Since agarotriose lingers as the unreacted residual oligomer in the currently available saccharification system using β-agarases and acid prehydrolysis, the agarotriose-hydrolyzing capability of this novel β-galactosidase offers an enormous advantage in the saccharification of agarose or agar in red macroalgae for its use as a biomass feedstock for fermentable sugar production.

Red algae (*Rhodophyta*) could be considered a potential source of renewable biomass for the production of fuels and chemicals because the monomeric sugars obtained by the hydrolysis of algal carbohydrates can be fermented by microorganisms (1, 2). The main constituent of red algae is agarose, which is also a cell wall component (3). Agarose consists of D-galactose and 3,6-anhydro-L-galactose (AHG), which are alternately bonded by β1-4 and α1-3 linkages (3). Agarose-derived oligosaccharides are classified into agarooligosaccharides (AOSs) and neoagarooligosaccharides (NAOSs) (Fig. 1). The nonreducing end of AOSs is the galactose unit, whereas that of NAOSs is the AHG unit (3, 4).

Three methods have primarily been used for agarose hydrolysis: acid hydrolysis, enzymatic hydrolysis, and enzymatic saccharification combined with acid prehydrolysis. Acid hydrolysis using a strong acid at a high temperature is a simple, less expensive, and rapid method (5, 6); however, this method produces sugar degradation products such as 5-hydroxymethylfurfural (6–9), which can inhibit fermentative microorganisms (10, 11).

This problem is circumvented by the enzymatic hydrolysis method because it is performed at a milder temperature. The β-agarase system that is commonly used by marine agarolytic bacteria has been well established for agarose hydrolysis (7, 8, 12–14). This system is capable of producing monomeric sugars from agarose and consists of β-agarases I and II (EC 3.2.1.81) and a neoagarooligosaccharide hydrolase (NAOS hydrolase) or a neoagarobiose hydrolase (NABH; EC 3.2.1.159), which was discovered in several agarolytic bacteria, including *Saccharophagus degradans* 2-40^T and *Zobellia galactanivorans* (12, 14). β-Agarases I and II cleave the β1-4 linkages of agarose to release NAOS and neoagarobiose, respectively, whereas NAOS hydrolase degrades neoagarobiose into AHG and galactose (15–17).

Agarose hydrolysis using enzymes alone results in a low yield of monomeric sugars owing to the low solubility of agarose in water as the substrate for the enzyme reaction (18). Therefore, enzymatic hydrolysis combined with weak acid prehydrolysis was recently developed for agarose saccharification (18–20), in which agarose is predominantly prehydrolyzed into even-numbered AOSs with galactose at the nonreducing ends since α1-3 linkages are preferentially cleaved by a weak acid. The AOSs are then hydrolyzed into monomeric sugars by a β-agarase II and an NABH. A major disadvantage of this method is that the smallest odd-numbered AOS, agarotriose [galactose-β1-4-AHG-α1-3-galactose] (Fig. 1), produced by a β-agarase II, cannot be further hydrolyzed into monomers by using typical β-agarases and a NAOS hydrolase (or an NABH) (20). For the cost-effective use of agarose as a biomass feedstock, a high yield of saccharification that does not leave unreacted oligomeric sugars is highly desirable. In our previous study (20), when an NABH was combined with the crude enzyme of *Vibrio* sp. strain EJY3, which was previously isolated from the coast of the West Sea of Korea (21), the crude enzyme was found to hydrolyze agarotriose into AHG and galactose, thus increasing the final yield of monomeric sugars compared to that achieved in experiments that did not include the crude enzyme. Therefore, we hypothesized that such an enzyme capable of cleaving the β linkage of agarotriose may be expressed from the genes that encode three putative β-galactosidases (EC 3.2.1.22) in *Vibrio* sp. EJY3, the nucleotide sequences of which were obtained from UniProt (22). Such an enzyme that can act on agarose-derived galactan to produce

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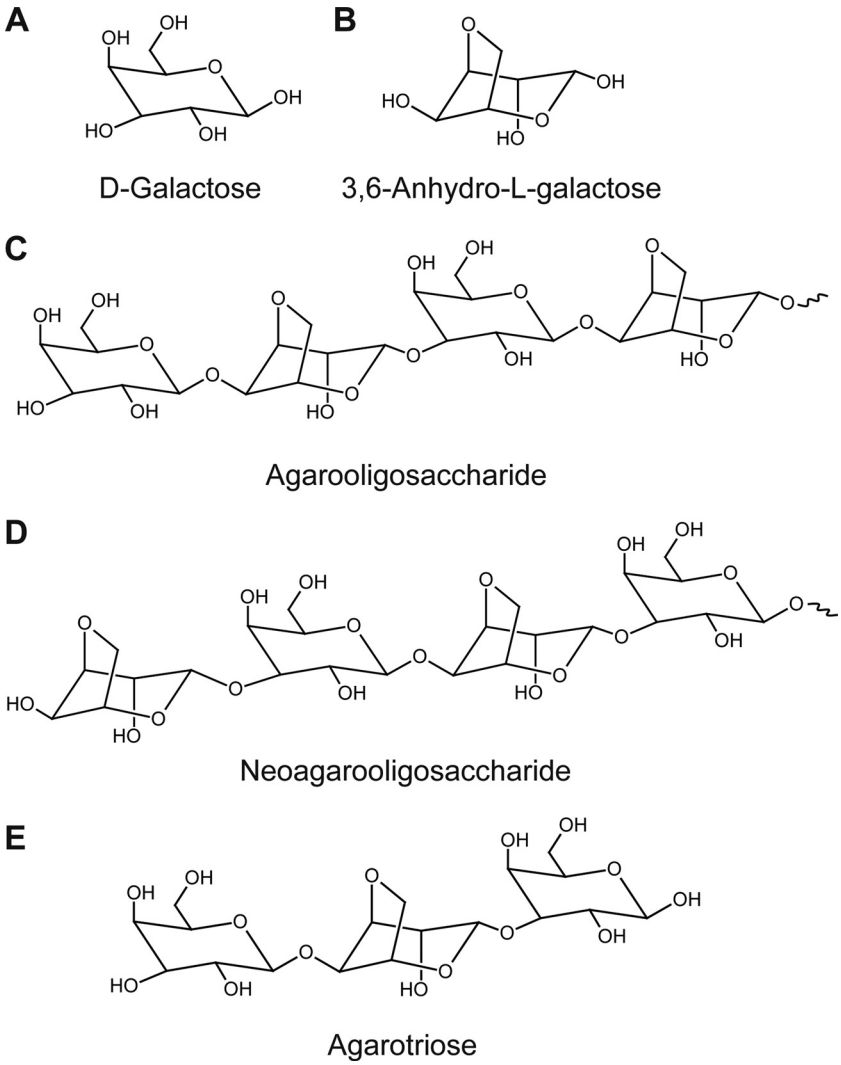

