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Analysis of oligoguluronic acids with NMR, electrospray ionization–mass spectrometry and high-performance anion-exchange chromatography☆

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Abstract

Oligoguluronates were prepared by enzymatic hydrolysis of homopolymeric blocks of guluronic acids. Two different oligosaccharides were prepared by separating final hydrolysates on Q-Sepharose FF ion chromatography. High-performance anion-exchange chromatography analysis showed the high purity of these oligosaccharides. The molecular masses of these two oligosaccharides, determined by electrospray ionization–mass spectrometry, were 396 and 594, respectively. ^{1}H, ^{13}C NMR, H-H COSY and HSQC analysis proved that they were the unsaturated dimer and trimer oligoguluronates. The ^{1}H and ^{13}C NMR chemical shifts of these two oligosaccharides are also reported.

Keywords: Oligoguluronic acids; Guluronic acids; Polysaccharides

1. Introduction

Poly-α-guluronic acid (PolyG) is one of the polysaccharides composed of alginate [1]. Once PolyG is hydrolyzed by alginate-degrading enzymes, alginate lyases, 4-deoxy-erythro-hex-4-en-poyranuronosyl groups are produced at the non-reducing ends of hydrolysate [2–4]. Recently, interest has been shown in the bioactivity of oligoguluronates, such as antiviral and antitumoral activity [5,6]. The study of these oligosaccharides involves explanation of the relationship between the structure and bioactivity. There are several methods to identify the structure of oligosaccharides. High resolution ^{1}H and ^{13}C NMR is a rapid and efficient technology to determine oligosaccharides [7,8]. However, the spectrograms are usually difficult to explain due to the complexity of the hydrolysates. Moreover, the difficulty of purifying oligosaccharides makes the study more arduous. This paper reports the purification of oligosaccharide hydrolysates by anion-exchange resins, the identification of purity by high-performance anion-exchange chromatography (HPAEC), the determination of molecular mass by electrospray ionization–mass spectrometry (ESI–MS), and the analy-

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sis of structure by ^{1}H, ^{13}C NMR, H-H COSY and HSQC techniques.

2. Methods

2.1. Preparation of unsaturated oligoguluronates

PolyG (10 g) was dissolved in 1000 ml of 50 m*M* Tris–HCl buffer at pH 7.5, and 50 units alginate enzyme (supplied by the Microbiology Laboratory of the Food Science Department of the Ocean University of Qingdao) 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 230 nm [1]. The reaction was carried out at 30 °C for 10 h till the absorption at 230 nm did not change. The enzyme reaction was stopped by heating the solution in boiling water for 10 min. After the solution was filtered, 10 ml of concentrated supernatant were placed on a column (30×1.6 cm) of Q-Sepharose FF, which had previously been equilibrated with 0.5 *M* NaAc. The gradient buffer (0.5–1.0 *M* NaAc) with a flow-rate of 1.5 ml/min was used to wash out the sample. The absorbance of the eluted solution was measured by UV detection at 230 nm. There were two major oligoguluronates found in the eluent in terms of elution profile. After concentration and precipitation of the eluents, respectively, two oligoguluronates were obtained and ready for further study.

Table 1
Eluent conditions for HPAEC

Time (min)	A (%)	B (%)	C (%)
Init	80.00	10.00	10.00
0.00	80.00	10.00	10.00
1.00	80.00	10.00	10.00
2.00	80.00	10.00	10.00
14.00	0.00	10.00	90.00
22.00	0.00	10.00	90.00
22.10	0.00	100.00	0.00
27.90	0.00	100.00	0.00
28.00	80.00	10.00	10.00
30.00	80.00	10.00	10.00

Eluent A, water; eluent B, 100 m*M* NaOH; eluent C, 1 *M* NaAc.

2.2. High-performance anion-exchange chromatography apparatus

The analytical system consisted of a Dionex DX-500 system (Sunnyvale, CA, USA) equipped with a CarboPac PA-1 guard precolumn (25×3 mm), a CarboPac PA-1 column (250×4 mm), a quaternary gradient pump, eluent degas (He) module, and a pulsed amperometric detector (with gold working electrode). For the eluent conditions see Table 1.

