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A simple method of preparing diverse neoagaro-oligosaccharides with β-agarase

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Abstract—In order to prepare pure and well-defined oligosaccharides from agarose in a rapid and simple manner, an enzymatic degradation method was developed, which includes degradation with either recombinant β-agarase (EC 3.2.1.81) AgaA or AgaB and gel permeation chromatography. Agarose was degraded with AgaA at the optimized conditions, yielding 47% and 45% of neoagarotetraose and neoagarohexaose, respectively. These neoagaro-oligosaccharides were conveniently separated by consecutive column chromatography on Bio-Gel P2 or P6 and were identified by FACE. The structure of these neoagaro-oligosaccharides was confirmed by MALDI-TOF MS and ^{13}C NMR spectroscopy.

Keywords: Agarose; Enzymatic hydrolysis; Neoagaro-oligosaccharides; Recombinant β-agarase; AgaA; AgaB

1. Introduction

Agarose, an important marine polysaccharide present in the cell walls of some red algae, is a linear chain composed of alternately arranged 3-O-linked β-D-galactopyranose and 4-O-linked 3,6-anhydro-α-L-galactopyranose residues.[1] Owing to its gelling ability, stabilizing properties, and high viscosity, agarose and its derivatives are widely used in food, cosmetics, and pharmaceutical industries. α-Agarase hydrolyzes α-(1→3) linkages of agarose, producing agaro-oligosaccharides, while β-agarase hydrolyzes β-(1→4) linkages, yielding neoagaro-oligosaccharides. Neoagaro-oligosaccharides inhibit the growth of bacteria and slow down the degradation of starch. As additives, they can reduce the caloric value of food. Neoagarobiose is a rare agent with moisturizing and whitening effects on melanoma cells.[2] Recently, the prebiotic effectiveness of neoagaro-oligosaccharides has been investigated both in vivo and in vitro.[3]

Agaro-oligosaccharides can be prepared conveniently through acid hydrolysis of agar. However, the hydrolysis product is not homogenous and the hydrolyzing reaction is not easily controllable. Therefore, acid hydrolysis is not appropriate for large-scale preparation of oligosaccharides with high purity. Enzymatic degradation of polysaccharides should be a promising alternative to acid hydrolysis; the enzyme degrades polysaccharides with high specificity and under mild conditions. So far, several types of β-agarase have been identified in *Pseudomonas*, *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, and *Bacillus*.[4–10] These β-agarases degrade agarose, usually generating neoagarobiose, neoagarotetraose, or neoagarohexaose as the main products. Recently, two novel β-agarase genes, *agaA* (GenBank access number: AY150179) and *agaB* (GenBank access number: AY293310), have been cloned from *Pseudoaltermonas* sp. CY24. AgaA is a new member of glycoside hydrolase family 16, and it degrades agarose to generate neoagarotetraose and neoagarohexaose as the main products. AgaB does not belong to any existing glycoside hydrolase family, and appears to represent a new family of glycoside hydrolases. AgaB decomposes agarose to generate mainly neoagaro-octaose and neoagarodecaose.[11]

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In this study, a simple method of preparing diverse neoagaro-oligosaccharides was developed, in which agarose was degraded with two recombinant β-agarases, AgaA and AgaB, yielding neoagarotetraose, neoagarohexaose, neoagaro-octaose, neoagarodecaose, and neoagarododecaose, which were then purified through gel-filtration chromatography and identified using fluorophore-assisted carbohydrate electrophoresis (FACE), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and ^{13}C NMR spectroscopy.

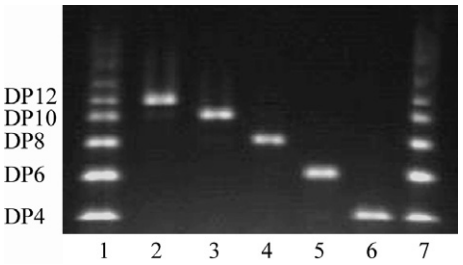

Figure 2. FACE analysis of the purified neoagaro-oligosaccharides. Lines 1 and 7, the ladder of neoagaro-oligosaccharides with different DP (4–14); Lanes 2–6, neoagaro-oligosaccharides with DP12, 10, 8, 6, and 4, respectively.

