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Preparation, purification and characterization of alginate oligosaccharides degraded by alginate lyase from *Pseudomonas* sp. HZJ 216

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ABSTRACT

Alginate lyase which was purified from the fermentation solution of marine bacteria *Pseudomonas* sp. HJZ216 was applied to hydrolyze algae alginate. Six oligosaccharides, including di- and trisaccharides, were isolated and purified through anion exchange chromatography. The oligosaccharide structures were elucidated based on electrospray ionization-mass spectrometry (ESI-MS) and 2D NMR spectra analysis.

1. Introduction

Alginate is a kind of acidic linear polysaccharide which consists of α-L-guluronate and its C-5 epimer, β-D-mannuronate, linking with 1,4-*O*-glycosidic bonds. It usually exists in three different ways: poly-α-L-guluronate (pG), poly-β-D-mannuronate (pM), and heteropolymeric regions (pMG). This polymer is produced by brown seaweed such as *Laminaria* or some bacteria belonging to the genera *Azotobacter* and *Pseudomonas*.[1,2] Alginate oligosaccharide and their derivatives have been widely used as releasing agents in pharmacy,[3] and additives in food industry.[4] Recently, alginate oligosaccharide and their derivatives have been attracting considerable attention due to their bioactivity of antitumor and promoting plant growth.[5–9] Identification of oligosaccharides derived from pM, pG, or pMG and their sequence determination are important for molecular understanding of structure-function relationships of alginate oligosaccharides. High resolution of ^{1}H and ^{13}C using NMR techniques are primary, rapid, and efficient technology for determining the structure of oligosaccharide and sequence determination of polysaccharides.[10–15]

Here, we used extracellular endo-alginate lyase from *Pseudomonas* sp. HZJ216 to hydrolyze algae alginate, and the oligosaccharide fractions were separated by anion exchange chromatography. Six alginate-derived oligosaccharides, namely DM, DMM, DGM, DG, DGG, and DMG were obtained. All the structures were determined using ESI-MS, ^{1}H, ^{13}C NMR, ^{1}H–^{1}H COSY, HMBC, and HMQC techniques.

2. Results and discussion

The algae alginate was degraded by alginate lyase from *Pseudomonas* sp. HZJ216 for 6 h. Oligosaccharide mixture A (OMA) and oligosaccharide mixture B (OMB) collected by acid adjustment and ethanol precipitation were further separated on Q-Sepharose F.F. column. The separation profile (Fig. 1A) of OMA showed three major oligosaccharide fractions based on both UV absorption and reducing sugar analysis, marked as **1**, **2,** and **3**, respectively. The separation profile (Fig. 1B) of OMB also showed three fractions and marked as **4**, **5,** and **6**. The ESI-MS spectrogram of sample **1** showed *175* and *351* corresponding to the molecular ion [M/2−1] and [M−1]. Therefore, the molecular mass of sample **1** was 352. Analyzed in the same way, ESI-MS spectra gave the molecular mass 352, 528, 352, 528, and 528 corresponding to sample **2**, **3**, **4**, **5,** and **6**, indicating disaccharide (352) and trisaccharide (528), respectively. (Data not shown.)

The enzymatic hydrolyzed oligosaccharides ended with 4-deoxy-*erythro*-hex-4-enopyranosyluronate group at the non-reducing ends.[11] In order to express the structure conveniently, 'D' was used to represent the non-reducing end residue, 'M' stood for middle residue near non-reducing end and 'R' represented for reducing end residue.

^{1}H–^{1}H COSY, HMBC and HMQC spectra of sample **1** are shown in Figures 2–4, respectively. Through ^{1}H–^{1}H COSY and HMQC analyses, all proton and carbon signals of sample **1** can be assigned.

