JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 12249−12256

pubs.acs.org/JAFC

Novel Two-Step Process Utilizing a Single Enzyme for the Production of High-Titer 3,6-Anhydro-L-galactose from Agarose Derived from Red Macroalgae

Dong Hyun Kim, Eun Ju Yun, Sang-Hyun Lee,* and Kyoung Heon Kim*

Department of Biotechnology, Graduate School, Korea University, Seoul 02841, South Korea

Supporting Information

ABSTRACT: 3,6-Anhydro-L-galactose (L-AHG), a major component of agarose derived from red macroalgae, has excellent potential for industrial applications based on its physiological activities such as skin whitening, moisturizing, anticariogenicity, and anti-inflammation. However, L-AHG is not yet commercially available due to the complexity, inefficiency, and high cost of the current processes for producing L-AHG. Currently, L-AHG production depends on a multistep process requiring several enzymes. Here, we designed and tested a novel two-step process for obtaining high-titer L-AHG by using a single enzyme. First, to depolymerize agarose preferentially into agarobiose (AB) at a high titer, the agarose prehydrolysis using phosphoric acid as a catalyst was optimized at a 30.7% (w/v) agarose loading, which is the highest agarose or agar loading reported so far. Then AB produced by the prehydrolysis was hydrolyzed into L-AHG and D-galactose (D-Gal) by using a recently discovered enzyme, Bgl1B. We suggest that this simple and efficient process could be a feasible solution for the commercialization and mass production of L-AHG.

KEYWORDS: *3,6-anhydro-L-galactose, agarose, red macroalgae, agarobiose, phosphoric acid, agarobiose hydrolase*

1. INTRODUCTION

Agar is the main carbohydrate of red macroalgae[1] and consists of neutral agarose and charged agaropectin.[2] The unique gelling property of agar makes agar one of the most important gelling agents in the food industry[2] and is conferred by agarose.[3] Agarose consists of D-galactose (D-Gal) and 3,6-anhydro-L-galactose (L-AHG).[4] To form the straight chain of agarose, agarobiose (AB), the disaccharides of D-Gal and L-AHG linked via a β-1,4-glycosidic linkage, are repeatedly linked via α-1,3-glycosidic linkages.[5] L-AHG derived from agarose has received increasing attention as a functional food or cosmetic material due to its physiological activities, such as skin whitening,[6] moisturizing,[6] antiinflammation,[6] and anticariogenicity.[7] However, L-AHG is not yet commercially available because of its complex, inefficient, and costly production process.

Thus far, three types of saccharification processes have been developed to produce L-AHG and D-Gal from agarose or agar. The first type is the acid saccharification process utilizing strong acids, such as sulfuric acid, at a high temperature.[8] Although the acid saccharification process without using any enzyme is simple to perform, process conditions require high concentration acid and high temperature. It is because this process is intended to depolymerize agarose or agar directly into monomeric sugars at harsh conditions. Therefore, produced monomeric sugars further undergo degradation. For example, during the acid saccharification process, high amounts of 5-hydroxymethylfurfural (5-HMF) are inevitably produced from hexose sugars, particularly from L-AHG because of its low thermal stability.[8−10]

The second type is the enzymatic saccharification process. In this process, agarose is depolymerized into neoagarobiose (NAB) by endo- and exotype β-agarases, and NAB is then hydrolyzed intoL-AHG and D-Gal by α-neoagarobiose hydrolase (α-NABH).[11] The advantage of the enzymatic saccharification process is high L-AHG yields that can be obtained by complete hydrolysis of agarose due to the high substrate specificity of those enzymes. However, because agarose is insoluble in water at most temperatures other than in boiling water, only low loadings of agarose can be applied to the enzymatic saccharification process,[12] thus resulting in low L-AHG titers after enzymatic saccharification.

As the third saccharification type, the chemical prehydrolysis and enzymatic saccharification processes are combined to overcome the drawbacks of the acid and the enzymatic saccharification processes each described above. For the liquefaction of agarose, agarose is prehydrolyzed into agarooligosaccharides (AOSs) through the preferential cleavage of α-1,3-linkages in agarose by using chemicals such as weak acid[13] (Figure 1a) or Tris−HCl buffer[14] (Figure 1b). Then AOSs are depolymerized into neoagarooligosaccharides (NAOSs) or NAB by β-agarase. NAOSs and NAB are then depolymerized into L-AHG and D-Gal by α-NAOS or α-NAB hydrolase. Such a combined process is relatively complex because it is a four-step process that involves a prehydrolysis step utilizing acid or chemical and three consecutive enzymatic saccharification steps (Figure 1a,b). The requirements to produce three agarolytic enzymes and to perform multiple enzymatic reactions make this process difficult to commerci-

Received: August 2, 2018
Revised: October 21, 2018
Accepted: October 25, 2018
Published: October 25, 2018