FIG 1 Chemical structures of agarose-derived oligosaccharides. (A) D-Galactose (Gal); (B) 3,6-anhydro-L-galactose (AHG); (C) agarooligosaccharide (AOS); (D) neoagarooligosaccharide (NAOS); (E) agarotriose. The nonreducing end of agarooligosaccharide is the galactose unit, whereas that of neoagarooligosaccharide is the AHG unit. Agarotriose is the smallest odd-numbered agarooligosaccharide.

galactose is considered a novel β-galactosidase capable of hydrolyzing agarose in agarooligosaccharides.

In this study, we describe the identification, expression, and biochemical characterization of this novel β-galactosidase isolated from *Vibrio* sp. EJY3 and its role in the agar degradation process. This novel agarolytic enzyme is extremely important, not only due to its novelty but also by virtue of its industrial application. This enzyme can supplement the missing function of the currently available β-agarase system in the saccharification of agarose by completely hydrolyzing the entrapped, odd-numbered short-chain AOSs.

MATERIALS AND METHODS

Cloning and expression of putative genes of agarolytic β-galactosidase. *Vibrio* sp. EJY3, which was previously isolated from the coast of the West Sea of Korea (21), was grown in a sea salt minimal medium containing 23 g of synthetic sea salt (Aquarium Systems), 50 mmol Tris-HCl, 2 g agarose (Invitrogen), 1 g yeast extract, and 0.5 g ammonium chloride in 1 liter of water at 30°C for 4 h (20, 21). The genomic DNA was extracted using a commercial DNA isolation kit (Qiagen). Eight putative genes annotated as encoding an α- or β-galactosidase (VEJY3_05670 [UniProt accession no. H2IA24], VEJY3_09135 [UniProt accession no. H2IFD0], VEJY3_09170 [UniProt accession no. H2IFD7], VEJY3_09535 [UniProt accession no. H2IG01], VEJY3_21461 [UniProt accession no. H2IN31], VEJY3_22581 [UniProt accession no. H2ILJ6], VEJY3_22596 [UniProt accession no. H2ILJ9], and VEJY3_24051 [UniProt accession no. H2INB0]) were amplified using Phusion thermostable polymerase (Thermo Scientific). The primers used for the amplification of these genes are described in Table 1. The restriction sites of BamHI and NotI were added at the 5′ and 3′ regions of the N- and C-terminal ends, respectively. The PCR product and pET21a (Novagen) were digested by BamHI and NotI and ligated together using T4 DNA ligase (BioLabs). The resulting pET21a vectors harboring each putative gene were transformed into *Escherichia coli* BL21(DE3) (Novagen).

To produce recombinant proteins from each gene, cells were grown at 37°C in Luria-Bertani broth (BD) containing 50 μg/ml of ampicillin until the mid-exponential phase and then induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) at 16°C for 16 h. These cells were harvested by centrifugation at 5,000 × *g* for 15 min at 4°C

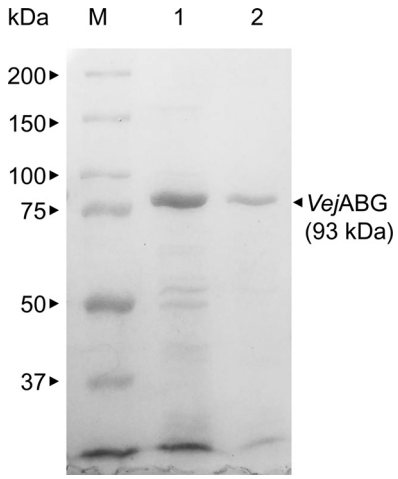

FIG 2 SDS-PAGE analysis of the purified recombinant *Vej*ABG. Lanes: M, protein markers; 1, *Vej*ABG purified by His-tag affinity chromatography; 2, *Vej*ABG purified by His-tag and anion-exchange chromatography.

and resuspended in ice-cold lysis buffer, composed of 20 mM sodium phosphate and 500 mM NaCl (pH 7.4). Each cell suspension was disrupted by a sonicator (Branson), and the supernatant was collected by centrifugation at 16,000 × *g* for 1 h at 4°C. The recombinant proteins were purified by two consecutive chromatographic separations using a His-trap column (GE Healthcare) and an anion-exchange column (GE Healthcare). The purified proteins were concentrated using an Amicon ultrafiltration membrane (Millipore), and the protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce).

Determination of enzymatic activity of agarolytic β-galactosidase. To determine the enzyme activity of the agarolytic β-galactosidase from *Vibrio* sp. EJY3 (*Vej*ABG) on agarotriose, 9.14 pmol of protein was incubated in 100 μl of 20 mM Tris-HCl buffer (pH 7) containing 2.5 mM agarotriose (Beijing Ad Hoc International Technologies) at 30°C for 25 min. After the reaction was stopped by boiling the reaction mixture, galactose was quantified by high-performance liquid chromatography (HPLC) as described later in the section "Analyses of the enzymatic reaction products by TLC and HPLC." A commercial β-galactosidase from *E. coli* (i.e., *lacZ*-encoded β-galactosidase; catalog no. G4155; Sigma) (23) was also tested using agarotriose as the substrate. One unit of agarolytic β-galactosidase on agarotriose was defined as the amount of enzyme required to produce 1 μmol of galactose per min using the conditions described above.

