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Preparation and structure elucidation of alginate oligosaccharides degraded by alginate lyase from *Vibro* sp. 510

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Abstract—Alginate that was purified from the fermentation solution of marine bacteria *Vibro* sp. 510 under specific reaction conditions was hydrolyzed by alginate lyase. Seven oligosaccharides, including di-, tri- and tetrasaccharides, were isolated through low-pressure, gel-permeation chromatography (LP-GPC) and semipreparative strong-anion exchange (SAX) fast-protein liquid chromatography (FPLC). The oligosaccharide structures were elucidated based on ESIMS and 2D NMR spectral analysis. The hydrolytic specificity of this alginate lyase to alginate is discussed.

Keywords: Alginate lyase; Guluronic acid; Mannuronic acid; Alginate; Oligosaccharides

1. Introduction

Alginate, a water-soluble linear polymer from brown algae, is composed of (1→4)-β-D-mannuronic acid (M) and α-L-guluronic acid (G) units in the form of a homopolymeric (MM- or GG-blocks) and heteropolymeric sequences (MG- or GM-blocks).[1,2] Owning to its gelling ability, stabilizing properties and high viscosity, alginate and derivatives are widely used in the food, cosmetics and pharmaceutical industries.[3,4] Recent studies have revealed that alginate hydrolysates and their derivatives exhibit many important bioactivities, such as stimulating human keratinocytes,[5] accelerating plant root growth[6] and enhancing penicillin production from cultures of *Penicillium chrysogenum*.[7] Enzymatically depolymerized alginate oligosaccharides can cause cytotoxic cytokine production in human mononuclear cells.[8] PSS (propylene glycol alginate sulfate sodium) and PGMS (propylene glycol mannuronate sulfate), which are two kinds of low-molecular-weight alginate derivatives, showed antioxidation and prevention of cardiovascular and cerebrovascular diseases.[9,10]

A number of alginate lyases from decayed brown algae, mollusks and bacteria have been described.[11,12] These lyases usually hydrolyze alginate by β-elimination, and the mechanism is similar to that of alkaline degradation to glycuronans.[13,14] Technically, gel-permeation chromatography, strong-anion exchange chromatography, ESIMS and 2D NMR spectral analysis have been successfully used in the sequential structure determination of alginate-derived oligosaccharides.[15–21] In this paper, we use extracellular endoalginate lyase from *Vibro* sp. 510 to hydrolyze alginate, and the oligosaccharide fractions were separated on BioGel P4 column. The uniform-sized oligosaccharides with different M/G ratios were further isolated by SAX-FPLC, and seven alginate-derived oligosaccharides, namely ΔG, ΔGG, ΔMG, ΔGGG, ΔMGG, ΔGMG and ΔMMM, were obtained.[10,15,22] All the structures were elucidated using ESIMS, ^{1}H, ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC and HMBC techniques.

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2. Results and discussion

The alginate was hydrolyzed by alginate lyase of *Vibro* sp. 510 for 24 h, and a higher-molecular-weight component was precipitated by adding ethanol to the extracts. The supernatant containing alginate-derived oligosaccharides (account for ~28%) was evaporated off and separated on a BioGel P4 column. The separation profile (Fig. 1) of the oligosaccharides showed four major fractions based on UV absorption and uronic acid analysis, marked as Fr I, Fr II, Fr III and Fr IV in yields of 40%, 45%, 13% and 2%, respectively. ESIMS gave 352.5, 528.5, 704.8 and 881.2 corresponding to Fr I, Fr II, Fr III and Fr IV (Table 1), indicating di-, tri-, tetra- and pentasaccharide fragments, respectively.

The uniform sized fractions of Fr I, Fr II and Fr III were further separated by SAX-FPLC. Fr I showed one symmetric peak (compound **1**), but Fr II and Fr III showed two (compounds **2** and **3**) and four (compounds **4**–**7**) peaks. Each fraction was then purified by semi-preparative SAX-FPLC using a gradient elution program. Seven pure oligosaccharides (compounds **1**–**7**) were thus obtained. Their precise structures were further determined from their 2D NMR spectra. The structure of Fr IV was not established due to the limited amount of this sample.