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256

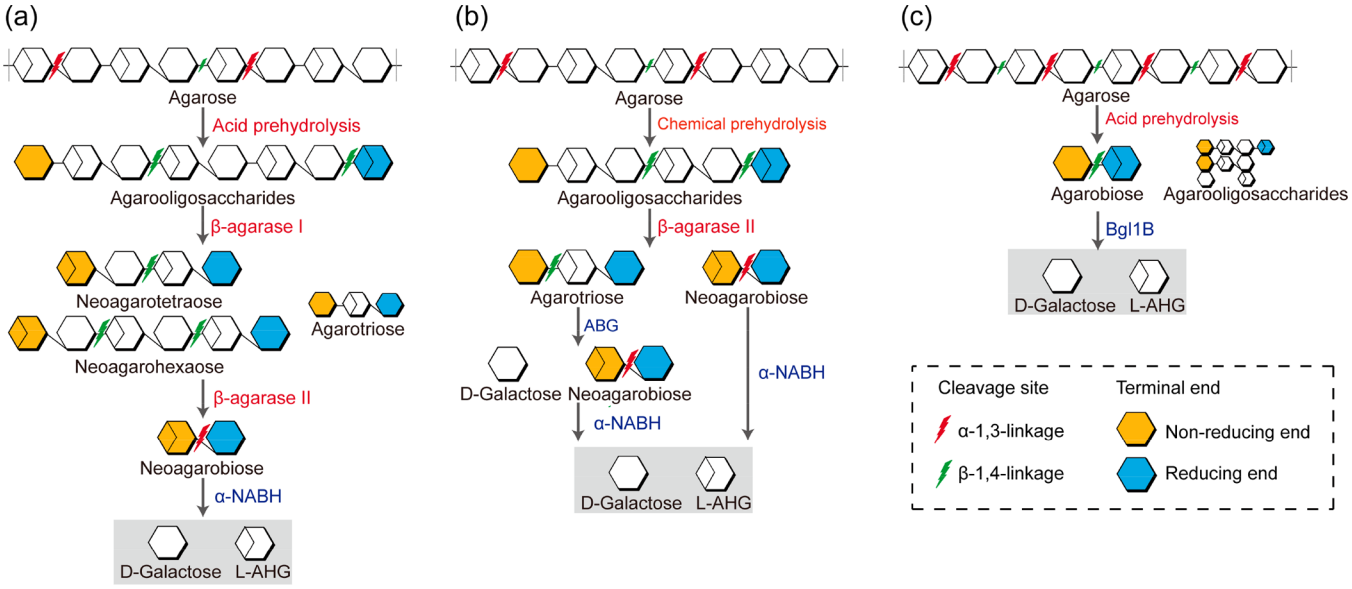

Figure 1. Combined process of acid or chemical prehydrolysis and enzymatic saccharification of agarose to produce L-AHG. (a) Acetic acid prehydrolysis,[13] (b) Tris-HCl buffer prehydrolysis,[14] and (c) phosphoric acid prehydrolysis (this study), followed by enzymatic saccharification.

alize. For the mass production of L-AHG from agarose or agar, it is necessary to develop a simple and efficient saccharification process.

In this study, we designed a novel L-AHG production process composed of two steps: the acid prehydrolysis of agarose into AB and the enzymatic hydrolysis of AB into L-AHG and D-Gal by using a single enzyme (Figure 1c). First, acid prehydrolysis conditions were optimized to produce AB from agarose with high titers and high yields by utilizing phosphoric acid as a catalyst. Second, a novel enzyme from the marine bacterium *Saccharophagus degradans* 2−40^T, called Bgl1B,[15] was exploited to hydrolyze AB into monomeric sugars. To the best of our knowledge, this is the first study on agarose or agar saccharification processes for producing monomeric sugars utilizing a single enzyme.

2. MATERIALS AND METHODS

2.1. Enzymes and Chemicals. The enzymes for DNA manipulation were purchased from New England BioLabs (Ipswich, MA). The plasmids for recombinant protein expression were obtained from Novagen (Darmstadt, Germany). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MI).

2.2. Acid Prehydrolysis of Agarose into AB. To produce AB from agarose at a high titer, agarose (Genomicbase, Seoul, Korea) was loaded at a final concentration of 16.8−36.8% (w/v) into a 100-mL vessel (Milestone, Shelton, CT) filled with diluted phosphoric acid solutions at various concentrations ranging from 0.5−2.0% (w/v). The charged vessel was then equipped with a thermocouple and loaded into a microwave digester (Milestone). Each agarose and phosphoric acid slurry sample was incubated at different temperatures ranging from 110−140 °C for 5−10 min for the prehydrolysis of agarose. The prehydrolysates were chilled on ice and neutralized to pH 6 by using 5 M NaOH before further enzymatic hydrolysis.

2.3. Analysis of Liquefaction and Saccharification of Prehydrolyzed Agarose. To determine the liquefaction yield of agarose after prehydrolysis, the unliquefied fraction was separated from the prehydrolysate, and its dry weight was measured. First, to separate the liquefied fraction, the agarose prehydrolysate was kept at 4 °C for 12 h following prehydrolysis and then centrifuged at 8000*g* and 4 °C for 30 min. The pellet obtained from the centrifuged prehydrolysate was considered as the unliquefied fraction. To determine the dry weight of the pellet, the pellet was dried at 105 °C for 12 h in a drying oven (Biofree, Seoul, South Korea). The liquefaction yield (%, w/w) was calculated as follows:

$$\text{Liquefaction yield (\%, w/w)} = \frac{\text{Amount of initial agarose (g)} - \text{Amount of unliquefied fraction (g)}}{\text{Amount of initial agarose (g)}} \times 100$$

To determine the saccharification yield after prehydrolysis of agarose, reducing sugar was quantified by using the dinitrosalicylic acid reagent. The absorbance was measured at 540 nm by using D-Gal as the monomeric sugar standard representing the reducing ends of both D-Gala and L-AHG.[12,13,16,17] The saccharification yield was calculated as follows, where 1.1 is the conversion factor of hydrolysis of agarose into the monomeric sugars, L-AHG and D-Gal:[13]

$$\text{Saccharification yield (\%, w/w)} = \frac{\text{Amount of reducing sugar produced (g)}}{\text{Amount of initial agarose} \times 1.1\ \text{(g)}} \times 100$$

2.4. Cloning and Expression of *bgl1B* Gene. Plasmid pET21a-*bgl1B*, which was initially constructed to contain the *bgl1B* gene from *S. degradans* 2−40^T,[15] was transformed into *Escherichia coli* BL21-(DE3). The transformed cells were grown to a cell density at 600 nm (OD_{600}) of 0.5 in 10 mL of Luria−Bertani medium (BD, Sparks, MD) containing 50 μg/mL of ampicillin (Sigma-Aldrich) at 37 °C. The recombinant protein expression of the *bgl1B* gene was induced by using 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich) at 16 °C for 12 h. The cells of *E. coli* BL21(DE3) harboring pET21a-*bgl1B* were harvested by centrifugation at 5000*g* for 30 min at 4 °C and stored at −20 °C prior to purification of the recombinant Bgl1B enzyme.

2.5. Purification of Recombinant Bgl1B Enzyme. The cells of *E. coli* BL21(DE3) harboring pET21a-*bgl1B* were suspended in a lysis buffer (20 mM Tris−HCl, pH 7.4) and disrupted by sonication. The disrupted cell suspension was centrifuged at 8,000 *g* for 30 min at 4 °C, and the supernatant was collected for protein purification. The Bgl1B enzyme was purified by affinity chromatography using a HisTrap column (GE Healthcare, Piscataway, NJ), and the purification was verified by 8% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (Sigma-Aldrich) as a standard.