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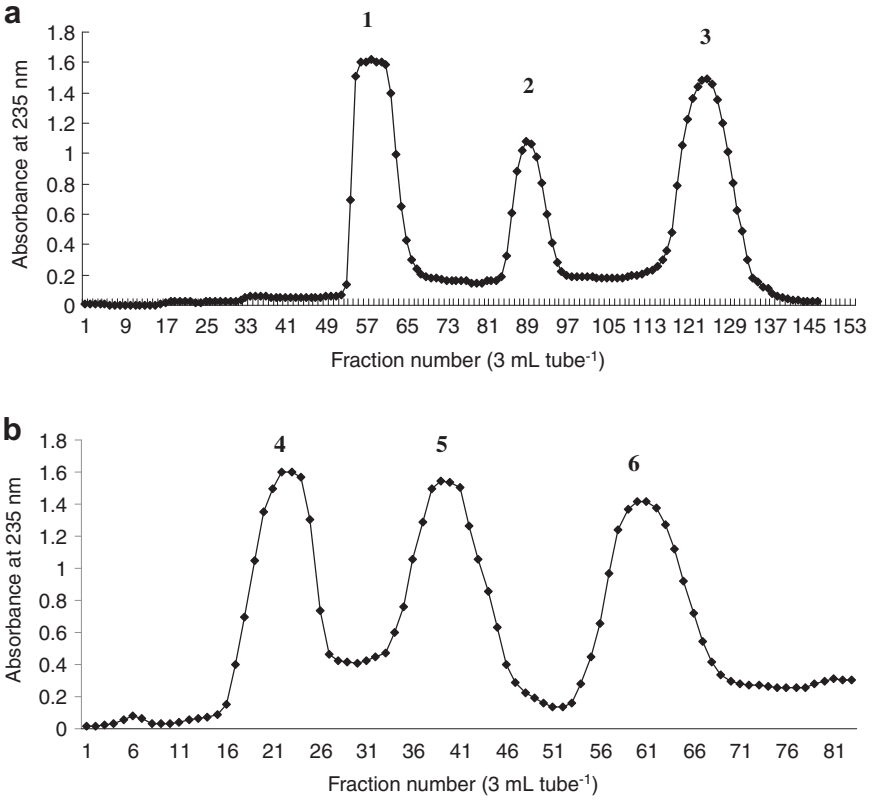

Figure 1. Separation graphs of alginate oligosaccharides OMA (A) and OMB (B) on a Q-Sepharose Fast Flow column. A: Gradient elution (0.2–1.2 M NaAc) was performed at a flow rate of 1.0 mL/min with detection at 235 nm and fractions were then detected by DNS method. 0.5 g of each sample was loaded on each column; B. Condition was same as A except that the gradient elution of NaAc was from 0.2 to 1.0 M.