Through ^{1}H–^{1}H COSY and HMQC analysis, all proton and carbon signals of **1** can be assigned. The chemical shifts of H-4^{II} (5.92 ppm) and C-4^{II} (110.96 ppm) of the nonreducing end were found in down-field in ^{1}H NMR and ^{13}C NMR spectra, while the nonreducing end H-1^{II} (α), reducing end H-1^{I} (β) and reducing end anomeric carbon (C-1^{I}) of guluronic acid residues appeared at 5.21, 4.87 and 96.04 ppm, respectively. Thus, **1** was determined as: 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate-(1 → 4)-α-L-gulopyranosyluronate (ΔG). All ^{1}H and ^{13}C NMR data are listed in Table 2.

Table 1. Selected ESIMS data of alginate-derived oligosaccharides

Parent ion	Fr I (dp 2) $[M-H]^{-1}$	Fr II (dp 3) $[M-H]^{-1}$	Fr III (dp 4) $[M-2H]^{-2}$	Fr IV (dp 5) $[M-2H]^{-2}$
m/z	351.5	527.5	351.4	439.6
M_r (tested)	352.5	528.5	704.8	881.2
M_r (calcd)	352.2	528.4	704.5	880.6

Compounds **2** and **3** were isolated from Fr II in a ratio of nearly 1:1, and their ^{1}H and ^{13}C NMR data are listed in Table 3. For compound **2**, the nonreducing end H-4^{III} appeared at 5.69 ppm, while the characteristic peaks for homoguluronic acids H-1s (H-1^{III}, H-1^{II}, H-1^{I}) appeared at 5.02, 4.85 and 4.72 ppm, respectively. All carbon and proton signals from the HMQC spectra (Fig. 2A) of **2** supported the following structure: 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate)-(1 → 4)-α-L-gulopyranosyluronate-(1 → 4)-α-L-gulopyranosyluronate (ΔGG). For compound **3**, three H-1s (H-1Gβ at 4.68 ppm, H-1Δ at 4.94 ppm and H-1M at 4.54 ppm) and further HMQC spectra assignment (Fig. 2B) gave **3** as following: 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate-(1 → 4)-β-D-mannopyranosyluronate-(1 → 4)-α-L-gulopyranosyluronate (ΔMG).

There are four compounds (**4**, **5**, **6** and **7**) in Fr III with **6** as the major fraction. ESIMS suggested that Fr III is a tetrasaccharide mixture. From the ^{1}H–^{1}H COSY, HMBC spectra (Fig. 3A and B), and in comparison with structures **1** (ΔG) and **2** (ΔGG), compound **4** was elucidated as a homoguluronic acid having the following