Screening for substrate specificity of agarolytic β-galactosidase. To examine the substrate specificity of *Vej*ABG, a total of 7 substrates were used. The first substrate group was composed of 3 AOSs, agarotriose, agaropentaose, and agaroheptaose (Beijing Ad Hoc International Technologies), whereas the second group consisted of common substrates of general β-galactosidases, lactose, lactulose (Sigma), lacto-*N*-neotetraose (LNT; Dextra Laboratories), and galactobiose (galactose-β1-4-galactose; Dextra Laboratories). To determine the enzymatic activity on the even-numbered AOSs, acetic acid-prehydrolyzed agarose was used as the substrate, which was prepared by dissolving agarose (Invitrogen) in a 3 N acetic acid solution to a final concentration of 7% (wt/vol), using a microwave digester (Milestone) at 130°C for 10 min. The acid-prehydrolyzed agarose was then lyophilized using a freeze dryer for 24 h. To screen for the substrate specificity of *Vej*ABG, 9.14 pmol of the purified recombinant protein was incubated in 100 μl of 20 mM Tris-HCl buffer (pH 7) containing 2.5 mM each substrate.

Characterization of agarolytic β-galactosidase. To study the temperature optimum of *Vej*ABG, this enzyme was incubated with 2.5 mM aga-

TABLE 1 Primers used for cloning the putative genes of α- and β-galactosidases from *Vibrio* sp. EJY3

Putative function	Gene name	Sequence (5′→3′)	
		Forward primer	Reverse primer
β-Galactosidase	VEJY3_09170	GCGGGATCCATGCACAATTCACCGAGAAGTAC	GCGGCGGCCGCAAGTAGTGAAAAAGAGGCTTTCAC
	VEJY3_22596	GCGGGATCCATGGCATTTTCAGATATTATTCAACG	GCGGCGGCCGCAGCGGGCTGAAACGATACTC
	VEJY3_24051	GCGGGATCCATGGCATCACAGATAGAAAAGAC	GCGGCGGCCGCGAAATGAAATTCGCCACAATCTG
α-Galactosidase	VEJY3_22581	GCGGTCGACATGACGGACAAAACGGCTGATTG	GCGGCGGCCGCTTCGTTTACGGGTTCAAACTTCAC
	VEJY3_09535	GCGGGATCCATGAATAATTTTGTACATCTGCAAAGT	GCGGCGGCCGCTATTTTTTGTAAATGAACAATCAGTGC
	VEJY3_21461	GCGGTCGACATGAAAGAAAAAAAGTTAGTTGAGTTAAG	GCGGCGGCCGCATTTATCTCTCGACTTTAATTAACATC
	VEJY3_09135	GCGGGATCCATGCAAAGAATCGTTCACCTAAAATC	GCGGCGGCCGCAAGTTTTTTCAGGTGAACAATAAGCG
	VEJY3_05670	GCGGGATCCATGACTCAAACCTTTCAGTTCGC	GCGGCGGCCGCTACCGTGACGATGGCTTTTGC

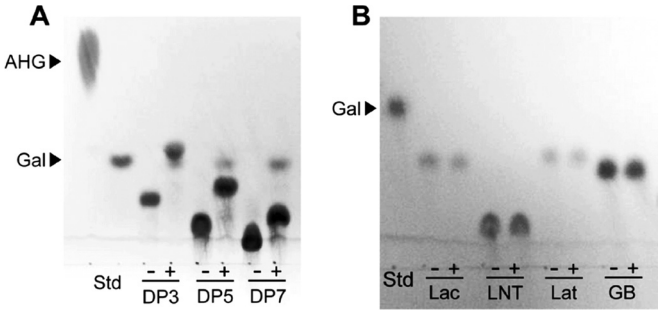

FIG 3 Substrate specificity of *Vej*ABG. TLC analysis of the reaction products of recombinant *Vej*ABG incubated with agarooligosaccharides with different DPs (A) and the general substrates of β-galactosidase (B). −, substrate only; +, substrate incubated with *Vej*ABG for 12 h. DP3, agarotriose; DP5, agaropentaose; DP7, agaroheptaose; Lac, lactose; LNT, lacto-*N*-neotetraose; Lat, lactulose; GB, galactobiose. Galactose (Gal) and AHG were used as markers for the reaction products. Std, standard.

rotriose at 10 to 50°C in 20 mM Tris-HCl (pH 7) for 25 min. The optimum pH of *Vej*ABG was also examined by incubation with 2.5 mM agarotriose contained in buffers of different pHs, such as 50 mM citric acid (pH 5 to 6), 20 mM Tris-HCl (pH 7 to 8), and glycine-NaOH (pH 9 to 10), at 35°C for 25 min. The kinetic parameters (V_{max} and K_m) of the enzyme for the different odd-numbered AOSs were determined from the Lineweaver-Burk plot, in which the enzyme activity was measured using agarotriose, agaropentaose, and agaroheptaose at concentrations ranging from 0.625 mM to 40 mM.

To verify whether it is possible to completely saccharify AOSs into monomeric sugars using *Vej*ABG and the NAOS hydrolase from *Saccharophagus degradans* 2-40^T (*Sd*NABH), 91.4 nmol of each enzyme was incubated in 100 μl of 20 mM Tris-HCl buffer (pH 7) containing 2.5 mM agarotriose, agaropentaose, or agaroheptaose at 35°C for 24 h.