2. Results and discussion

In order to prepare pure and well-defined neoagaro-oligosaccharides, an orthogonal design was applied to optimize the enzymatic degradation conditions, which are as follows: agarose (0.2% in water) was hydrolyzed with recombinant β-agarase AgaA (15 U/100 mL) or AgaB (20 U/100 mL) at 30 °C for 12 h. Eighty-one percent and 90% of agarose was degraded into oligosaccharides by AgaA and AgaB, respectively, under these conditions. The remaining agarose was removed easily with centrifugation at 4 °C because the polymer will gel at low temperature. The supernatant of AgaA degradation was concentrated by rotary evaporation and then purified with a Bio-Gel P2 column with detection by phenol–sulfuric acid analysis.[12] As shown in Figure 1a, most of the products of AgaA degradation were neoagarohexaose (45%) and neoagarotetraose (47%). On the other hand, the products of AgaB degradation were mainly neoagaro-octaose (36.9%) and neoagarodecaose (32.0%) with a small amount of neoagarododecaose (16.7%), neoagarohexaose (9.7%), and neoagarotetraose (3.5%). These neoagaro-oligosaccharides were eluted as five peaks from a Bio-gel P6 column (Fig. 1b). They were collected and concentrated separately and purified again with P2 or P6 columns. More than 90% of the oligosaccharides were recovered after two chromatography steps. After the second gel-filtration, the purity of the neoagaro-oligosaccharides reached 99% as this was determined by FACE analysis (Fig. 2). In order to determine whether Bio-gel column chromatography can be used for the preparation of neoagaro-oligosaccharides on a large scale or not, the AgaB hydrolyzate of 2 g agarose was applied on an enlarged P6 column (95 cm × 4.5 cm). It was found that this column could purify diverse oligosaccharides with similar recovery rates and resolutions comparable to the smaller column.

MS and NMR spectroscopy were used for the structure determination of the different oligosaccharides. MALDI-TOF MS confirmed the molecular weights of 630, 936, 1242, 1548, and 1854 Da for neoagarotetraose, neoagarohexaose, neoagaro-octaose, neoagarodecaose, and neoagarododecaose, respectively. The ^{13}C NMR spectrum of neoagarohexaose showed the typical pattern for neoagaro-oligosaccharides (Fig. 3). Resonances at about 97 and 93 ppm were characteristic of β and α anomeric forms of galactose residues at the reducing end of the neoagaro-oligosaccharides, respectively.[13]

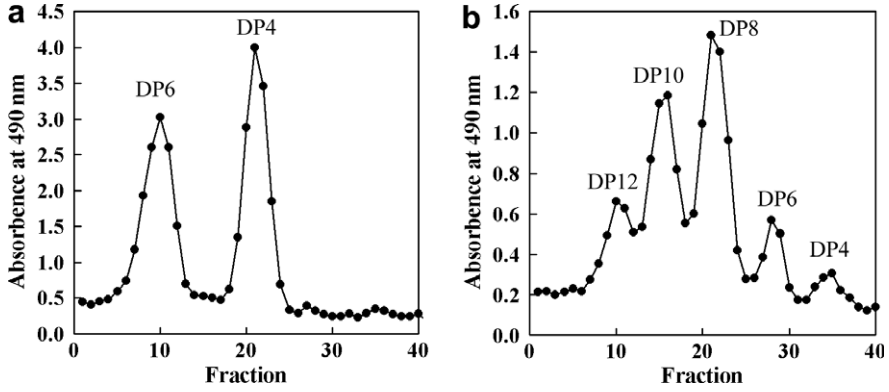

Figure 1. Gel-filtration on Bio-gel P2 or P6 of the neoagaro-oligosaccharides derived from crude agarase degradation. (a) Neoagaro-oligosaccharides derived from AgaA degradation (separated with a P2 column). DP6, neoagarohexaose, DP4, neoagarotetraose; (b) neoagaro-oligosaccharides derived from AgaB degradation (separated with a P6 column). DP12, neoagarododecaose, DP10, neoagarodecaose, and DP8, neoagaro-octaose.