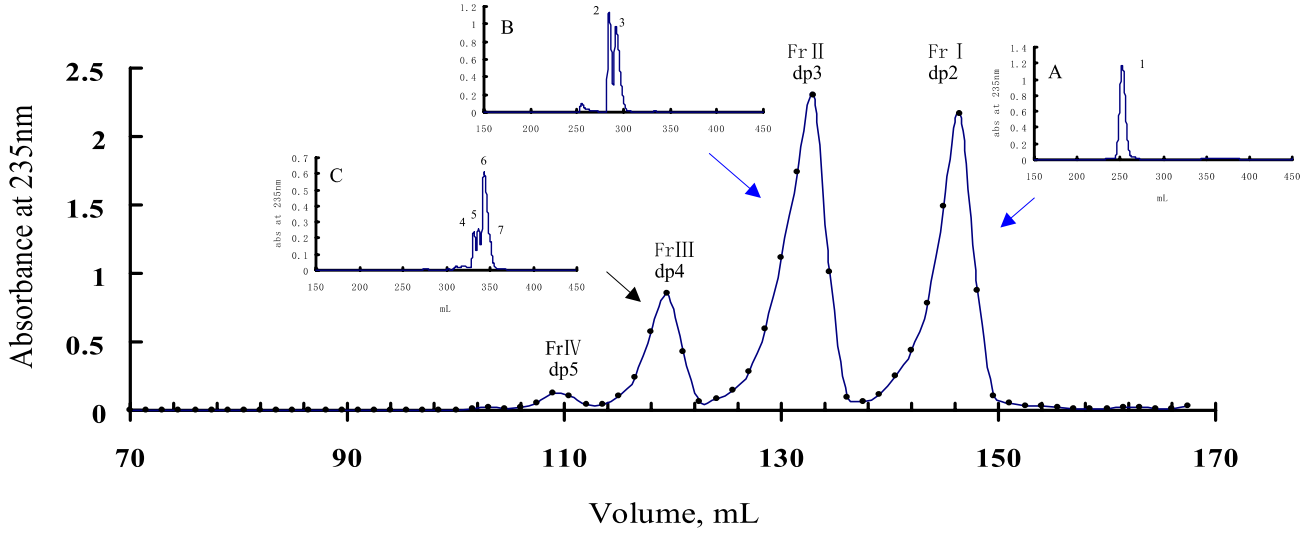

Figure 1. The separation graph of alginate oligosaccharides on a BioGel P4 column (V_0 = 72 mL, V_t = 178 mL). Eluted with 0.2 mol/L NH_4HCO_3 at a flow rate of 1.5 mL/h with detection at 235 nm. Panel A, B and C was the purification graph of Fr I, Fr II and Fr III , respectively, on SAX-FPLC. The gradient was 0–0.4 mol/L NaCl (pH 3.5) at 3 mL/min for 2.5 h. Numbers 1–7 correspond to compounds **1**–**7**, respectively.

Table 2. The NMR data of disaccharide **1**[a]

Compound	Terminals	^{13}C NMR				
		C-1	C-2	C-3	C-4	C-5
1 ΔG	I	96.04 (93.42)	71.61 (69.00)	72.66 (70.03)	82.39 (79.77)	75.92 (73.55)
	II	103.20 (100.39)	69.73 (67.07)	65.21 (62.39)	110.96 (107.99)	146.99 (144.61)
		^{1}H NMR				
		H-1	H-2	H-3	H-4	H-5
	I(α)	5.19	3.87	3.63	—	—
	I(β)	4.87 (4.85)	3.55 (3.53)	4.20 (4.14)	4.16 (4.11)	4.44 (4.39)
	II	5.21 (5.18)	3.92 (3.91)	4.32 (4.29)	5.92 (5.88)	—

[a]The chemical shifts in brackets are data of Ref. 10.

Table 3. The NMR data of trisaccharides **2** and **3**[a]

Compounds	Terminals	^{13}C NMR				
		C-1	C-2	C-3	C-4	C-5
2 ΔGG	I	93.29 (94.52)	69.13 (70.41)	70.09 (71.25)	80.35 (81.50)	73.45 (74.67)
	II	100.49 (101.99)	64.85 (65.97)	68.93 (70.11)	79.72 (80.90)	67.12 (68.32)
	III	100.83 (101.63)	66.91 (68.05)	62.72 (63.82)	107.77 (108.86)	44.71 (145.90)
3 ΔMG	I	93.25	68.83	70.29	80.15	73.68
	II	101.37	70.51	71.21	76.09	78.26
	III	100.13	66.69	63.67	107.52	145.18
		^{1}H NMR				
		H-1	H-2	H-3	H-4	H-5
2 ΔGG	I	4.72 (4.78)	3.45 (3.50)	3.96 (4.00)	3.86 (3.91)	4.24 (4.30)
	II	4.85 (4.90)	3.70 (3.76)	3.94 (4.00)	4.08 (4.13)	4.31 (4.36)
	III	5.02 (5.08)	3.75 (3.81)	4.24 (4.23)	5.69 (5.75)	—
3 ΔMG	I	4.68	3.53	4.15	3.94	4.21
	II	4.54	3.81	3.59	3.75	3.55
	III	4.94	3.77	4.28	5.57	—

[a]The chemical shifts in bracket are data of Ref. 15.