Analyses of the enzymatic reaction products by TLC and HPLC. To analyze the reaction products of *Vej*ABG by thin-layer chromatography (TLC), a 1-μl aliquot from each reaction mixture was loaded onto silica gel 60 TLC plates (Merck), and the plates were developed with *n*-butanol–ethanol–water (3:1:1, by volume). The plates loaded with sample were visualized with 10% (vol/vol) H_2SO_4 and 0.2% (wt/vol) naphthoresorcinol in ethanol (19).

The final products from the combined treatment of *Vej*ABG and *Sd*NABH were analyzed using an Agilent 1100 HPLC system (Agilent Technologies) equipped with a gel permeation column (KS-802; Shodex) and a refractive index detector. Analysis was conducted at 80°C using distilled water as the mobile phase at a flow rate of 0.5 ml/min. Molecular size markers (Waters) ranging from 106 Da to 4,270 Da were used to distinguish the degrees of polymerization (DPs) of products in the range of 1 to 7.

Analyses of the enzymatic reaction products by GC-MS and MALDI-TOF/TOF MS. To analyze monomeric sugars obtained from the enzymatic reaction on acid-prehydrolyzed agarose, gas chromatography-mass spectrometry (GC-MS) was performed according to the previously described methods (24). Briefly, the aldehyde group of the sample was derivatized by methoxyamination using 50 μl of 20 mg/ml methoxyamine hydrochloride in pyridine (Sigma) at 75°C for 30 min. The volatility of the sample was increased by adding 80 μl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Fluka). An Agilent 7890A GC/5975C MSD system (Agilent Technologies) equipped with a DB5-MS column (30 m by 0.25 mm [inner diameter], 0.25-μm film thickness; Agilent Technologies) was used. For the GC-MS analysis, the column temperature of the GC was initially set at 100°C for 3.5 min, increased to 160°C at 15°C/min, and maintained at 160°C for 20 min. The temperature was then increased to 200°C at 20°C/min, this temperature was maintained for 15 min, and finally, it was increased to 280°C at 20°C/min and held at 280°C for 5 min. The mass spectra were recorded at 70 eV of electron impact, with the temperature of the ion source of the MS being 230°C. Mass spectra were recorded in the range of 50 to 700 *m/z*.

The matrix-assisted laser desorption ionization–tandem time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis was performed in the positive ion reflectron mode using an ultrafleXtreme system (Bruker Daltonics). Purified reaction products were dissolved in water, and 1 μl of the solubilized reaction products was spotted onto a stainless steel target plate, followed by the addition of 0.3 μl of 0.01 M NaCl and 0.5 μl of 50 mg/ml 2,5-dihydroxybenzoic acid in 50% acetonitrile. The spot was rapidly dried under vacuum for homogeneous crystallization. Each acquired spectrum represented the combined signal from 800 laser shots at each of 3 random locations on the spot, totaling 2,400 laser shots, using a 1-kHz laser. The laser attenuator offset and range were set at 68% and 15%, respectively, and the laser focus was set at 50%. Mass spectra were recorded over the range of 0 to 5,100 *m/z*. To obtain high-resolution data, the detector sampling rate was set at a maximum rate of 4.00 gigasamples/s, and the detector gain was set at 4.0×. Mass spectra were externally calibrated using maltooligosaccharides isolated from commercial beer. A ladder of hexose polymers, spaced 1 hexose unit (162.053 Da) apart, provided comprehensive coverage of the entire mass acquisition range, enabling the accurate mass calibration of the MALDI-TOF/TOF MS just prior to the sample analysis. Raw MS data were processed with FlexAnalysis software (version 3.3; Bruker Daltonics). MS peaks were filtered with a signal-to-noise ratio of 3.0 and manually inspected to detect the formation of sodium, potassium, or other common adducts. All peaks were then deconvoluted, and a list of all neutral masses in the samples was generated, with the abundances represented by mass spectral peak intensities.

Phylogenetic analysis. To determine the amino acid sequence homology between *Vej*ABG and other β-galactosidases from glycoside hydrolase family 2 (GH2), a phylogenetic tree was drawn using the MEGA (version

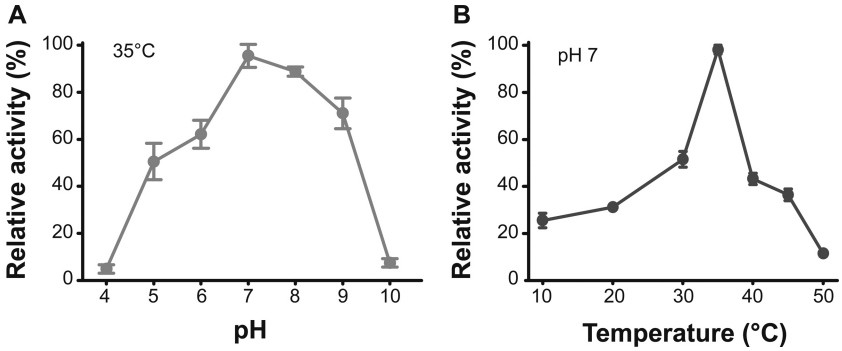

FIG 4 Effect of pH and temperature on the activity of *Vej*ABG. The optimum pH (A) and temperature (B) of *Vej*ABG in the hydrolysis of agarotriose were determined. The enzymatic reactions were performed using buffers of different pHs (pH 4 to 10) and different temperature (10 to 50°C) conditions for 25 min.

TABLE 2 Kinetic parameters of *Vej*ABG in the hydrolysis of different types of agarooligosaccharides[b]

Substrate	K_m (mM)	V_{max} (U[a]/mg)	k_{cat} (s^{-1})
Agarotriose (DP3)	3.25 ± 0.38	9.12 ± 0.54	14.14 ± 0.83
Agaropentaose (DP5)	1.73 ± 0.13	4.63 ± 0.06	7.17 ± 0.10
Agaroheptaose (DP7)	1.76 ± 0.24	3.92 ± 0.16	6.07 ± 0.25

[a] One unit of *Vej*ABG was defined as the amount of enzyme that releases 1 μmol/min of galactose.
[b] Experiments were performed in triplicate, and data are shown as means ± standard deviations.