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256

Table 1. Comparison of Processes for Prehydrolysis of Agarose or Agar

substrate	catalyst	solids loading	temp. (°C)	time (min)	saccharification yield (%, w/w)[a]	5-HMF yield (%, w/w)	ref
agarose	27.4% (w/v) acetic acid	15.0% (w/v)	80	180	16.7	NA[b]	13
agarose	3.0% (w/v) acetic acid	7.0% (w/v)	130	30	30.6	2.3	16
agar	0.3% (w/v) Tris−HCl	5.0% (w/v)	170	10	NA	1.3	14
agarose	2.0% (w/v) phosphoric acid	30.7% (w/v)	110	10	48.5	1.3	this study

[a]After acid or chemical prehydrolysis. [b]Not available.

2.6. Enzymatic Hydrolysis of AB. AB produced by acid prehydrolysis of agarose was hydrolyzed by using Bgl1B enzyme prepared as described above. To investigate the effect of different enzyme loadings on the hydrolysis of AB in agarose prehydrolysate, different amounts of Bgl1B at ranging from 10−100 nmol were incubated in 1 mL of neutralized and 100-fold diluted agarose prehydrolysate containing mainly AB at 30 °C for 12 h. In addition, to obtain high-titer L-AHG, 60 nmol of Bgl1B was incubated at 30 °C for 24 h with 1 mL of agarose prehydrolysate diluted by 2−100-fold. The reaction was halted by quenching the reaction mixture into boiling water for 3 min. The reaction products, including L-AHG and D-Gal, were analyzed by using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography/time-of-flight mass spectrometry (GC/TOF MS).

2.7. TLC Analysis. TLC was performed on silica gel 60 TLC plates (Merck, Darmstadt, Germany), and the plates were developed utilizing *n*-butanol-ethanol−water (3:1:1, v/v/v). The developed plates were visualized by using 10% (v/v) H_2SO_4 and 0.2% (w/v) naphthoresorcinol in ethanol. 3,6-Anhydro-D-galactose (D-AHG; Dextra Laboratories, Berkshire, UK) and D-Gal (Sigma-Aldrich) were utilized as the standards of a degree of polymerization 1 (DP1). Additionally, NAB as a DP2 standard, and neoagarotetraose and neoagarohexaose as DP4 and DP6 standards, respectively, were produced in-house from agarose by using Aga50D and Aga16B enzymes, respectively, as described previously.[12,18]

2.8. HPLC Analysis. The concentration of 5-HMF was measured by using an HPLC system (Agilent 1100, Agilent Technologies, Wilmington, DE) equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, CA) at 65 °C by using 0.01 N H_2SO_4 at a flow rate of 0.5 mL/min as the mobile phase. The concentrations of monomeric sugars and oligosaccharides were measured by using the HPLC equipped with a gel permeation column (Shodex KS-802, Shodex, Japan). As the mobile phase, distilled water with a flow rate of 0.5 mL/min was used, and the column temperature was maintained at 80 °C. All HPLC analyses were performed by using a refractive index detector (Agilent). Because the standards of AOSs with high DPs over 6 are not commercially available, the yields of AOSs with high DPs were calculated as follows:

$$\text{AOS (DP} \geq 6\text{) yield (\%, w/w)} = \frac{\text{Amount of initial agarose (g)} - \text{Amount of DP1} - \text{DP5 and 5-HMF}}{\text{Amount of initial agarose (g)}} \times 100$$

2.9. GC/TOF MS Analysis. To identify L-AHG and to quantify its amount after enzymatic saccharification of agarose prehydrolysates, GC/TOF MS analysis was performed after some minor modifications of a previous method.[19] The reaction mixtures were centrifuged at 16 000*g* for 10 min at 4 °C, and 20 μL of supernatants was then vacuum-dried by utilizing a speed vacuum concentrator (Labconco, Kansas City, MO). For the derivatization of reaction products, the dried samples were incubated with 5 μL of 40 mg/mL methoxyamine hydrochloride in pyridine (Sigma-Aldrich) at 30 °C for 90 min and then incubated with 45 μL of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (Fluka, Buchs, Switzerland) at 37 °C for 30 min. The derivatized samples were then analyzed by using an Agilent 7890B GC (Agilent Technologies) with an RTX-5Sil MS column (30 m × 0.25 mm, 0.25-μm film thickness; Restek, Bellefonte, PA) and an additional 10-m guard column coupled to a Pegasus HT TOF MS (LECO, St. Joseph, MI). Each aliquot of 0.5 μL of the derivatized samples was injected into the GC in splitless mode. The initial oven temperature was set to 50 °C for 1 min, then ramped to 330 °C at 20 °C/min and held at 330 °C for 5 min. The temperatures of the ion source and transfer line were 250 and 280 °C, respectively. The samples were ionized by electron impact at 70 eV. The mass spectra of the enzymatic reaction samples were in the mass range of 85−500 *m*/*z* at an acquisition rate of 10 spectra/s.

3. RESULTS AND DISCUSSION

3.1. Effects of Agarose Loading on Liquefaction Yield. Achieving high biomass loadings in prehydrolysis and saccharification is essential for obtaining high-titer sugar and reducing operation costs related to water usage, equipment size, and product purification.[20−22] High-titer production of L-AHG is largely hampered by low agarose or agar loadings due to the insolubility of agarose or agar in water.[11] To date, loadings of agarose have remained at less than 15% (w/v) in hydrolysis reactions[13,14,16] (Table 1). Therefore, it is necessary to develop a new saccharification process that is suitable for high loadings of agarose for the industrial saccharification of agar or red macroalgae.