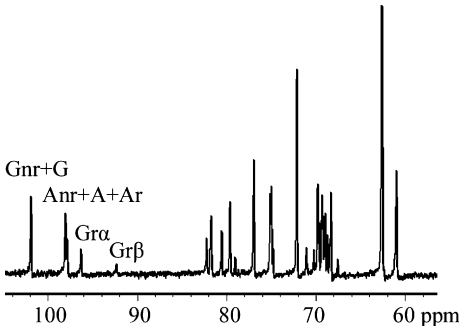

Figure 3. ^{13}C NMR spectrum of neoagarohexaose prepared with AgaA from agarose. Resonances at about 97 and 93 ppm correspond to neoagaro-oligosaccharides. G represents the 3-O-linked β-D-galactopyranose; A represents the 4-O-linked 3,6-anhydro-α-L-galactopyranose; r and nr denote residues at the reducing and non-reducing end, respectively; α/β for anomer.

The signal at 90.72 ppm, characteristic of agaro-oligosaccharides, the main products of α-agarase degradation of agarose was not found.[14] The spectra for the other neoagaro-oligosaccharides showed similar patterns, but with different intensities (data not shown). Therefore, the obtained neoagaro-oligosaccharides had their structure maintained during the various steps of preparation.

The two new β-agarases, AgaA and AgaB, are highly active and specific. Furthermore, gel-filtration can separate neoagaro-oligosaccharides with a good resolution. These advantages allowed us to successfully prepare five neoagaro-oligosaccharides. FACE is a useful tool for carbohydrate analysis.[15] To our knowledge, it is the first time that FACE is used in the analysis of neutral neoagaro-oligosaccharides.

The entire procedure including pre-cultivation of the β-agarases producing strain can be completed in one week. The real working time is less than 30 h since most of the steps, such as degradation, chromatography, and lyophilization, do not require constant attention. Preparative scales over 10 mg can be easily reached in one experiment and each step of the method can be scaled up to some degree. The establishment of this simple and fast method will open a new avenue for the research on structure–function relationship of neoagaro-oligosaccharides and their industrial preparation.

3. Experimental

3.1. Preparation of recombinant β-agarase

We ourselves prepared the recombinant β-agarases, AgaA and AgaB. Briefly, *Escherichia coli* BL21 (DE3) carrying the AgaA or AgaB encoding gene in the plasmid pET24a (+), respectively, were cultivated. One liter of cell free supernatant was mixed with solid ammonium sulfate till 70% saturation was reached. The mixture was stirred at 4 °C for 1 h and centrifuged at 12,000*g* and 4 °C for 20 min. The precipitate was dissolved in 50 mL 50 mmol/L phosphate buffer (pH 7.0) and the crude enzyme was used in agarose degradation. Agarase activity was determined by measuring the increase in the concentration of reducing sugar as described by Von Borel et al.[16] One unit (U) of agarase activity was defined as the amount of enzyme that released 1 μmol of D-galactose per minute.

3.2. Preparation of neoagarosaccharides

Agarose (200 mg) was dissolved in 100 mL hot water (99 °C). The soln was cooled down in a 40 °C water bath, mixed with 0.1 mL crude agarase and incubated at 30 °C and 150 rpm for 12 h. The hydrolytic reaction was stopped by heating the mixture in a boiling water bath for 10 min. After being cooled down to 4 °C, the hydrolyzate was centrifuged at 12,000*g* and 4 °C for 10 min with the supernatant concentrated to 2 mL through rotary evaporation at 45 °C.

3.3. Purification of neoagarosaccharides

The neoagaro-oligosaccharides solns (2 mL) were loaded onto a Bio-gel P2 or P6 column (fine, 95 cm × 1.5 cm, Bio-Rad Laboratories, USA). NH_4HCO_3 (0.5 mol/L) was used as eluent at a flow rate of 7 mL/h. Detection used the classical phenol–H_2SO_4 method. Fractions (1.7 mL) were collected. The salt (NH_4HCO_3) was removed by repeated evaporation under diminished pressure at 50 °C. After a second column chromatography using the same conditions, the recovered neoagaro-oligosaccharides were lyophilized.