5) program with the neighbor-joining method (25), and the GH2 β-galactosidases were selected from the CAZy database (26).

RESULTS

Identification of a β-galactosidase that acts on agarotriose. We previously showed that agarotriose, consisting of two units of galactose and one unit of AHG in alternating β1-4 and α1-3 glycosidic linkages, can be depolymerized using a NAOS hydrolase and the crude enzyme of *Vibrio* sp. EJY3 (20). To search for the enzyme responsible for this β-galactosidase activity against agarotriose, eight putative genes encoding α- or β-galactosidases in *Vibrio* sp. EJY3 were cloned, expressed, and tested for the activities of their recombinant enzymes. Among these, the crude enzyme obtained from *E. coli* BL21(DE3) harboring a plasmid containing the VEJY3_09170 gene was found to have the ability to hydrolyze agarotriose into galactose and neoagarobiose (data not shown). Therefore, the purified enzyme with a theoretical mass of 93 kDa (Fig. 2), encoded by VEJY_09170, was named agarolytic β-galactosidase (*Vej*ABG) and was then examined as the potential β-galactosidase acting an agarotriose.

Substrate specificity of agarolytic β-galactosidase. The specificity of *Vej*ABG was tested on 7 different substrates. *Vej*ABG was found to release galactose from odd-numbered AOSs, such as agarotriose, agaropentaose, and agaroheptaose (Fig. 3A). However, lactose, lacto-*N*-neotetraose, lactulose, and galactobiose, which are the common substrates hydrolyzable by the general β-galactosidases, such as the *lacZ*-encoded β-galactosidase of *E. coli*, were not hydrolyzed by *Vej*ABG (Fig. 3B). Although the *lacZ*-encoded β-galactosidase did not hydrolyze the AOSs with DP3, DP5, and DP7, which indicate agarotriose, agaropentaose, and agaroheptaose, respectively, it was capable of hydrolyzing all the general substrates (data not shown).

Optimum pH and temperature of *Vej*ABG for agarotriose hydrolysis. To determine the optimum pH and temperature of *Vej*ABG for the hydrolysis of agarotriose, 2.5 mM agarotriose was incubated with *Vej*ABG at different temperatures (10 to 50°C) and pHs (pH 4 to 10). The enzyme showed the highest activity at pH 7 and maintained approximately 70% of its maximum activity over a broad pH range from 6 to 9 (Fig. 4A). The optimum temperature for *Vej*ABG was determined to be approximately 35°C in 20 mM Tris-HCl buffer (pH 7), and its

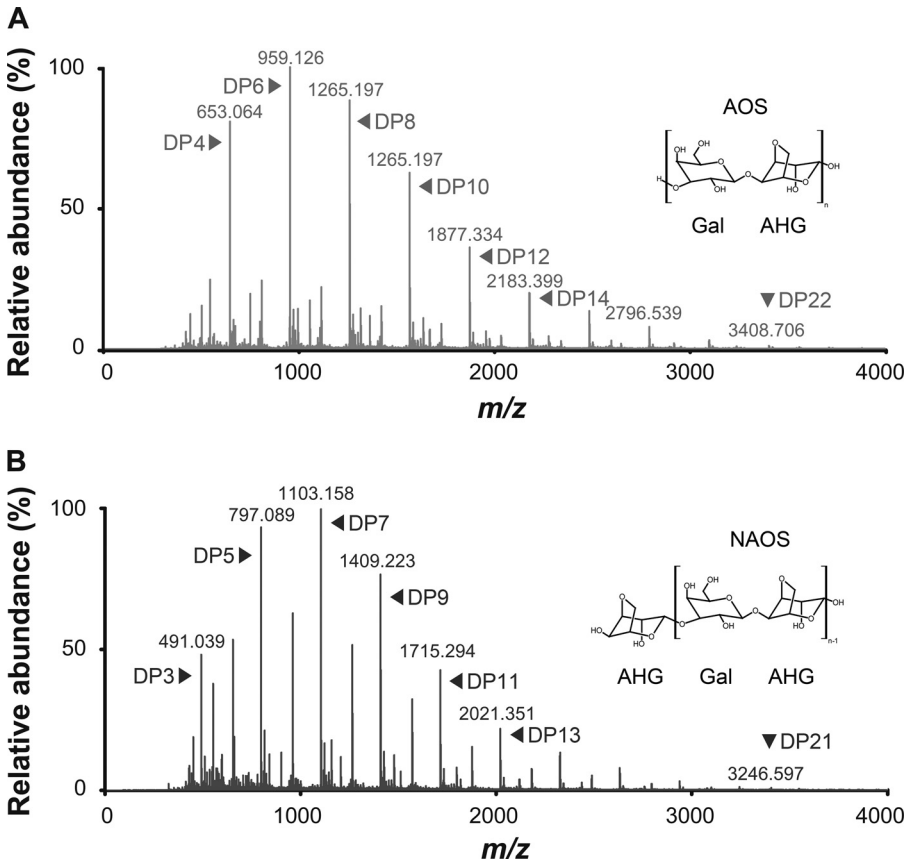

FIG 5 MALDI-TOF/TOF MS of AOSs before and after reaction with *Vej*ABG. (A) AOSs were prepared by acid prehydrolysis of agarose; (B) AOSs were further hydrolyzed predominantly to NAOSs by *Vej*ABG.