In this study, we chose phosphoric acid as the catalyst for the prehydrolysis of agarose mainly into AB, which can be simply hydrolyzed to monomeric sugars, L-AHG and D-Gal, by using an enzyme such as Bgl1B.[15] Under strong acidic conditions, L-AHG is readily degraded into 5-HMF.[25] Thus, to liquefy agarose, acetic acid was previously used as a weak acid catalyst.[13,16] However, acetic acid cannot efficiently break α-glycosidic linkages of agarose, resulting in low liquefaction and saccharification yields.[13,16] In addition, as the agarose loading increases, it is more difficult for acetic acid to liquefy agarose. Previously, even at a 7% (w/v) agarose loading, only a 9.6% saccharification yield was obtained by using 3% (w/v) acetic acid at 120 °C for 10 min.[16] Although the acetic acid concentration, reaction time, and temperature increased to 3% (w/v), 30 min, and 130 °C, only 30.6% saccharification yield was obtained in a previous study[16] (Table 1). Therefore, in this study, phosphoric acid, which is a stronger acid than acetic acid, was chosen as a weak acid catalyst. Another advantage of choosing phosphoric acid was to utilize sodium phosphate, which is produced as the neutralization product of sodium hydroxide, as an enzymatic reaction buffer during enzymatic hydrolysis of agarose prehydrolysate for producing monomeric sugars.

In this study, the potential of prehydrolysis of agarose at high loadings by using phosphoric acid as a catalyst was examined. To accomplish this, prehydrolysis of agarose at various loadings, 16.8%, 23.8%, 30.7%, and 36.8% (w/v), was performed in a 0.5% (w/v) phosphoric acid solution at 140 °C for 10 min by using a microwave digester. At agarose loadings

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256

of 16.8−30.7%, more than 98% liquefaction yields were obtained (Figure 2a), although a significant amount of 5-HMF

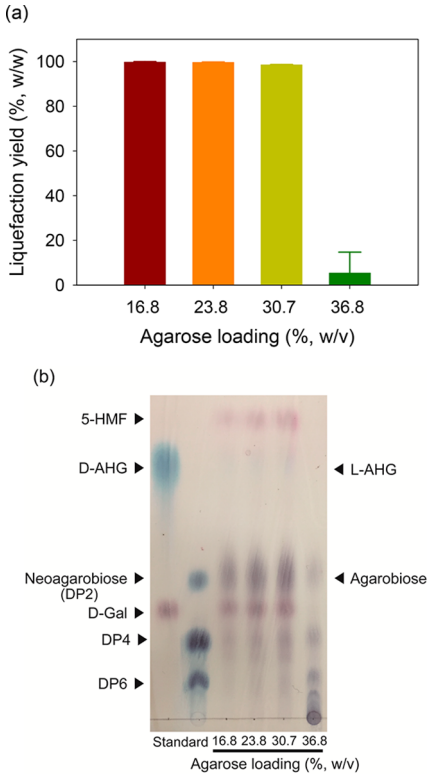

Figure 2. Effects of agarose loadings on the liquefaction yields of agarose. (a) Liquefaction yields of agarose and (b) TLC analysis of agarose prehydrolysate after acid prehydrolysis at 140 °C for 10 min using 0.5% (w/v) phosphoric acid.

(i.e., 4.2% (w/v)) was produced at an agarose loading of 30.7% (Table 2). However, when agarose loading was increased to 36.8%, agarose was only liquefied by 5.4% (Figure 2a) and strong spots were observed over DP6 in the TLC analysis (Figure 2b).

In this study, there was a significant difference in liquefaction yield between the prehydrolysis of agarose at loadings of 30.7% and 36.8%. Agarose exists in water as a solid in either the sol or gel state.[23] To efficiently liquefy agarose, agarose should be in the sol state so that agarose can be more accessible to enzyme or a chemical catalyst during liquefaction.[12] The sol−gel transition temperature of agarose differs depending on the concentration of agarose in water. In the case of agar at 1% (w/v), agar melts in the range from 80 to 92 °C by going through a gel-to-sol transition.[24] As the agarose concentration increases, the melting temperature of agarose increases. Therefore, at a higher agarose loading, it becomes more difficult to liquefy agarose. In this study, the agarose loading of 30.7% (w/v) resulted in the agarose liquefaction yield of 98.5%; however, the higher agarose loading of 36.8% gave the agarose liquefaction yield of only 5.4% (Figure 2a). This liquefaction yield difference may have been caused by the differences in sol−gel transition temperature and accessibility of agarose to the chemical catalyst of the two different loadings of agarose during the liquefaction processes.

Liquefaction is an important indicator in biomass saccharification processes because unliquefied solids result in a decrease in saccharification yield. Therefore, we concluded that an agarose loading of 36.8% is too high as a solids loading for effective agarose prehydrolysis. Additionally, to reduce the formation of 5-HMF and to increase saccharification yields, further process optimization was performed at an agarose loading of 30.7%.

3.2. Effects of Acid Concentration, Temperature, and Time on Liquefaction Yield. First, at 0.5% (w/v) phosphoric acid, liquefaction yields were examined at various temperatures and for various time lengths (Figure 3a). At 120 °C, the liquefaction yields were 0.0% and 18.5% after 5 and 10 min, respectively. However, when the temperature was increased to 130 °C, the liquefaction yields increased significantly to 91.1% and 99.3% after 5 and 10 min, respectively. At 140 °C, the liquefaction yields were over 99.0% after both 5 and 10 min.

Second, at 1.0% (w/v) phosphoric acid, liquefaction yields were examined at various temperatures and time lengths (Figure 3b). At 110 °C, the liquefaction yields were 64.4% and 99.4% after 5 and 10 min, respectively. When temperature increased to 120 °C, 130 °C, or 140 °C, the liquefaction yields were all over 93.0% after 5 or 10 min. Finally, at 2.0% (w/v) phosphoric acid, the liquefaction yields were over 98% under all reaction conditions (Figure 3c). We can conclude that as the concentration of phosphoric acid, reaction temperature,