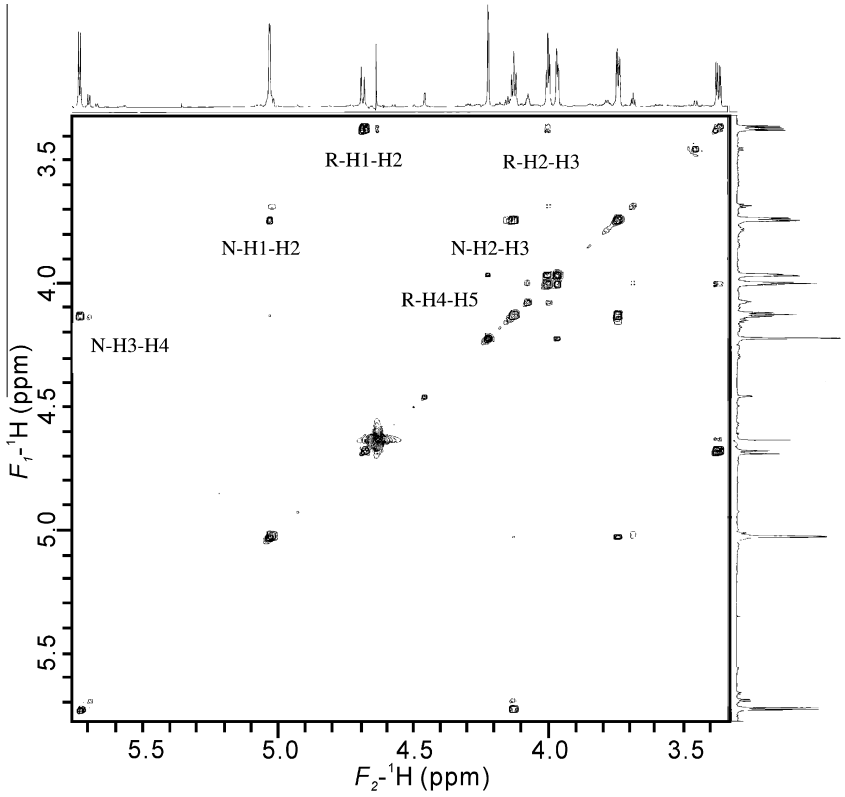

Figure 2. ^{1}H–^{1}H COSY spectrum of sample **1**. N, Non-reducing end; R, reducing end; Numbers are the positions in pyranosyluronic acids.

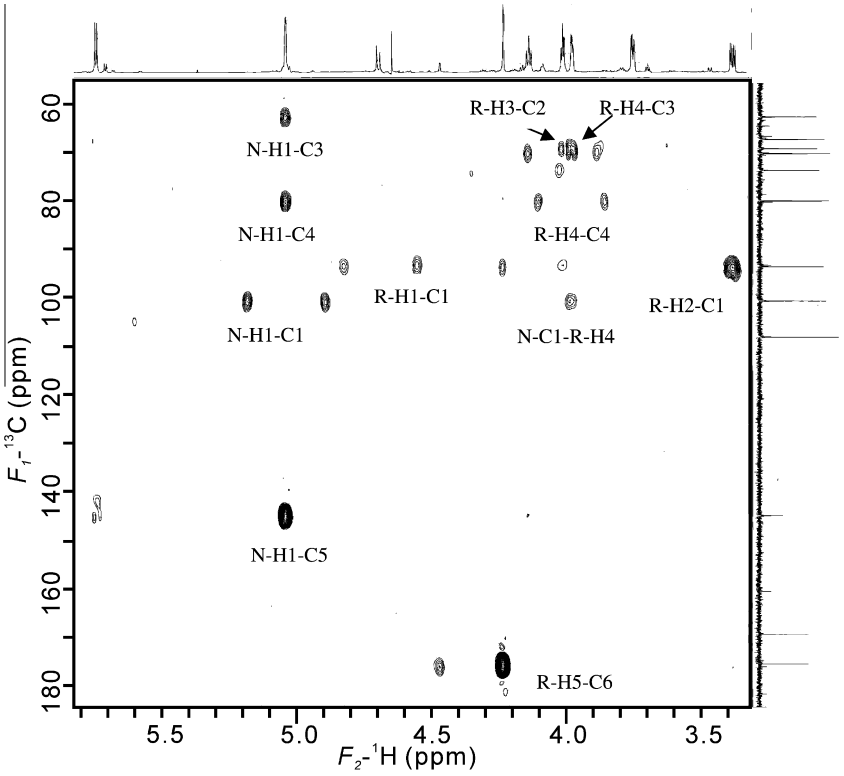

Figure 3. HMBC spectrum of sample **1**. N, non-reducing end; R, reducing end; numbers are the positions in pyranosyluronic acids.