structure: 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate-(1→4)-α-L-gulopyranosyluronate-(1→4)-α-L-gulopyranosyluronate-(1→4)-α-L-gulopyranosyluronate (ΔGGG). In the HMBC spectrum of ΔGGG (Fig. 3B), the cross-peaks of (H-1^{IV},C-4^{III}), (H-1^{III},C-4^{II}), (C-1^{I},H-2^{I}), (C-1^{I},H-3^{I}), (C-1^{I},H-5^{I}), (C-1^{I},H-1^{II}), combined with the ^{1}H–^{1}H COSY spectrum (Fig. 3A), facilitated the full assignments of **4**. From the ^{1}H–^{1}H COSY, HMBC spectra (Fig. 4A and B), and in comparison with the structure of **3** (ΔMG), compound **5** was determined as the following tetrasaccharide: 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate-(1→4)-β-D-mannopyranosyluronate-(1→4)-α-L-gulopyranosyluronate-(1→4)-α-L-gulopyranosyluronate (ΔMGG). As shown in the HMQC and ^{1}H–^{1}H COSY spectra (Fig. 4A) of **5**, upfield H-1^{III} (4.52 ppm) indicates that residue III is a mannuronic acid. In the HMBC spectrum of **5** (Fig. 4B), the cross-peaks between H-1^{IV} (4.92 ppm) and C-4^{III} (78.28 ppm), H-1^{III} and C-3^{II}, H-1^{II} and C-4^{I}, C-1^{I} and H-2^{I}, C-1^{I} and H-3^{I}, C-1^{I} and H-5^{I}, C-1^{I} and H-1^{II}, H-4^{II} and C-1^{III}, H-4^{I} and C-1^{II} are important correlations that confirm the linkage sequence. Similar NMR spectral analyses of **6** and **7** (Table 4, Fig. 5A and B) suggested the following structures: 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate-(1→4)-α-L-gulopyranosyluronate-(1→4)-β-D-mannopyranosyluronate-(1→4)-α-L-gulopyranosyluronate (ΔGMG, **6**) and 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronate-(1→4)-β-D-mannopyranosyluronate-(1→4)-β-D-mannopyranosyluronate-(1→4)-β-D-mannopyranosyluronate (ΔMMM, **7**).

In this paper, seven oligosaccharides were purified from alginate oligosaccharide mixture, and the yields of compounds **1**–**7** are 40.2%, 23.4%, 21.4%, 1.9%, 2.0%, 7.8% and 0.8%, respectively. Structural elucidation showed that only one disaccharide (ΔG, 40.2%) and two trisaccharides (ΔGG, 23.4%; ΔMG, 21.4%) were separated from the mixture. Based on the fact that no ΔM, ΔMM or ΔGM fractions were separated from the mixture, we postulated that the hydrolysis mainly occurred between two guluronic acids (–G–G–) making one

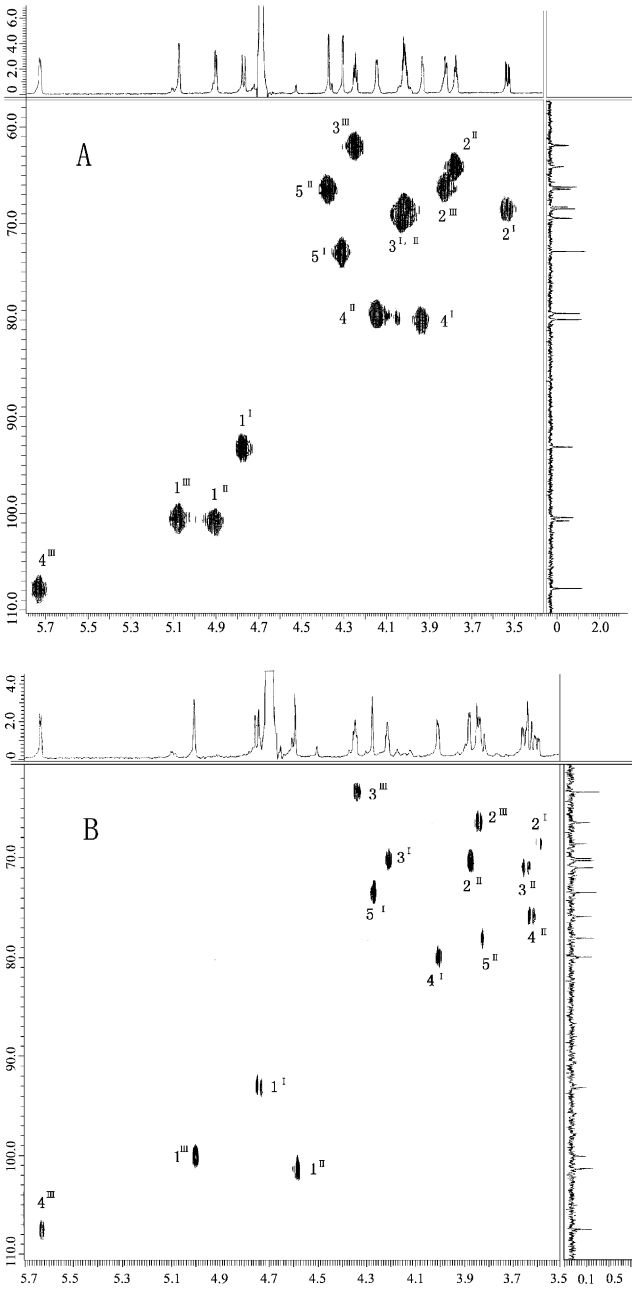

Figure 2. HMQC of trisaccharide **2** (A) and **3** (B). I: reducing end, II: middle residues; III: nonreducing end; and the numbers are the positions in pyranosyluronic acids.