2.3. Electrospray ionization–mass spectrometry

Negative ion electrospray mass spectra were obtained using a Micromass Quattro GC–MS/MS instrument, with ion-spray source (Analytic, USA). Data acquisition and manipulation was accomplished using the Micromass MassLynx software package, including MaxEnt data analysis software. Sample solution (in MeOH–water, 1:1) was directly injected and the flow-rate was 0.16 ml/h. The scanning range was from 40 to 2000 u; the scanning speed was 100 u/min. Nitrogen was used to keep the pressure at 0.5–0.7 mPa.

2.4. NMR spectrometry studies

The Inova 500 console was used, which was a two-channel system equipped with a triple axis pulse field gradient amplifier. The solvent was $^{2}H_{2}O$. The experiment was done at ambient temperature.

3. Result and discussion

3.1. Diguluronate (ΔG) analysis by HPAEC, ESI–MS and NMR

HPAEC is widely used in the analysis of polysaccharides due to its great sensitivity and resolution [9]. There was only one main peak on the HPAEC spectrograph of the first sample (retention time at 11.34 min, profile not shown), therefore, the obtained oligoguluronate was a pure sample, which could be used to determine molecular mass by ESI–MS.

Producing multiple ion peaks is a property of ESI–MS. The major peaks are the multiple ion peaks, which can be used to further interpret the

molecular mass [10]. The ESI–MS spectrogram of the first sample is shown in Fig. 1. The peaks at 397, 419, 793 and 816 correspond to the molecular ion peak [M+1], [M+Na], [2M+1], and [2M+Na]. Therefore, the molecular mass of this sample was 396, which corresponded to an unsaturated dimer with two sodium ions (diguluronate, ΔG). The alginate lyases cleave the glycosidic linkage of PolyG and produce oligoguluronic acids with a 4,5-unsaturated uronic acid residue at the non-reducing end. ''Δ'' is used to represent non-reducing end residue. ''G'' is used to represent other residue units.

High resolution ^{1}H and ^{13}C NMR spectroscopy have been widely used in the structure and sequence analysis of polysaccharides. Two-dimensional NMR spectroscopy, such as COSY, HSQC, and HMQC, makes this analysis much easier than before [7,8,11]. Fig. 2 is the HSQC spectrum of ΔG. The corresponding chemical shift on HSQC and HMQC could be easily read from spectrograms of ^{1}H and ^{13}C NMR (spectrograms not shown). Since it was an unsaturated oligoguluronate, the signals of the H-4 proton and C-5 resonance at the non-reducing end were easily found in the down-field region, at a chemical shift of 5.88 ppm for H-4, and 144.61 ppm for C-5. From the HSQC spectrum, H-4 should relate to C-4, at a chemical shift of 107.99 ppm. It was reported that the anomeric proton chemical shifts were found in the range 4.4–5.5 ppm and the chemical shifts at the reducing end were smaller than that of the non-reducing end [11,12]. Therefore, the chemical shift of H-1 at the reducing end was 4.85 ppm (related C-1 at 93.42 ppm), that at the non-reducing end was 5.18 ppm (related C-1 at 100.39 ppm). Carboxyl chemical shifts were found in the range 165–176 ppm [12]. However, the unsaturated bond at C-4–C-5 puts the carboxyl at C-6 in the high-field region, therefore, it was easy to tell that the chemical shift of C-6 at the non-reducing end was 169.38 ppm, and that at the reducing end was 175.48 ppm. Other protons could also be found according to Ref. [11], and the related carbon