3.4. FACE

The oligosaccharide was modified by mixing 5 μL carbohydrate (10 μg) with 5 μL 0.2 mol/L AGA (dissolved in 3:17 AcOH–water) and 5 μL 1 mol/L $NaCNBH_3$ followed by incubation at 45 °C overnight (>8 h) and then vortexing and brief centrifugation. The modified labeled oligosaccharide was mixed with an equal vol of 50% sucrose and separated on polyacrylamide gel buffered with the discontinuous Tris–glycine/Tris–HCl system. Electrophoresis was performed at 300 V for 120 min using a Hofer miniVE apparatus (Amersham Phamarcia Biotech, Sweden). The gel was photographed and analyzed with a Bio Imaging System (Syngene, Cambridge, UK).

3.5. MALDI-TOF mass spectrometry

It was performed on a Biflex III equipment (Bruker Daltonics, Inc., Billerica, MA) set in positive-ion mode with 2,5-dihydroxybenzoic acid (DHB) as the matrix. All detected oligosaccharide ions were observed as pseudomolecular ions, $[M+Na]^+$ and $[M+K]^+$.

3.6. ^{13}C NMR spectroscopy

Samples (20 mg) were dissolved in D_2O (0.5 mL) in 5 mm NMR tubes. Spectra were recorded on a JNM-ECP600 (JEOL, Japan) apparatus set at 150 MHz with 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as the internal standard.

Acknowledgments

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References

1. Kloareg, B.; Quatrano, R. S. *Oceanogr. Mar. Biol. Annu. Rev.* **1988**, *26*, 259–315.
2. Kobayasbi, R.; Takisada, M.; Suzuki, T.; Kirimura, K.; Usami, S. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 162–163.
3. Hu, B.; Gong, Q. H.; Wang, Y.; Ma, Y. M.; Li, J. B.; Yu, W. G. *Anaerobe* **2006**, *12*, 260–266.
4. Groleau, D.; Yaphe, W. *Can. J. Microbiol.* **1977**, *23*, 672–679.
5. Potin, P.; Richard, C.; Rochas, C.; Kloareg, B. *Eur. J. Biochem.* **1993**, *214*, 599–607.
6. Leon, O.; Quintana, L.; Peruzzo, G.; Slese, J. C. *Appl. Environ. Microb.* **1992**, *58*, 4060–4063.
7. Vera, J.; Alvarez, R.; Murno, E.; Slebe, J. C.; Leon, O. *Appl. Environ. Microb.* **1998**, *64*, 4378–4383.
8. Sugano, Y.; Terada, I.; Arita, M.; Noma, M.; Matsumoto, T. *Appl. Environ. Microb.* **1993**, *59*, 1549–1554.
9. Araki, T.; Hayakawa, M.; Lu, Z.; Kaita, S.; Morishita, T. *J. Marine Biotechnol.* **1998**, *6*, 260–265.
10. Kim, B. J.; Kim, H. J.; Ha, S. D.; Hwang, S. H.; Byun, D. S.; Lee, T. H.; Kong, J. Y. *Biotechnol. Lett.* **1999**, *21*, 1011–1105.
11. Ma, C.; Lu, X.; Shi, C.; Li, J.; Gu, Y.; Ma, Y.; Chu, Y.; Han, F.; Gong, Q.; Yu, W. *J. Biol. Chem.* **2006**, *282*, 3747–3754.
12. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1965**, *28*, 350–356.
13. Rochas, C.; Lahaye, M.; Yaphe, W.; Phan Viet, M. T. *Carbohydr. Res.* **1986**, *148*, 199–207.
14. Phaye, M.; Yaphe, W.; Phan Viet, M. T.; Rochas, C. *Carbohydr. Res.* **1989**, *190*, 249–265.
15. Jackson, P. *Biochem. J.* **1990**, *270*, 705–713.
16. Von Borel, E.; Hostettler, F.; Deuel, H. *Helv. Chim. Acta* **1952**, *35*, 115–120.