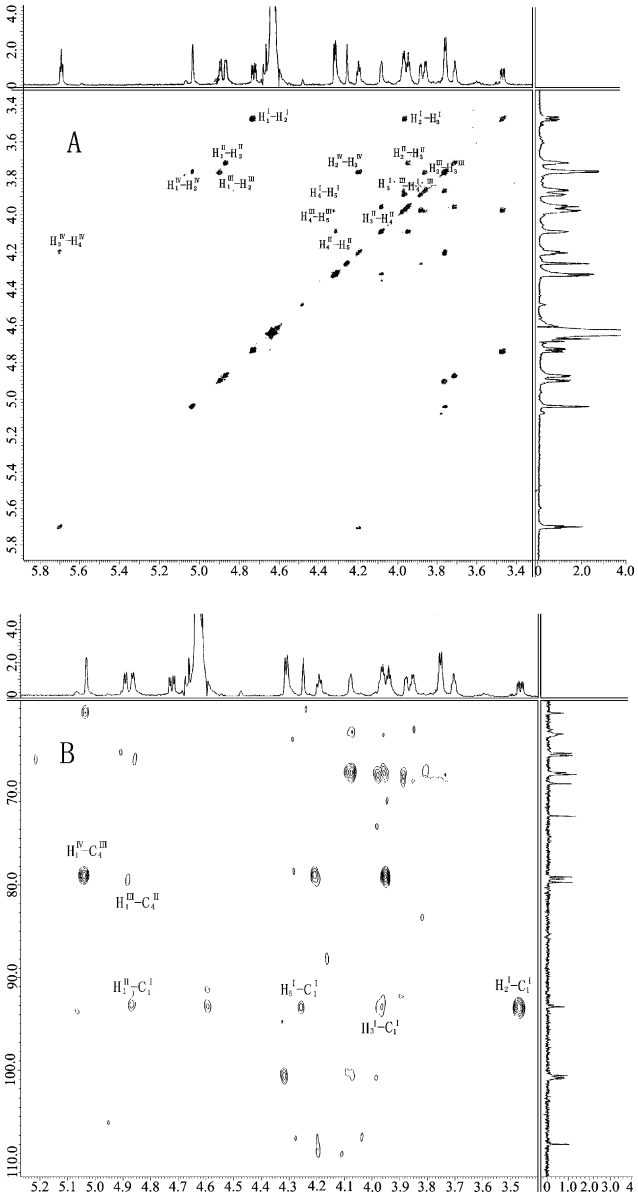

Figure 3. ^{1}H–^{1}H COSY (A) and HMBC (B) of tetrasaccharide **4**. IV: nonreducing end; III: middle residues near to nonreducing end; II: middle residues near to reducing end; I: reducing end, and the numbers are the positions in pyranosyluronic acids.

guluronic acid (G) residue on the reducing end and an unsaturated guluronic acid (Δ) in the nonreducing end for all products. These results proved that the alginate lyase, purified from *Vibro* sp. 510, was α-(1→4)-L-guluronic acid lyase. Furthermore, a high content of di- to tetrasaccharides (98%), a very low yield of pentasaccharide (1.99%) and no trace amount of hexasaccharide in the solution, indicated that the minimal recognition oligosaccharide of this enzyme should be one of the following pentasaccharides: GGGGG, GGGMG, MGGMG or GGGMG. The hydrolysis mechanism of this enzyme is illustrated in Figure 6. This result is similar to α-L-guluronate lyase purified from *Corynebacterium* sp. ALY-1.[23]

In conclusion, the alginate was hydrolyzed by alginate lyase of *Vibro* sp. 510, furnishing oligosaccharide mixtures that were separated on a BioGel P4 column. Each uniform-sized fraction was further purified on a SAX-FPLC column, and seven oligosaccharides were obtained, the structures of which were determined by ESIMS and 2D NMR techniques. All of these oligosaccharides will be used as building blocks in the chemical synthesis of neoglycoprotein, neoglycolipid or in the alginate lyase–oligosaccharide crystal studies.[24,25]