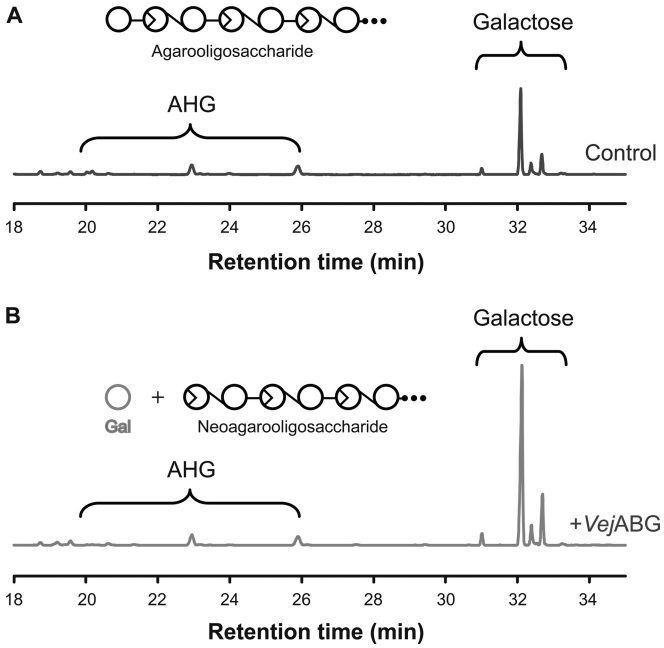

FIG 6 GC-MS of agarooligosaccharides before and after reaction with *Vej*ABG. Agarooligosaccharides were prepared by acid prehydrolysis of agarose (A) and were further hydrolyzed using *Vej*ABG (B).

activity decreased significantly at temperatures higher or lower than 35°C (Fig. 4B).

Kinetic parameters of *Vej*ABG. The kinetic parameters of *Vej*ABG were determined from the Lineweaver-Burk plot by using agarotriose, agaropentaose, and agaroheptaose as the substrates at pH 7 and 35°C. To determine the kinetic parameters of *Vej*ABG for these AOSs, the amount of galactose produced was measured by HPLC. The K_m for agarotriose was approximately 2 times higher than the K_ms for agaropentaose and agaroheptaose. However, the V_{max} value for agarotriose was 1.9 and 2.3 times higher than the V_{max} values for agaropentaose and agaroheptaose, respectively. As a result, the k_{cat}/K_m values were similar for all three substrates (Table 2).

Mode of action of *Vej*ABG toward AOSs and NAOSs. MALDI-TOF/TOF MS analysis showed that even-numbered AOSs were predominantly produced by the acid prehydrolysis of agarose due to the preferential cleavage action on α1-3 linkages, which did not affect the β1-4 linkages in agarose (6) (Fig. 5A). The sum of the abundances of agarotetraose (DP4), agarohexaose (DP6), and agarooctaose (DP8) was more than 70% of the abundance of all the AOSs from the acid prehydrolysate of agarose (Fig. 5A). Incubation of the AOSs with *Vej*ABG clearly showed that *Vej*ABG primarily hydrolyzed them into galactose and NAOSs (Fig. 5B). The AOSs with a wide DP range containing galactose at their nonreducing ends were hydrolyzed by *Vej*ABG, thus producing the corresponding NAOSs with one less galactose unit and the addition of one water molecule (Fig. 5B). The GC-MS analysis over the range of 50 to 700 *m/z* showed that the amount of galactose increased after the incubation of AOSs prepared by acid prehydrolysis with *Vej*ABG (Fig. 6B) compared to the amount obtained before the reaction with *Vej*ABG (Fig. 6A). However, the amount of AHG did not change after the reaction with *Vej*ABG.

The possibility of the complete hydrolysis of agarose-derived oligosaccharides into monomeric sugars by combining *Vej*ABG and a NAOS hydrolase (i.e., *Sd*NABH) was examined. When agarotriose (Fig. 7A), agaropentaose (Fig. 7B), or agaroheptaose (Fig. 7C) was incubated with *Vej*ABG and *Sd*NABH in a single reaction, only AHG and galactose were formed, without any significant amount of residual AOSs or NAOSs being found.

DISCUSSION

In this study, we discovered a novel agarolytic β-galactosidase, *Vej*ABG, originating from *Vibrio* sp. EJY3. *Vej*ABG is capable of cleaving the β1-4 linkage at the nonreducing end of AOS to release galactose and NAOS, as β-galactosidases can cleave β1-4 linkages to release galactose as a product (27). *Vej*ABG is the first β-galactosidase identified that is capable of hydrolyzing AOSs. This novel enzyme acts on the β1-4 linkage only at the nonreducing end of AOSs, as shown by the release of galactose only from the odd-numbered (Fig. 3A) or even-numbered (Fig. 5 and 6) AOSs. However, *Vej*ABG cannot cleave the β1-4 linkage at the reducing end and is unable to release AHG. Therefore, the hydrolysis of AOSs by *Vej*ABG did not increase the amount of AHG (Fig. 6).

The activity of *Vej*ABG can explain several heretofore unknown mechanisms of agar degradation in agarolytic bacteria. The first role of *Vej*ABG is in the conversion of AOSs into NAOSs, by which the α-agarase can be linked to the β-agarase system in the hydrolysis of agarose into monomeric sugars. AOSs produced by α-agarases (28, 29) can be converted to NAOSs by *Vej*ABG, and

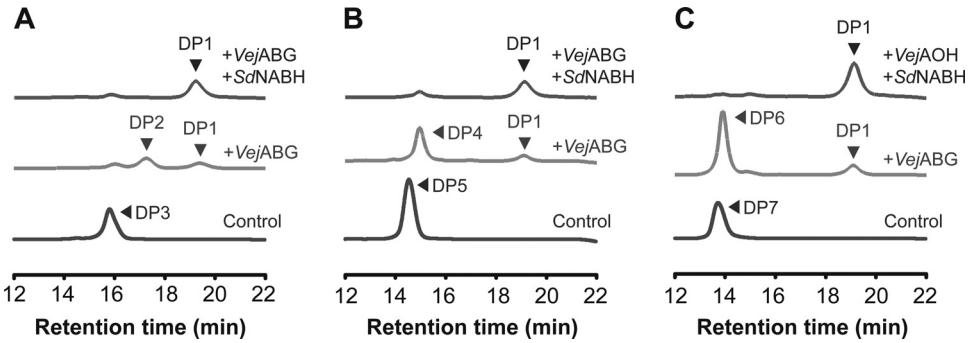

FIG 7 Reaction products from various agarooligosaccharides treated with *Vej*ABG and *Sd*NABH. Agarotriose (DP3) (A), agaropentaose (DP5) (B), and agaroheptaose (DP7) (C) were treated with *Vej*ABG alone and with *Vej*ABG and *Sd*NABH. Control, substrate only; +*Vej*ABG, substrate incubated with *Vej*ABG for 12 h; +*Vej*ABG and +*Sd*NABH, substrate incubated with *Vej*ABG and *Sd*NABH for 12 h. The reaction products were analyzed by gel permeation chromatography.