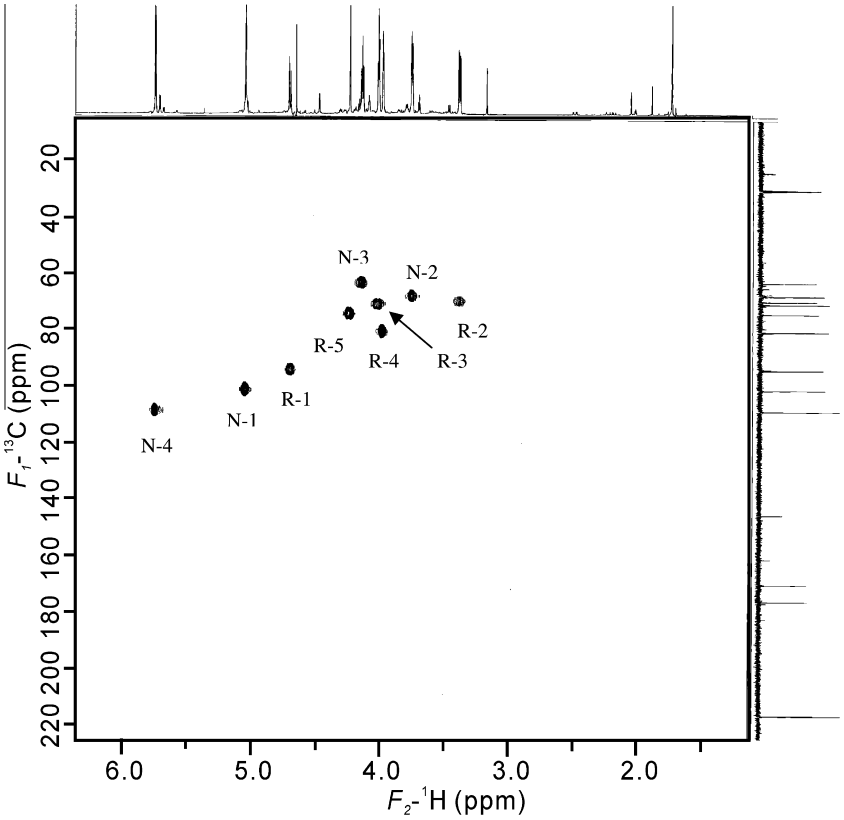

Figure 4. HMQC spectrum of sample **1**. N, Non-reducing end; R, reducing end; numbers are the positions of pyranosyluronic acids.

The chemical shifts of H-4 (5.76 ppm) and C-4 (108.90 ppm) of the non-reducing end were found in down-field in ^{1}H NMR and ^{13}C NMR spectra, while the non-reducing end H-1 and reducing end anomeric carbon (C-1) of mannuronic acid residues appeared at

4.72 and 93.56 ppm, respectively. Thus, sample **1** was determined as (4-deoxy-α-L-erythro-hex-4-enopyranosyluronate)-(1→4)-(β-D-mannopyranosyluronate) (DM). All ^{1}H and ^{13}C NMR chemical shifts of DM are listed in Table 1.

The ^{1}H and ^{13}C NMR data of sample **2** and **3** are also listed in Table 1. ^{1}H–^{1}H COSY, HMBC, and HMQC spectra of sample **2** are shown in Figures 5–7, respectively. For sample **2**, the non-reducing end H-4 appeared at 5.72 ppm, while the characteristic peaks for homo-mannuronic acids H-1s (H-1^{N}, H-1^{M}, H-1^{R}) appeared at 5.05, 4.87, and 4.74 ppm, respectively. The chemical shifts of C-4 related to H-4^{N}, H-4^{M}, and H-4^{R} were observed at 107.95, 79.83, and 80.64 ppm in the HMQC spectra, respectively. All carbon and proton signals from the COSY, HMBC, and HMQC spectra supported the following structure: (4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate)-(1→4)-(β-D-mannopyranosyluronate)-(1→4)-(β-D-mannopyranosyluro-nate) (DMM). For sample **3**, three H-1s (H-1^{N} at 5.05 ppm, H-1^{M} at 4.94 ppm, and H-1^{R} at 4.51 ppm), non-reducing end H-4 at 5.59 ppm and carbon and proton signals from HMQC spectra supported the following structure: (4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate)-(1→4)-(α-L-gulopyranosyluronate)-(1→4)-(β-D-mannopyranosyluronate) (DGM). (Spectra not shown.)