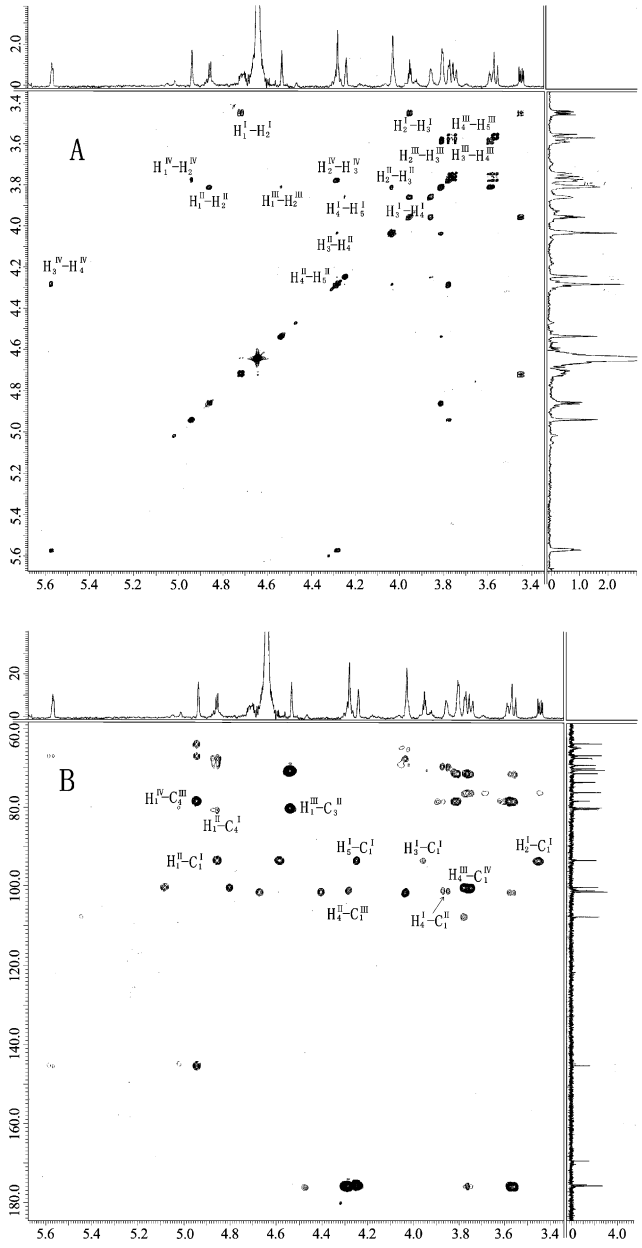

Figure 4. ^{1}H–^{1}H COSY (A) and HMBC (B) of tetrasaccharide **5**. IV: nonreducing end; III: middle residues near to nonreducing end; II: middle residues near to reducing end; I: reducing end. The numbers are the positions in pyranosyluronic acids.

3. Experimental

3.1. Materials

Alginate from *L. Japonica* was purchased from Huahai Pharmaceutical industry (Qingdao, China), and the ratio of M/G is 2.28. BioGel P4 (extra fine, 45 μm) was from BioRad (Richmond, CA). Sephadex G-10 was from Amersham Biosciences (Uppsala, Sweden). SAX-FPLC was performed on a Spherisorb S5 SAX (20×250 mm) column (Clwyd, UK). D_2O was from Cambridge Isotope Laboratories Inc., and the other reagents are G.R. or A.R.