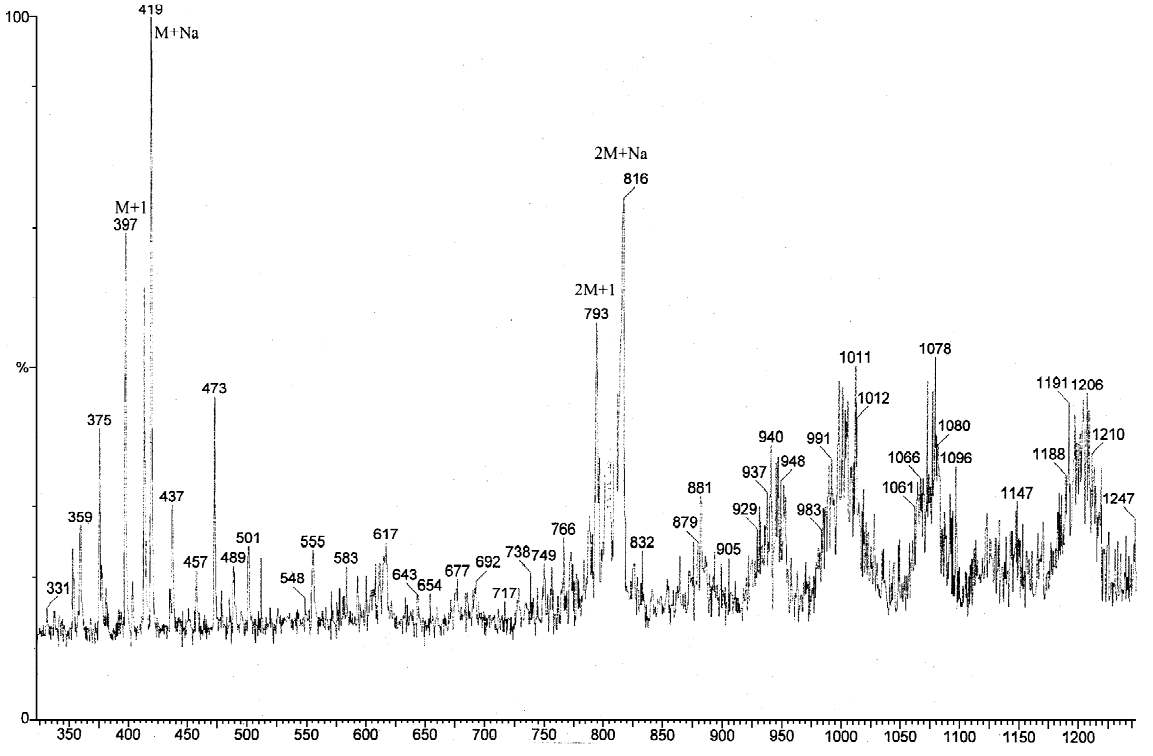

Fig. 1. ESI–MS of diguluronate (ΔG). *x*-Axis, *m*/*z*.