Analyzed in the same way, samples **4**, **5,** and **6** were identified as DG, DGG, and DMG, respectively. The data are presented in Table 2. These results are in accordance with the previous studies reported by Liu et al.[11] and Zhang et al.[12] (Spectra not shown.)

In this study, six oligosaccharides, including DM, DMM, and DGM from oligosaccharide mixture A (OMA), DG, DGG, and DMG from oligosaccharide mixture B (OMB), were purified. Based on the fact that they were all the di- and trioligosaccharides which can be derived from alginate, we can deduce that the enzymatic hydrolysis occurred between two random guluronic acid (G) or/and mannuronic acid (M), and produced one G residue or M residue on reducing end and an unsaturated residue named 'D' mentioned above on non-reducing end for all products. It suggests that the alginate lyase isolated from *Pseudomonas* sp. HZJ216 could hydrolyze the alginate thoroughly into disaccharides and trisaccharides.

Table 1
^{1}H and ^{13}C NMR chemical shifts of DM, DMM and DGM isolated from OMA

Compound	Terminal	^{13}C NMR, δ					^{1}H NMR, δ				
		C-1	C-2	C-3	C-4	C-5	H-1	H-2	H-3	H-4	H-5
DM	R (M)	93.56	69.21	70.23	79.98	73.68	4.72	3.41	4.04	4.01	4.26
	N (D)	100.63	67.29	62.64	108.09	144.87	5.06	3.78	4.17	5.76	–
DMM	R (M)	93.47	69.31	70.18	80.64	73.65	4.74	3.47	3.97	3.86	4.27
	M (M)	101.07	64.70	68.91	79.83	67.21	4.87	3.73	3.96	4.09	4.32
	N (D)	100.53	67.05	62.66	107.95	144.83	5.05	3.78	4.20	5.72	–
DGM	R (M)	99.60	71.19	70.24	78.19	75.62	4.51	3.61	3.87	3.80	3.62
	M (G)	99.89	69.86	68.76	72.47	77.75	4.94	3.79	3.84	3.98	3.83
	N (D)	93.38	66.46	63.39	107.34	144.98	5.05	3.77	4.28	5.59	–

OMA: oligosaccharide mixture A; N, non-reducing end; M, the middle residue near the non-reducing end; R, reducing end.