Table 4. The NMR data of tetrasaccharides **4**, **5**, **6** and **7**

Compounds	Terminals	^{13}C NMR				
		C-1	C-2	C-3	C-4	C-5
4 ΔGGG	I	93.31	69.15	70.11	80.35	73.45
	II	100.69	64.89	68.95	80.02	67.16
	III	100.86	65.01	69.15	79.79	67.16
	IV	100.56	66.92	62.71	107.75	144.75
5 ΔMGG	I	93.30	69.16	70.09	80.40	73.47
	II	100.93	64.79	69.16	67.34	63.68
	III	101.28	70.50	71.25	78.28	76.10
	IV	100.17	64.79	67.34	107.55	145.19
6 ΔGMG	I	93.30	68.88	70.29	80.22	73.75
	II	101.34	70.93	71.46	77.37	76.08
	III	99.47	64.82	69.06	79.84	67.25
	IV	100.49	71.22	62.56	107.80	144.73
7 ΔMMM	I	93.09	66.70	71.46	77.37	76.21
	II	100.17	70.18	71.22	78.16	76.21
	III	101.34	71.22	71.46	78.01	76.21
	IV	100.03	66.93	63.66	107.63	145.22
		^{1}H NMR				
		H-1	H-2	H-3	H-4	H-5
4 ΔGGG	I	4.71	3.46	3.96	3.87	4.24
	II	4.86	3.70	3.93	4.07	4.30
	III	4.88	3.75	3.84	3.96	4.31
	IV	5.02	3.74	4.19	5.68	—
5 ΔMGG	I	4.69	3.43	3.94	3.85	4.23
	II	4.84	3.79	4.02	4.27	4.23
	III	4.52	3.79	3.56	3.73	3.54
	IV	4.92	3.76	4.28	5.55	—
6 ΔGMG	I	4.68	3.53	4.14	3.93	4.21
	II	4.52	3.76	3.59	3.82	3.57
	III	4.83	3.69	3.91	4.06	4.61
	IV	5.02	3.74	4.17	5.69	—
7 ΔMMM	I	5.04	3.81	3.58	3.70	3.55
	II	4.48	3.84	3.59	3.73	3.55
	III	4.53	3.81	3.59	3.79	3.60
	IV	4.93	3.78	4.28	5.57	—

3.2. Alginate lyase

The alginate lyase was purified from the fermentation solution of a marine bacterium (*Vibro* sp. 510). The activity of the enzyme was measured by monitoring the increase of absorbance at 235 nm at 28 °C using a UV–vis-PC 2101 spectrometer (equipped with thermostatted cell and enzyme dynamic analysis software, UNICO Company, Shanghai, China) in a 2.8-mL reaction mixture containing 0.2% (w/v) sodium alginate, 200 mmol/L NaCl, 50 mmol/L Tris–HCl buffer (pH 7.5) and 0.2 mL of enzyme solution. One unit of the enzyme activity is defined as the amount of enzyme required to cause an increase of one unit per min of optical density at 235 nm.