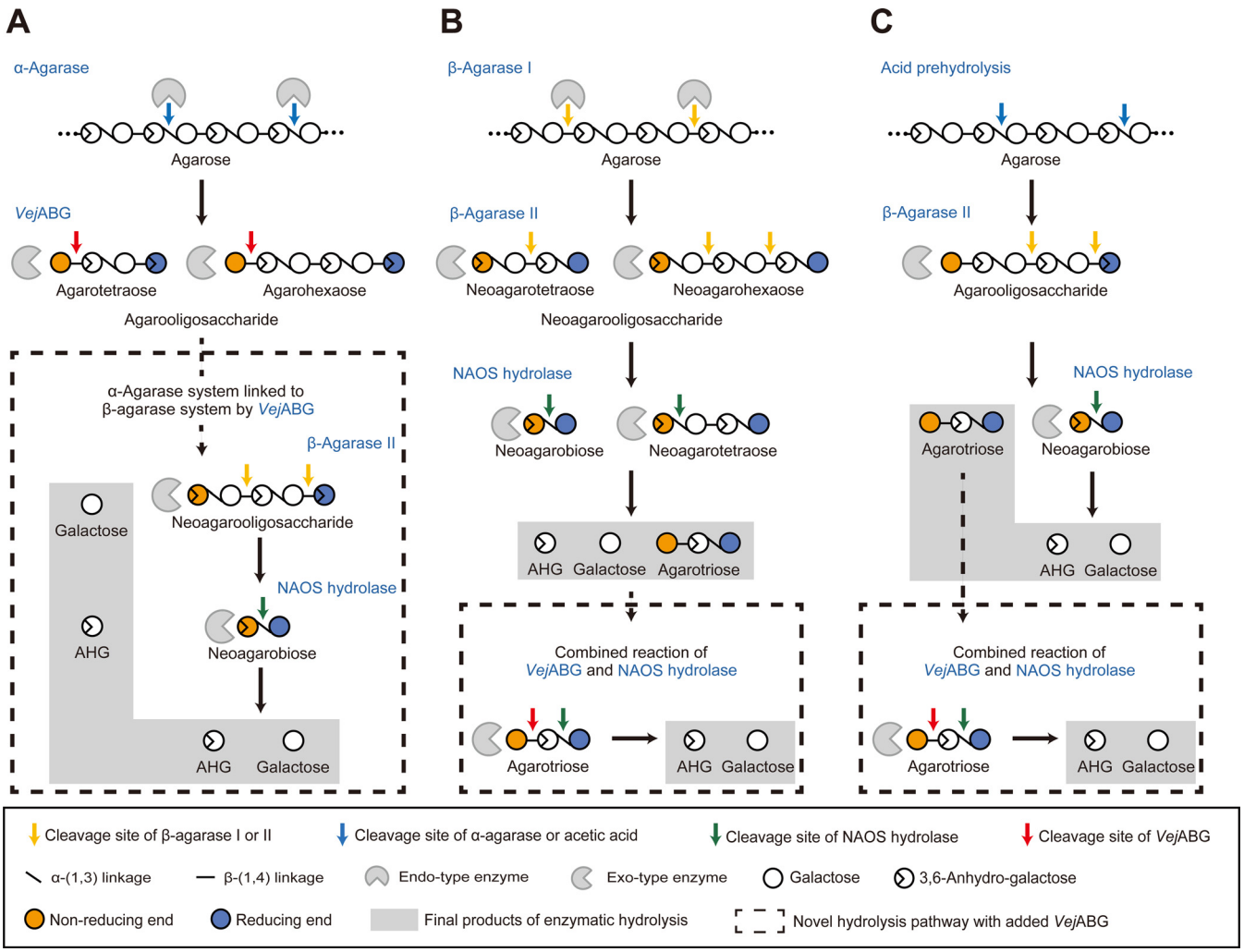

FIG 8 Schematic diagram representing the key modes of action of *Vej*ABG in the enzymatic hydrolysis of agarose. (A) The α-agarase system for producing monomeric sugars from agar is relatively unclear. *Vej*ABG converts agarooligosaccharides (AOSs) into neoagarooligosaccharides (NAOSs), thereby linking the α-agarase and β-agarase systems. (B) Some NAOSs, such as neoagarotetraose, remain unhydrolyzed in the β-agarase system and are further hydrolyzed at the α1-3 and β1-4 linkages into monomers by treatment with a NAOS hydrolase and *Vej*ABG, respectively. (C) In the saccharification of agarose for producing monomeric sugars using acid prehydrolysis and enzymatic hydrolysis, agarotriose remains unhydrolyzed. This problem can be resolved by the additional application of *Vej*ABG.

the NAOSs can then be hydrolyzed by a β-agarase II into neoagarobiose and AHG (Fig. 8A).

The second function of *Vej*ABG is the direct production of monomeric sugars from NAOSs through treatment of NAOSs with a combination of a NAOS hydrolase and the β-agarase system (Fig. 8B). Neoagarohexaose and neoagarotetraose are predominantly produced from agarose by a β-agarase I (30–32) and then primarily hydrolyzed into neoagarobiose by a β-agarase II. However, in some cases, residual NAOSs, such as neoagarotetraose, persist in the reaction (33); therefore, the additional degradation of these residual NAOSs is necessary. By the combined action of a NAOS hydrolase (or an NABH) and *Vej*ABG on the α1-3 and β1-4 linkages at the nonreducing end of a neoagarotetraose molecule, respectively, two molecules of AHG and galactose are successively produced (Fig. 8B).