Table 2. Compositions of Agarose Prehydrolysates at Various Conditions[a]

hydrolysis condition			product yield[b] (%, w/w)						
phosphoric acid (%, w/v)	temp. (°C)	time (min)	DP1	DP2	DP3	DP4	DP5	≥DP6	5-HMF
0.5	140	10	13.0 ± 0.8	57.2 ± 0.2	7.7 ± 0.1	12.0 ± 0.6	5.9 ± 0.5	ND[c]	4.2 ± 0.3
1.0	130	5	7.8 ± 1.3	66.1 ± 1.1	4.8 ± 0.2	14.7 ± 1.6	5.4 ± 1.2	ND	1.2 ± 0.3
		10	20.3 ± 5.5	49.3 ± 10.4	9.4 ± 0.8	8.3 ± 1.2	2.9 ± 0.4	ND	7.3 ± 3.3
	140	5	12.5 ± 1.3	62.5 ± 1.1	7.2 ± 0.2	10.6 ± 0.5	4.2 ± 0.4	ND	2.9 ± 0.6
		10	29.3 ± 0.0	32.4 ± 0.8	11.1 ± 0.2	5.9 ± 0.3	2.9 ± 0.0	ND	11.1 ± 2.2
2.0	110	10	8.0 ± 2.4	70.0 ± 1.2	4.4 ± 0.5	12.5 ± 1.7	4.7 ± 1.4	ND	1.3 ± 0.6
	120	10	12.1 ± 3.4	64.4 ± 3.9	7.4 ± 1.0	9.8 ± 0.7	3.8 ± 0.5	ND	2.5 ± 1.3
	130	5	11.7 ± 1.5	65.4 ± 2.1	7.8 ± 0.4	9.7 ± 0.2	3.3 ± 0.1	ND	2.1 ± 0.5
		10	24.8 ± 3.0	44.6 ± 6.8	10.0 ± 0.2	7.2 ± 0.8	3.0 ± 0.2	ND	9.0 ± 2.3
	140	5	20.5 ± 2.7	53.1 ± 6.9	9.3 ± 0.7	9.1 ± 0.9	0.1 ± 0.0	ND	5.7 ± 1.4
		10	32.0 ± 1.6	25.2 ± 3.0	9.7 ± 0.5	4.5 ± 0.6	2.4 ± 0.3	ND	16.9 ± 1.9

[a]Experimental data are expressed as means ± standard deviations from triplicate experiments. [b]Production yield was calculated as the percentage of product produced from the initial input of agarose. [c]Not detected.

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256

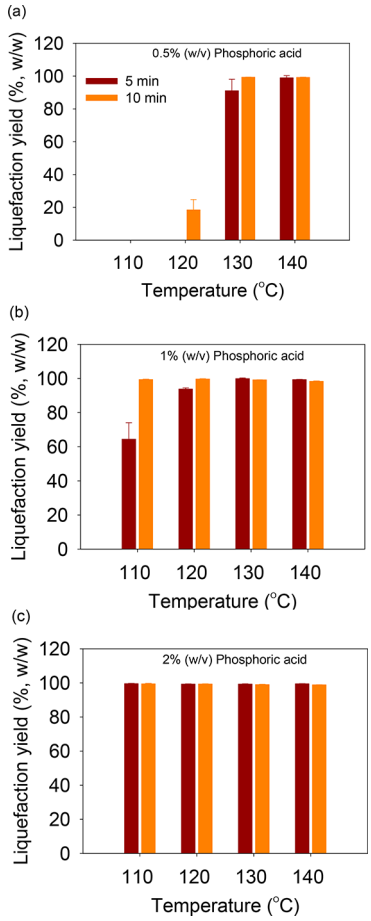

Figure 3. Effects of various prehydrolysis conditions, such as acid concentration, temperature, and time, on the liquefaction yields of agarose. Liquefaction yields of agarose at (a) 0.5%, (b) 1%, and (c) 2% (w/v) phosphoric acid at different temperatures and for different time lengths at a 30.7% (w/v) agarose loading.

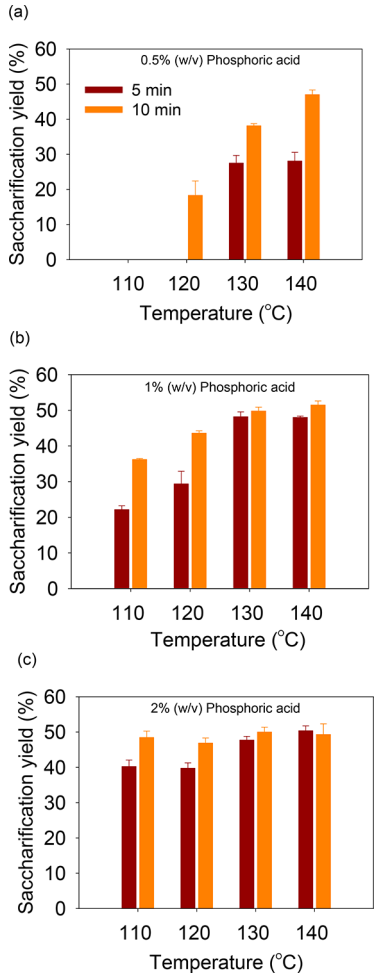

Figure 4. Effects of various prehydrolysis conditions, such as acid concentration, temperature, and time, on the saccharification yields of agarose. Saccharification yields of agarose at (a) 0.5%, (b) 1%, and (c) 2% (w/v) phosphoric acid at different temperatures and for different time lengths at a 30.7% (w/v) agarose loading.

and reaction time increase, the liquefaction yield of agarose increases.

3.3. Effects of Acid Concentration, Temperature, and Time on Saccharification Yield of Agarose. At a 0.5% (w/v) phosphoric acid, when prehydrolysis was performed for 5 min at 130 or 140 °C, the saccharification yields remained at less than 30% (Figure 4a), although most of agarose was liquefied (Figure 3a). However, when the reaction time increased from 5 to 10 min, the saccharification yields increased to 38.1% and 47.0%, respectively (Figure 4a). These results indicate that at a phosphoric acid concentration lower than 0.5%, prehydrolysis should be performed for sufficient time lengths at high temperatures, such as 130 or 140 °C.

At 1.0% (w/v) phosphoric acid, when the temperature was 110 °C, low saccharification yields, 22.3% and 36.5%, were observed after 5 and 10 min (Figure 4b), respectively, although the liquefaction yields were 62.8% and 93.8%, respectively (Figure 3b). However, when the temperature was over 130 °C, the saccharification yield reached approximately 50% after 5 or 10 min (Figure 4b). These results imply that even at 1.0% phosphoric acid, a high temperature is required to achieve acceptable saccharification yields. At 2.0% phosphoric acid, when prehydrolysis was performed for 10 min, the saccharification yields reached approximately 50% at all temperatures.

To determine the optimal prehydrolysis conditions, the prehydrolysis conditions indicating more than 95.0% liquefaction yields and 45% saccharification yields were selected from Figures 3 and 4, and the selected prehydrolysates were analyzed by using HPLC (Table 2).