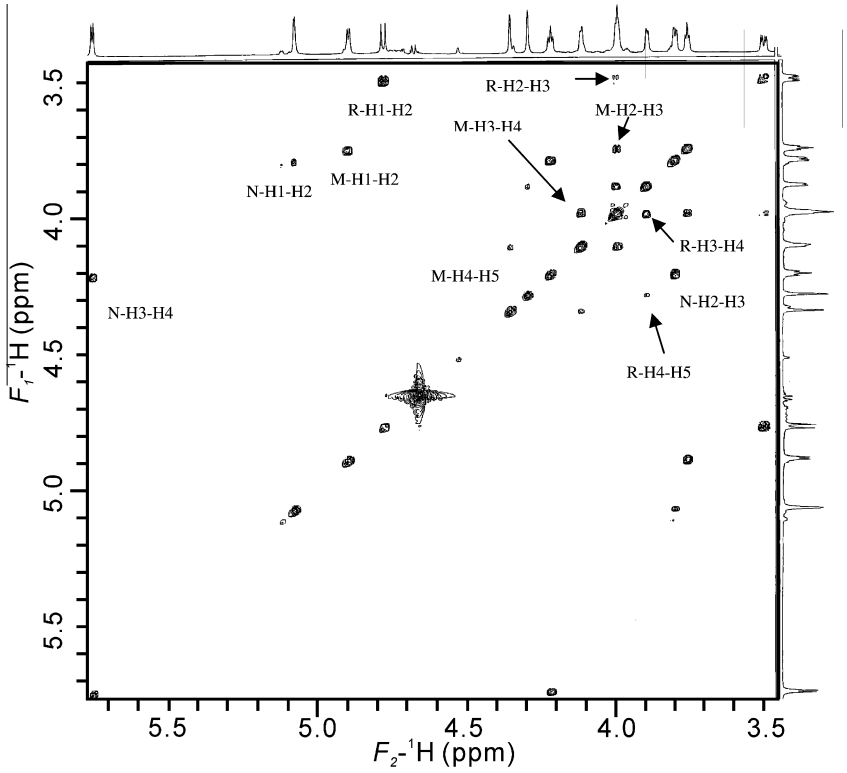

Figure 5. ^{1}H–^{1}H COSY spectrum of sample **2**. N, Non-reducing end; M, the middle residue near the non-reducing end; R, reducing end; the numbers are the positions in pyranosyluronic acids.

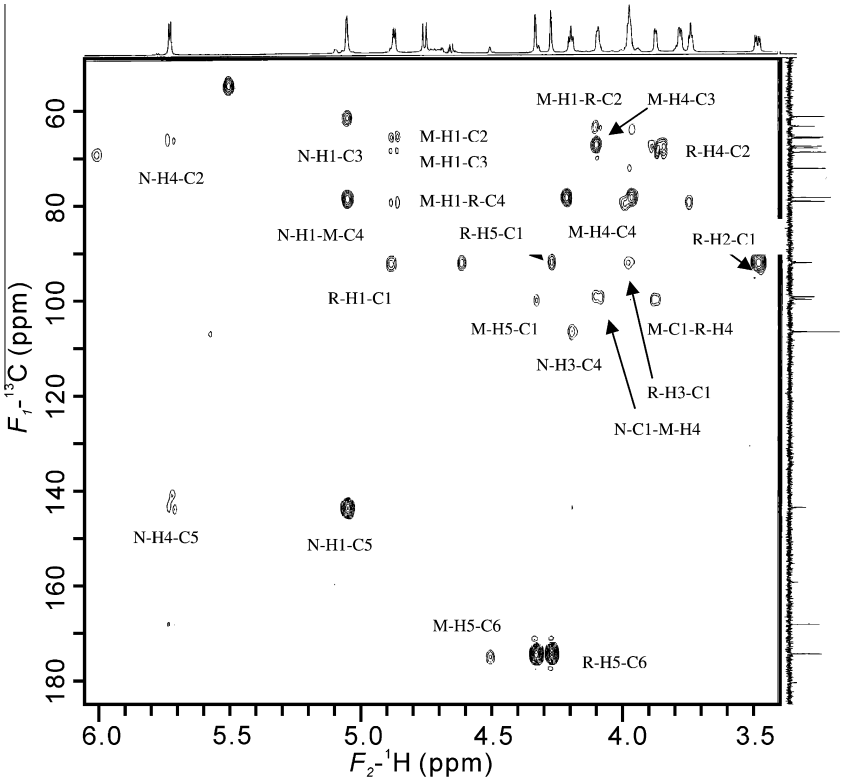

Figure 6. HMBC spectrum of sample **2**. N, Non-reducing end; M, the middle residue near the non-reducing end; R, reducing end; the numbers are the positions in pyranosyluronic acids.