Although *Vej*ABG was putatively annotated as a β-galactosidase and classified into glycoside hydrolase family 2 (GH2) on the basis of its amino acid sequence, this study revealed that its functional properties are significantly different from those of general GH2 β-galactosidases. In the phylogenetic analysis of the β-galactosidases belonging to GH2, *Vej*ABG locates distantly from other β-galactosidases and does not belong to any other group of previously characterized β-galactosidases (Fig. 9). While the commercial *lacZ*-encoded β-galactosidase from *E. coli* hydrolyzed all the general substrates of a β-galactosidase, including lactose and lactulose, *Vej*ABG did not hydrolyze these substrates (Fig. 3B). Conversely, AOSs were hydrolyzed by *Vej*ABG (Fig. 3A) but not by the *E. coli* β-galactosidase. Based on these results, we speculated that the differential substrate specificity of *Vej*ABG and the *E. coli lacZ*-encoded β-galactosidase is attributed to the difference in their ability to recognize galactose and AHG linked by the β1-4 bond located at the nonreducing end of AOSs. AHG is a rare sugar with a bicyclic structure that is not observed among the general substrates of β-galactosidases. There-

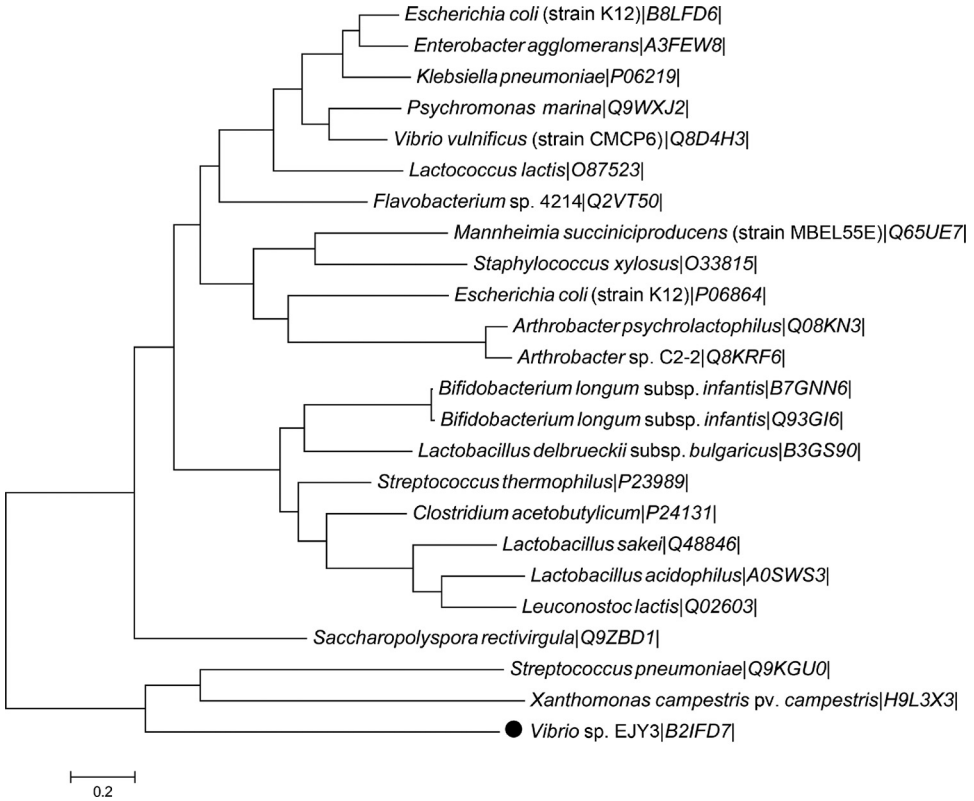

FIG 9 Phylogenetic tree of the host microorganisms of *Vej*ABG and other functionally characterized β-galactosidases in the GH2 family. Genetic information was obtained from the CAZy database and UniProt (22). The amino acid sequences of the enzymes were aligned by the ClustalW program, and the tree was constructed using the MEGA (version 5) program (25). The UniProt entries are indicated after the organism names.

fore, the unique mode of action of *Vej*ABG may partly be due to the presence of AHG in AOSs.

In the industrial application of red algae as the biomass feedstock for fuel and chemical production, the complete hydrolysis of agarose into monomeric sugars is important (20). Acid-prehydrolyzed agarose can be further enzymatically hydrolyzed into neoagarobiose, agarotriose, and AHG by the action of a β-agarase II (Fig. 8C). Among these products, neoagarobiose can be hydrolyzed into monomeric sugars by a NAOS hydrolase, but agarotriose cannot be further hydrolyzed by either β-agarases or a NAOS hydrolase (20). Additional application of *Vej*ABG in this case leads to the hydrolysis of agarotriose into monomeric sugars by the combined action of *Vej*ABG and a NAOS hydrolase or an NABH (i.e., *Sd*NABH; Fig. 8C). *Vej*ABG not only is a novel β-galactosidase with a unique function but also is industrially important for producing monomeric sugars from agar.

In conclusion, *Vej*ABG (agarolytic β1-4-D-galactosidase), isolated from *Vibrio* sp. EJY3, is a novel β-galactosidase with a distinct substrate specificity, unlike all other previously characterized β-galactosidases. This novel enzyme cleaves the β1-4 linkage between galactose and AHG only at the nonreducing end of an AOS. This reaction can link the products of the α-agarase system to the β-agarase system. Moreover, this enzyme can also be used to increase the yield of monomeric sugars in the saccharification process by using agar or red algae as biomass.

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