3.4. Optimal Conditions for Preferential Production of AB from Agarose. In this study, the optimal prehydrolysis conditions for preferential production of AB (AOS with a DP2) from agarose were selected based on the AB yield. This is because AB is a suitable substrate for the Bgl1B enzyme to effectively produce L-AHG.[15] Because α-glycosidic linkages are preferentially cleaved during acid hydrolysis,[16,25] oligosaccharides produced from the prehydrolysis of agarose in this experiment were presumed to be all AOSs, where AOSs with a DP2 were considered to be AB.

On the basis of the agarose prehydrolysates selected earlier, the yields of AOSs with DP < 6, which were defined as [wt. of AOSs with each DP]/[wt. of agarose] × 100 (%), as well as the yields of 5-HMF, were compared (Table 2). Among various conditions listed in Table 2, the highest yield of AB (70.0%) was obtained under prehydrolysis conditions of 2% (w/

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256

v) phosphoric acid, 110 °C, and 10 min. The final titer of AB in the postreaction mixture was 224.3 g/L. Under these conditions, the yield of 5-HMF was as low as 1.3%. When increasing the temperature to 140 °C and decreasing the phosphoric acid concentration to 0.5% (w/v) for 10 min of prehydrolysis, the yield of AB decreased to 57.2% and the yield of 5-HMF increased to 4.2%. This is likely caused by the degradation of heat-labile L-AHG into 5-HMF at the high temperature of 140 °C (Table 2). At other phosphoric acid concentrations (1.0% and 2.0%), similar trends of reduced AB yields and increased 5-HMF yields were observed when increasing the temperature from 110 to 120 °C, 130 °C, or 140 °C. Consequently, the prehydrolysis conditions of 2% (w/v) phosphoric acid, 110 °C, and 10 min were selected as the optimal conditions for producing AB. The prehydrolysate obtained from these conditions was utilized as the substrate for further enzymatic hydrolysis to produce monomeric sugars from AB.

Interestingly, odd-numbered oligosaccharides with DP3 and DP5, as well as DP1, were also obtained during the acid prehydrolysis of agarose (Table 2). This is likely because L-AHG residues at the reducing end of even-numbered AOSs are unstable under acidic conditions, and β-1,4-linkages at the reducing ends are easily cleaved.[25] Interestingly, in the TLC analysis of all prehydrolysates, the spot of L-AHG was not detected due to the low acid and thermal stabilities of L-AHG (Figure S1). Therefore, DP1 produced by acid prehydrolysis was considered D-Gal.

Previously, the primary purpose of acid prehydrolysis was to only liquefy agarose or agar. This is because substrate accessibility to enzyme is higher in the sol state than in the gel state.[12] Therefore, following the agarose liquefaction by acid prehydrolysis into AOSs, multiple enzymes are still required to convert AOSs into NAOSs and to completely depolymerize NAOSs into monomeric sugars. Specifically, the AOSs produced by acid prehydrolysis are depolymerized into NAOSs by using endo-type β-agarase such as Aga16B or DagA (Figure 1a).[13] The NAOSs are then depolymerized into NAB by utilizing exo-type β-agarase such as Aga50D.[18] The NAB is finally depolymerized into L-AHG and D-Gal by α-NABH[26] (Figure 1a,b). In this process, agarotriose is produced as a byproduct of the reaction with exo-type β-agarase and remains unhydrolyzed.[16] To depolymerize agarotriose, agarolytic β-galactosidase (ABG) is required to hydrolyze agarotriose into D-Gal and NAB (Figure 2b).[14,27] Overall, these processes require four steps to completely depolymerize agarose into monomers: a one-step prehydrolysis process utilizing acid or chemicals and three-step enzymatic saccharification using three enzymes. These complex procedures make the production of L-AHG difficult to commercialize. However, unlike previous acid liquefaction and enzymatic processes, if optimal prehydrolysis conditions for producing AB are adopted, one-step production of AB and another one-step enzymatic hydrolysis process for turning AB into L-AHG and D-Gal are made possible. The remaining task was to find the proper enzyme to hydrolyze AB into monomeric sugars.

3.5. Enzymatic Hydrolysis of AB into L-AHG and D-Gal.

Recently, Bgl1B was discovered in the marine bacterium *S. degradans* 2−40^T and annotated as a novel β-glucosidase.[15] According to this previous report, Bgl1B cleaves the β-1,3-glycosidic linkage of laminaribiose and the β-1,4-glycosidic linkages of cellobiose, lactose, and AB.[15] Therefore, Bgl1B was exploited to saccharify AB produced from prehydrolysis of agarose.

In this study, to investigate the utilization of Bgl1B for the hydrolysis of AB in agarose prehydrolysate, the enzymatic hydrolysis yield of AB by Bgl1B was tested by increasing the loading of Bgl1B. Different loadings of Bgl1B ranging from 10−100 nmol were incubated with 1 mL of neutralized and 100-fold diluted agarose prehydrolysate in which the AB concentration was 1.9 g/L at 30 °C for 12 h. The hydrolysis yield of AB increased with an increase in Bgl1B loading (Figure 5a). For example, AB was not completely hydrolyzed by 10 nmol of Bgl1B after 12 h, resulting in the hydrolysis yield of 73.3%. However, when the Bgl1B loadings were increased over 20 nmol, the hydrolysis yield over 94% was obtained after 12 h. In addition, AB was completely hydrolyzed into L-AHG and D-Gal by Bgl1B of 60 nmol after 12 h by TLC analysis (Figure S2). L-AHG and D-Gal produced from AB by Bgl1B were identified by using GC/TOF MS (Figure S3). We confirmed that the mass spectra of L-AHG and D-Gal acquired by GC/