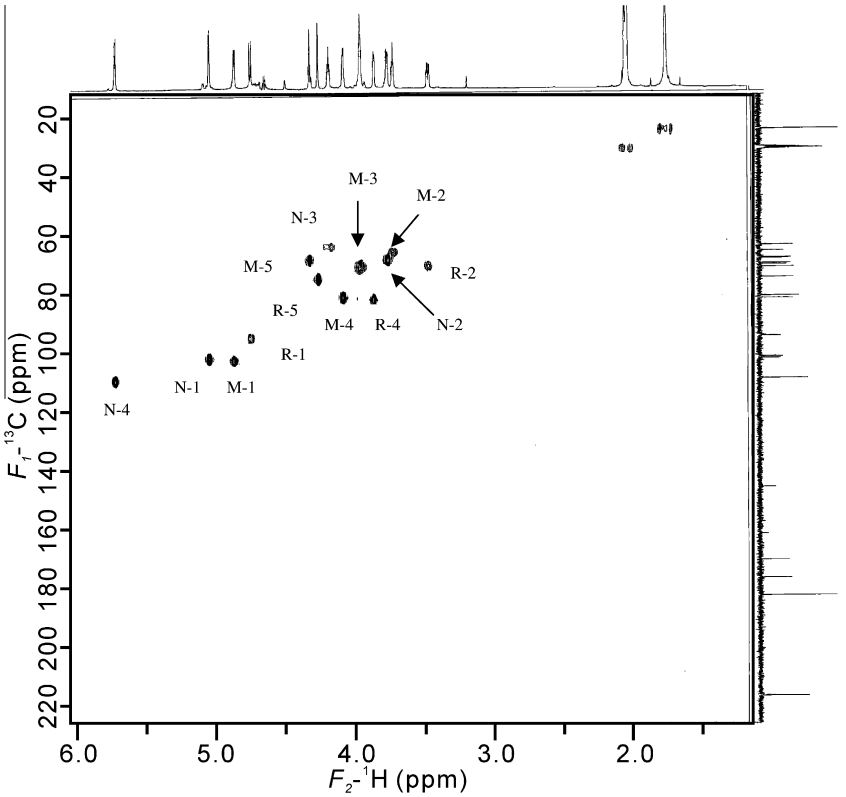

Figure 7. HMQC spectrum of sample **2**. N, Non-reducing end; M, the middle residue near the non-reducing end; R, reducing end; the numbers are the positions in pyranosyluronic acids.

Table 2
^{1}H and ^{13}C NMR chemical shifts of DG, DGG and DMG isolated from OMB

Compound	Terminal	^{13}C NMR, δ					^{1}H NMR, δ				
		C-1	C-2	C-3	C-4	C-5	H-1	H-2	H-3	H-4	H-5
DG	R (G)	93.36	71.50	70.11	79.60	73.43	4.82	3.50	4.10	4.05	4.33
	N (D)	100.20	67.11	62.39	107.89	144.55	5.18	3.89	4.26	5.88	—
DGG	R (G)	93.39	70.11	69.19	80.58	73.60	4.80	3.52	4.01	3.91	4.38
	M (G)	101.05	64.63	68.85	79.70	67.17	4.98	3.80	4.251	4.19	4.44
	N (D)	100.50	66.89	62.60	107.88	144.76	5.16	3.78	4.29	5.83	—
DMG	R (G)	93.15	68.78	70.22	80.09	73.68	4.68	3.50	4.11	3.94	4.21
	M (M)	101.29	70.45	71.12	76.02	78.26	4.51	3.81	3.52	3.75	3.55
	N (D)	100.13	66.60	63.67	107.52	145.01	4.94	3.77	4.28	5.57	—

OMB: oligosaccharide mixture B; N, non-reducing end; M, the middle residue near the non-reducing end; R, reducing end.

On the other hand, DM, DMM, and DGM from OMA ended with M units on reducing end, while those from OMB ended with G units. The different reducing ends may lead to different properties for these oligosaccharides. Firstly, OMA and OMB were separated from final hydrolysate by acid adjustment at pH 2.85 according to protocol provided by Haug et al.[16] Secondly, OMA was collected by five times volume of ethanol, while for OMB it just needed three times volume of ethanol. The mechanism of ethanol precipitation was described before that it could decrease the polarity and solubility of oligosaccharide and causes it to precipitate.[17] Thus, the polarity of the oligosaccharides from OMA was possibly much higher than that from OMB. Finally, the monomeric oligosaccharide peaks of OMA (Fig. 1A) delayed comparing with those of OMB (Fig. 1B). It indicates that the oligosaccharides ended with M unit may need a higher ionic strength for separation.