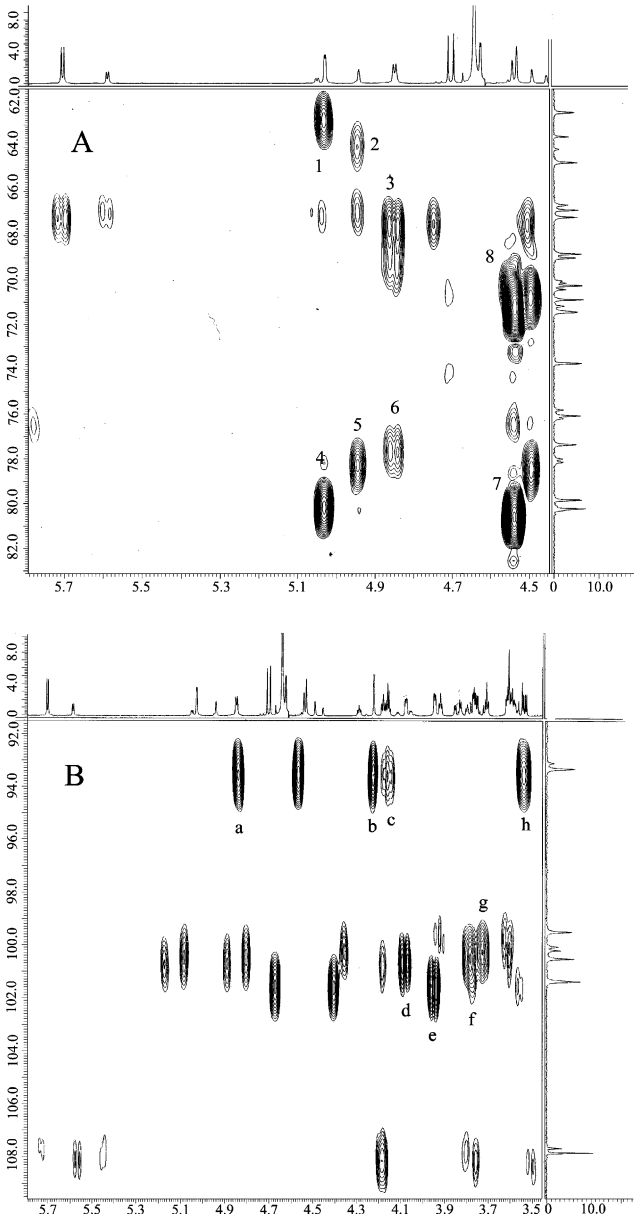

Figure 5. HMBC spectra of tetrasaccharide **6** (A) and **7** (B). The cross-peaks of **6** in panel A are assigned as: 1, H_1^{IV}–C_3^{IV}; 2, H_1^{IV}–C_3^{IV}; 3, H_1^{III}–C_5^{III}; 4, H_1^{IV}–C_4^{III}; 5, H_1^{IV}–C_4^{III}; 6, H_1^{III}–C_4^{II}; 7, H_1^{II}–C_4^{I}; 8, H_1^{II}–C_2^{II}; I: reducing end; II: middle residues near to reducing end; III: middle residues near to nonreducing end; IV: nonreducing end. Square brackets stand for contaminates signals of ΔMMM in **6**; The cross-peaks of **7** in panel **B** are assigned as: a, H_1^{III}–C^{11}; b, H_5^{I}–C_1^{I}; c, H_3^{I}–C_1^{I}; d, H_4^{III}–C_1^{IV}; e, H_4^{I}–C_1^{II}; f, H_4^{I}–C_1^{III}; g, H_4^{III}–C_1^{IV}; h, H_2^{I}–C_1^{I}; I: reducing end; II: middle residues near to reducing end; III: middle residues near to nonreducing end; IV: nonreducing end.

3.3. Preparation of alginate oligosaccharides

Alginate (5 g) was dissolved in 1 L of 50 mmol/L Tris–HCl buffer (pH 7.5), and 50 units alginate lyase were added. One unit of enzyme activity was defined as the amount of enzyme required to cause an increase of one unit of optical density at 235 nm.[10] The reaction was carried out at 28 °C for 24 h, and then the solution was heated to 100 °C and kept for 10 min to stop the hydrolysis reaction. Ethanol was added to the solution until the content of ethanol reached 50% (v/v), then the mixture was centrifuged to get rid of the precipitate. The supernatant was collected and evaporated, the solution was filtered though a 0.45-μm membrane and lyophilized, and the oligosaccharide mixture was stored at −20 °C.

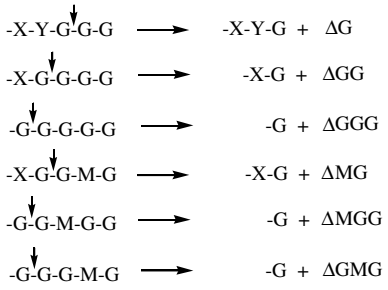

Figure 6. Possible modes for alginate hydrolysis with alginate lyase *Vibro* sp. 510. X = M or G; Y = G or M; G: guluronic acid; M: mannuronic acid; ↓: hydrolysis sites.

3.4. Low-pressure gel-permeation chromatography

The alginate oligosaccharides were size-fractioned by low-pressure gel-permeation chromatography (LP-GPC). The oligosaccharide mixture (100–150 mg/2 mL) was loaded on BioGel P4 Econo-column (16 × 1700 mm, Extra-fine, 45 μm, BioRad, USA) and eluted with 0.2 mol/L NH_4HCO_3 solutions at a flow rate of 1.5 mL h^{-1}, and the separation was carried out at rt. Each fraction (1.5 mL/tube) was gathered using a fraction collector (RediFrac, Pharmacia), and the uronate content was measured at 235 nm.

3.5. Strong anion-exchange fast-protein liquid chromatography (SAX-FPLC)

The uniform sized fractions that separated from the BioGel P4 column, were further separated on the SAX-FPLC system (ÄKTA™ FPLC equipped with P-920 pumps, conductivity and UV-monitor, and UNICORN™ V1.11 data processing software). The column was Spherisorb S5 SAX (20 × 250 mm), and was eluted with a linear gradient from 0 to 0.4 mol/L NaCl solution at a flow rate of 2 mL/min for 2.5 h.

3.6. Desalting

Each fraction was rotary evaporated and desalted on a Sephadex G-10 column (10 × 1000 mm), eluted with double distilled water.

3.7. ESIMS

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

3.8. NMR spectroscopy

The pure samples (1–5 mg) were dissolved in 1 mL of D_2O (99.96%) and freeze-dried two times to remove exchangeable protons, and then dissolved in 0.5 mL of D_2O (99.96%) for NMR analysis. All NMR analyses such as ^1H, ^{13}C NMR, ^1H–^1H COSY, HMQC and HMBC were performed on JEOL ECP 600 MHZ spectrometer at 298 K.

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