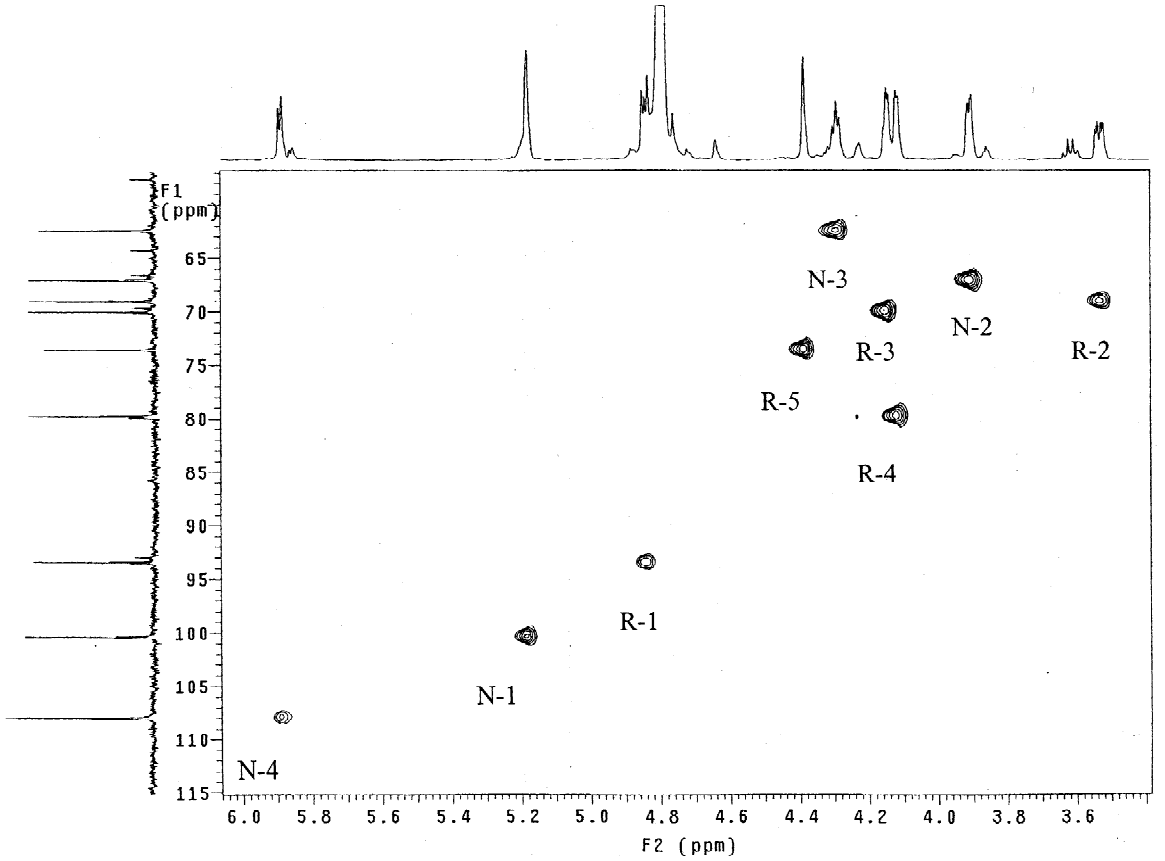

Fig. 2. HSQC spectrum of ΔG. N, non-reducing end; R, reducing end; the numbers are the positions in pyranosyluronic acids.

Table 2
^{1}H NMR chemical shifts of ΔG

	Chemical shifts (ppm)				
	H-1	H-2	H-3	H-4	H-5
Reducing end	4.85	3.53	4.14	4.11	4.39
Non-reducing end	5.18	3.91	4.29	5.88	–

resonance can be obtained by HSQC as well (Fig. 2). The ^{1}H and ^{13}C NMR chemical shifts of ΔG are shown in Tables 2 and 3.

3.2. Triguluronate (ΔGG) analysis by HPAEC, ESI–MS and NMR

The high purity of the second sample was identified by HPAEC because there was only one peak on the profile at 12.71 min (profile not shown). The peaks at 595.6, 617.2, and 1211.7 correspond to the molecular ion peaks [M+1], [M+Na], and [2M+Na], respectively (Fig. 3). Therefore, the molecular mass of this sample was 594, which was the unsatu-

Table 3
^{13}C NMR chemical shifts of ΔG

	Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
Reducing end	93.42	69.00	70.03	79.77	73.55	175.48
Non-reducing end	100.39	67.07	62.39	107.99	144.61	169.38