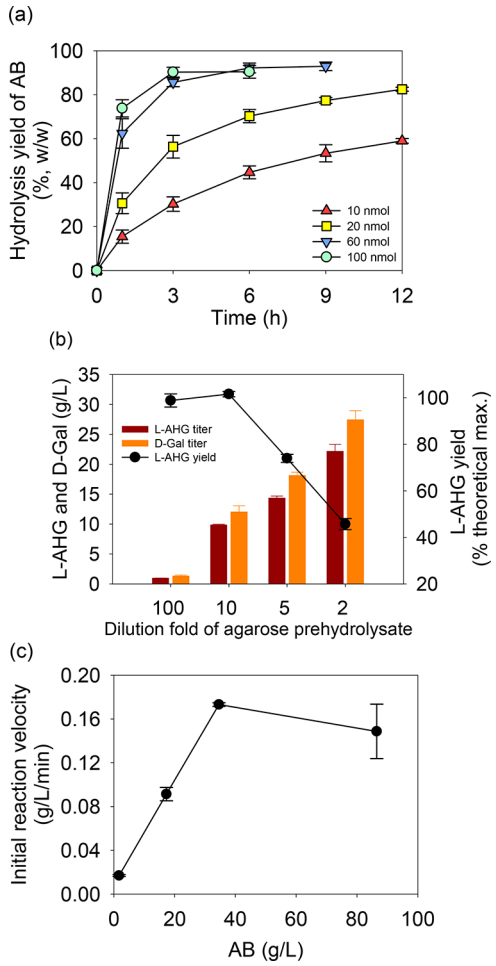

Figure 5. Enzymatic hydrolysis of AB in agarose prehydrolysate by using Bgl1B. (a) Effect of different enzyme loadings on the AB hydrolysis. (b) Effect of different agarose prehydrolysate concentrations on the AB hydrolysis. (C) Effect of agarose prehydrolysate concentrations on the initial reaction velocity of the AB hydrolysis. To prepare the prehydrolysate used in the AB hydrolysis, agarose at 30.7% (w/v) agarose loading were prehydrolyzed by using 2% (w/v) phosphoric acid at 110 °C for 10 min and then neutralized to pH 6 by using 5 M NaOH.

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256

TOF MS analysis were identical with those obtained from their authentic standards (Figure S3).

Additionally, to obtain high-titer L-AHG from agarose prehydrolysate, the enzyme reactions were performed with increased concentrations of agarose prehydrolysates. One milliliter agarose prehydrolysates diluted by 2−100-fold were incubated with 60 nmol of Bgl1B at 30 °C for 24 h. When agarose prehydrolysate concentrations increased from 100-fold to 2-fold dilution, the final L-AHG titer obtained increased from 1.0 to 22.2 g/L (Figure 5b). However, the L-AHG yield significantly decreased (Figure 5b), and it is probably due to the fact that Bgl1B has a higher K_m value, in other words, a lower affinity to AB than to other disaccharides.[15] In addition, the activity of Bgl1B was inhibited by its substrate, AB, at a high concentration probably due to the substrate inhibition. The initial reaction velocity of Bgl1B increased until the AB concentration in agarose prehydrolysate increased to 34.6 g/L (Figure 5c). However, at an AB concentration of 86.5 g/L, the initial reaction velocity of Bgl1B decreased. These results indicate that the hydrolysis of AB by Bgl1B follows the typical substrate inhibition mode.[28] This is why Bgl1B did not completely hydrolyze AB in high-concentration agarose prehydrolysates (Figure S4).

In this study, this enzymatic process utilizing Bgl1B was combined with the prehydrolysis of agarose into AB by using phosphoric acid to eventually produce L-AHG and D-Gal from agarose by using a single enzyme. From the final post-reaction mixture containing L-AHG, D-Gal, 5-HMF, and AOSs, L-AHG can be separated and purified by using silica gel column chromatography based on the polarity differences between L-AHG and others.[6] This is the first report to simply and efficiently produce L-AHG via AB from agarose using a single enzyme. We suggest that this process concept and demonstration for the high L-AHG titer production are well worth if enzyme capable of more efficiently hydrolyzing AB is found and applied.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04144.

TLC analyses of agarose prehydrolysates; reaction product of agarose prehydrolysates, enzymatically hydrolyzed by Bgl1B; GC/TOF MS analysis of reaction products of AB in agarose prehydrolysate hydrolyzed by Bgl1B(PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: khekim@korea.ac.kr. Phone: +82-2-3290-3028.
*E-mail: ribozyme@korea.ac.kr.

ORCID

Kyoung Heon Kim: 0000-0003-4600-8668

Funding

This research was supported by the Basic Research Laboratory Program through the National Research Foundation of Korea funded by the MSIT (2018R1A4A1022589).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Facility support from the Institute of Biomedical and Food Safety at CJ Food Safety Hall, Korea University is acknowledged.

■ ABBREVIATIONS USED

AB, agarobiose; ABG, agarolytic β-galactosidase; α-NABH, α-neoagarobiose hydrolase; AOSs, agarooligosaccharides; D-Gal, D-galactose; DPs, degree of polymerization; GC/TOF MS, gas chromatography/time-of-flight mass spectrometry; 5-HMF, 5-hydroxymethylfurfural; HPLC, high-performance liquid chromatography; L-AHG, 3,6-anhydro-L-galactose; NAB, neoagarobiose; NAOSs, neoagarooligosaccharides; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography

■ REFERENCES

(1) Wei, N.; Quarterman, J.; Jin, Y.-S. Marine macroalgae: an untapped resource for producing fuels and chemicals. *Trends Biotechnol.* **2013**, *31*, 70−77.

(2) Glicksman, M. Utilization of seaweed hydrocolloids in the food industry. *Hydrobiologia* **1987**, *151-152*, 31−47.

(3) Lahaye, M.; Rochas, C. Chemical structure and physico-chemical properties of agar. *Hydrobiologia* **1991**, *221*, 137−148.

(4) Kloareg, B.; Quatrano, R. S. Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. *Oceanogr. Mar. Biol. Annu. Rev.* **1988**, *26*, 259−315.

(5) Araki, C.; Arai, K. Studies on the chemical constitution of agar-agar. XX. Isolation of a tetrasaccharide by enzymatic hydrolysis of agar-agar. *Bull. Chem. Soc. Jpn.* **1957**, *30*, 287−293.

(6) Yun, E. J.; Lee, S.; Kim, J. H.; Kim, B. B.; Kim, H. T.; Lee, S. H.; Pelton, J. G.; Kang, N. J.; Choi, I.-G.; Kim, K. H. Enzymatic production of 3,6-anhydro-L-galactose from agarose and its purification and in vitro skin whitening and anti-inflammatory activities. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 2961−2970.