3. Experimental section

3.1. Materials

Sodium alginate from brown seaweed *Laminaria* (viscosity, 500 cps grade) was provided from Qingdao Bright Moon Seaweed Group Co. Ltd (Qingdao, China). The ratio of M/G applied in this study was 2.28. Q-Sepharose Fast Flow was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and Sephadex G-10 was obtained from Amersham Biosciences (Uppsala, Sweden). D_2O was presented from School of medicine and pharmaceutics, Ocean University of China. The other reagents are G.R. or A.R.

3.2. Alginate lyase

The enzyme was purified from the fermenting solution of a marine bacterium *Pseudomonas* sp. HZJ216 and verified to be a mixture of three monomeric alginate lyases with molecular masses of 60.25, 36, and 23 kDa. The activity of the enzyme was measured by monitoring the increase of absorbance at 235 nm.[18] One unit of enzyme activity was defined as an increase of 0.1 in absorbance of the reaction mixture at 235 nm per min.

3.3. Preparation of unsaturated alginate oligosaccharides

Alginate (5 g) was dissolved in 500 mL of 50 mmol L^{-1} Tris–HCl buffer (pH 7.0), by adding 200 units alginate lyase. The reaction was carried out at 30 °C for 6 h until the absorption level at 235 nm was not changed, and then the solution was heated by boiled water for 5 min to stop the enzymatic reaction. After the enzyme-degraded solution had been filtered, the pH of the supernatant was adjusted to 2.85 to separate alginate oligosaccharides preliminarily. The supernatant was collected by ethanol and marked as oligosaccharide mixture A (OMA). The pellet named as oligosaccharide mixture B (OMB) was dissolved in deionization water and then collected by ethanol. OMA and OMB were stored at 4 °C.

3.4. Anion-exchange chromatography

The oligosaccharide mixture A (OMA) was dissolved with 0.2 M NaAc buffer (pH 8.10) and filtered through 0.45 μm filter membrane. Two milliliters of oligosaccharide solution (0.25 mg mL^{-1}) was loaded into Q-Sepharose F.F. column (2 cm × 35 cm, Pharmacia), which previously equilibrated with 0.2 M NaAc. The gradient buffer (0.2–1.2 M NaAc) with a flow-rate of 1.0 mL min^{-1} was used for elution of sample. The separation was carried out at room temperature and each fraction was collected using a fraction collector. The oligosaccharide mixture B (OMB) was isolated and purified under the condition mentioned above except that the gradient elution was from 0.2 to 1.0 M NaAc.

Since there is a maximum absorbance at 220 nm for NaAc which could affect the detection of oligosaccharide at 235 nm, the uronic acid was measured by UV detection (UV-752 spectrometer) at 235 nm for non-reducing end and DNS (3,5-dinitrisalicylic acid) method[19] for reducing end to make sure that the fractions were unsaturated oligosaccharide. For the latter, the reducing sugar was measured at 520 nm.

3.5. Desalting

Each fraction was concentrated using rotary evaporator, freeze-dried, and desalted using a Sephadex G-10 column (10 × 1000 mm) and finally eluted with double-distilled water.

3.6. Electrospray-ionization mass spectroscopy (ESI-MS)

Electrospray-ionization mass spectroscopy (TSQ LC/MS/MS, Finnegan Company) was applied for determination of the molecular mass of each oligosaccharide. Negative-ionization mode was considered to get the mass spectra. The sample was dissolved in 1:1 MeOH–H_2O (10 pmol mL^{-1}), and was delivered to the electrospray source using a syringe pump at a flow rate of 5 μL min^{-1}. The mass scans range was from 100 to 1100 Da. The capillary temperature was kept at 250 °C, and nitrogen was used as nebulizing and desolvation gas.

3.7. NMR spectroscopy

The pure oligosaccharide samples (30 mg) from sample **1** to **6** were dissolved in 1 mL of D_2O (99.96%), respectively, for NMR analysis at ambient temperature. All NMR analyses data, such as ^{1}H, ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, and HMBC were performed on JEOL ECP 600 MHZ spectrometer at 298 K. Acetone was used as the internal standard (δ_H = 2.10 ppm, δ_C = 31.45 ppm and 216 ppm).

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