(7) Yun, E. J.; Lee, A. R.; Kim, J. H.; Cho, K. M.; Kim, K. H. 3,6-Anhydro-L-galactose, a rare sugar from agar, a new anticariogenic sugar to replace xylitol. *Food Chem.* **2017**, *221*, 976−983.

(8) Park, J. H.; Hong, J. Y.; Jang, H. C.; Oh, S. G.; Kim, S. H.; Yoon, J. J.; Kim, Y. J. Use of *Gelidium amansii* as a promising resource for bioethanol: a practical approach for continuous dilute-acid hydrolysis and fermentation. *Bioresour. Technol.* **2012**, *108*, 83−88.

(9) Kim, C.; Kim, H. S.; Ryu, H. J.; Kim, S. H.; Yoon, J. J.; Kim, Y. J. Acidity tunable ionic liquids as catalysts for conversion of agar into mixed sugars. *Bull. Korean Chem. Soc.* **2010**, *31*, 511−514.

(10) Stevenson, T. T.; Furneaux, R. H. Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydr. Res.* **1991**, *210*, 277−298.

(11) Seo, Y. B.; Park, J.; Huh, I. Y.; Hong, S. -K.; Chang, Y. K. Agarose hydrolysis by two-stage enzymatic process and bioethanol production from the hydrolysate. *Process Biochem.* **2016**, *51*, 759−764.

(12) Kim, J. H.; Yun, E. J.; Seo, N.; Yu, S.; Kim, D. H.; Cho, K. M.; An, H. J.; Kim, J. H.; Choi, I.-G.; Kim, K. H. Enzymatic liquefaction of agarose above the sol−gel transition temperature using a thermostable endo-type β-agarase, Aga16B. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 1111−1120.

(13) Kim, H. T.; Lee, S.; Kim, K. H.; Choi, I.-G. The complete enzymatic saccharification of agarose and its application to simultaneous saccharification and fermentation of agarose for ethanol production. *Bioresour. Technol.* **2012**, *107*, 301−306.

(14) Lee, C. H.; Yun, E. J.; Kim, H. T.; Choi, I.-G.; Kim, K. H. Saccharification of agar using hydrothermal pretreatment and enzymes supplemented with agarolytic β-galactosidase. *Process Biochem.* **2015**, *50*, 1629−1633.

(15) Kim, D. H.; Kim, D. H.; Lee, S.-H.; Kim, K. H. A novel β-glucosidase from *Saccharophagus degradans* 2−40^T for the efficient

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256

hydrolysis of laminarin from brown macroalgae. *Biotechnol. Biofuels* **2018**, *11*, 64.
(16) Kim, H. T.; Yun, E. J.; Wang, D.; Chung, J. H.; Choi, I.-G.; Kim, K. H. High temperature and low acid pretreatment and agarase treatment of agarose for the production of sugar and ethanol from red seaweed biomass. *Bioresour. Technol.* **2013**, *136*, 582−587.
(17) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426−428.
(18) Kim, H. T.; Lee, S.; Lee, D.; Kim, H.-S.; Bang, W.-G.; Kim, K. H.; Choi, I.-G. Overexpression and molecular characterization of Aga50D from *Saccharophagus degradans* 2−40: an exo-type β-agarase producing neoagarobiose. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 227−234.
(19) Lee, S.-H.; Kim, S.; Kwon, M.-A.; Jung, Y. H.; Shin, Y.-A.; Kim, K. H. Atmospheric vs. anaerobic processing of metabolome samples for the metabolite profiling of a strict anaerobic bacterium, *Clostridium acetobutylicum*. *Biotechnol. Bioeng.* **2014**, *111*, 2528−2536.
(20) Stickel, J. J.; Knutsen, J. S.; Liberatore, M. W.; Luu, W.; Bousfield, D. W.; Klingenberg, D. J.; Scott, C. T.; Root, T. W.; Ehrhardt, M. R.; Monz, T. O. Rheology measurements of a biomass slurry: an inter-laboratory study. *Rheol. Acta* **2009**, *48*, 1005−1015.
(21) Modenbach, A. A.; Nokes, S. E. The use of high-solids loadings in biomass pretreatment—a review. *Biotechnol. Bioeng.* **2012**, *109*, 1430−1442.
(22) Viamajala, S.; McMillan, J. D.; Schell, D. J.; Elander, R. T. Rheology of corn stover slurries at high solids concentrations−Effects of saccharification and particle size. *Bioresour. Technol.* **2009**, *100*, 925−934.
(23) Zha, J.; Roggendorf, H. In *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*; Brinker, C. J., Scherer, G. W., Eds.; Academic Press: Boston, 1990.
(24) Medina-Esquivel, R.; Freile-Pelegrin, Y.; Quintana-Owen, P.; Yáñez-Limón, J. M.; Alvarado-Gil, J. J. Measurement of the sol−gel transition temperature in agar. *Int. J. Thermophys.* **2008**, *29*, 2036−2045.
(25) Yang, B.; Yu, G.; Zhao, X.; Jiao, G.; Ren, S.; Chai, W. Mechanism of mild acid hydrolysis of galactan polysaccharides with highly ordered disaccharide repeats leading to a complete series of exclusively odd-numbered oligosaccharides. *FEBS J.* **2009**, *276*, 2125−2137.
(26) Ha, S. C.; Lee, S.; Lee, J.; Kim, H. T.; Ko, H.-J.; Kim, K. H.; Choi, I.-G. Crystal structure of a key enzyme in the agarolytic pathway, α-neoagarobiose hydrolase from *Saccharophagus degradans* 2−40. *Biochem. Biophys. Res. Commun.* **2011**, *412*, 238−244.
(27) Lee, C. H.; Kim, H. T.; Yun, E. J.; Lee, A. R.; Kim, S. R.; Kim, J.-H.; Choi, I.-G.; Kim, K. H. A novel agarolytic β-galactosidase acts on agarooligosaccharides for complete hydrolysis of agarose into monomers. *Appl. Environ. Microbiol.* **2014**, *80*, 5965−5973.
(28) Reed, M. C.; Lieb, A.; Nijhout, H. F. The biological significance of substrate inhibition: a mechanism with diverse functions. *BioEssays* **2010**, *32*, 422−429.

DOI: 10.1021/acs.jafc.8b04144
J. Agric. Food Chem. 2018, 66, 12249−12256