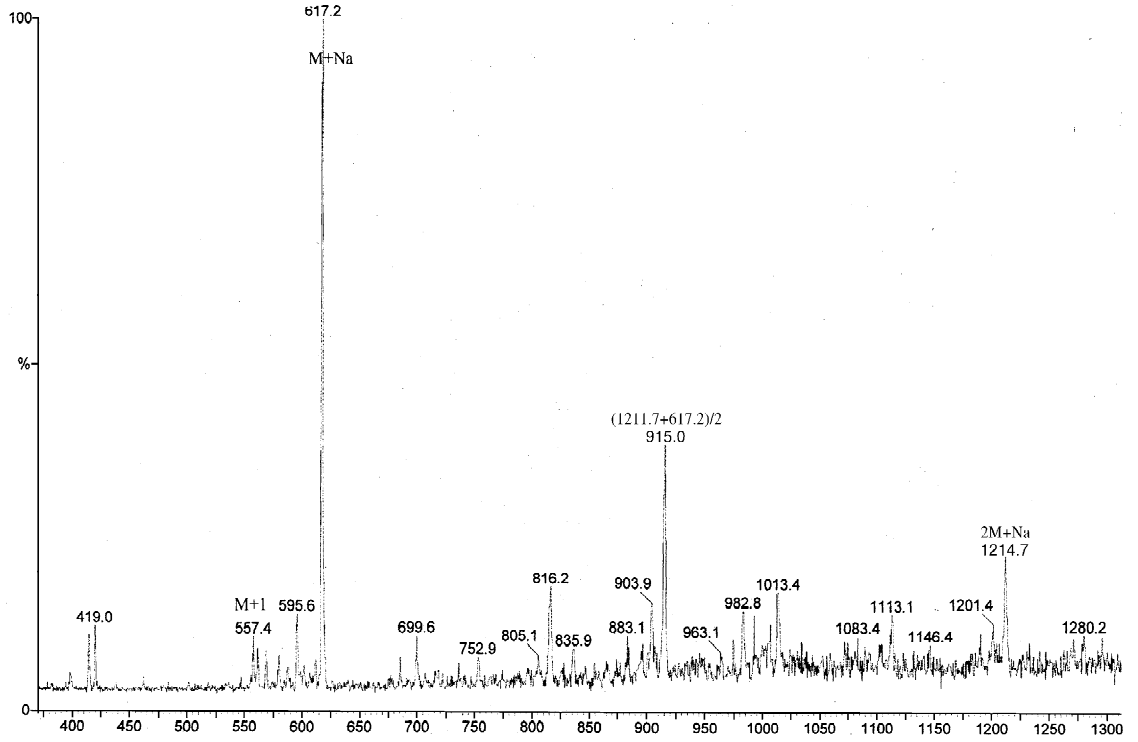

Fig. 3. ESI–MS of triguluronate (ΔGG). *x*-Axis, *m*/*z*.

rated trimer of guluronate (triguluronate, ΔGG), with three sodium ions.

Due to the influence of an unsaturated bond at C-4–C-5 of the non-reducing end, the signals of the H-4 proton and C-5 at the non-reducing end were easily found in the down-field region, at a chemical shift of 5.83 ppm for H-4, and 144.76 ppm for C-5. Because there was no H-5 proton at the non-reducing end, the position of H-3 (4.31 ppm) was easily found from the H-H COSY spectrum according to the position of the H-4 proton (Fig. 4). Since H-3 is related to H-2 and H-2 is related to H-1, the chemical shifts of protons at the non-reducing end could be found from the COSY spectrum and ^{1}H NMR. The chemical shifts of anomeric protons were assigned according to previous data for alginate polymer and oligomers [11]. The chemical shift of the anomeric proton at the reducing end was 4.87 ppm and that of the middle residue was at 4.98 ppm, which was used to analyze the position of other protons according to the relationship of protons from the COSY spectrum (Fig. 4). Thus, the chemical shifts of protons could be obtained. Since the proton positions were known, the ^{13}C NMR chemical shifts could be taken from the HSQC spectrum (Fig. 5) and ^{13}C NMR spectrum (profile not shown). ^{13}C NMR chemical shifts of carboxyl (C-6) were found at 175.61 ppm, and that at the non-reducing end was 169.34 ppm. The ^{1}H NMR and ^{13}C NMR chemical shifts of ΔGG are listed in Tables 4 and 5 and the structures of the two samples are shown in Fig. 6.

Table 4
^{1}H NMR chemical shifts of ΔGG

	Chemical shifts (ppm)				
	H-1	H-2	H-3	H-4	H-5
Reducing end	4.87	3.59	4.08	3.98	4.38
Middle	4.98	3.84	4.07	4.20	4.44
Non-reducing end	5.16	3.88	4.31	5.83	–

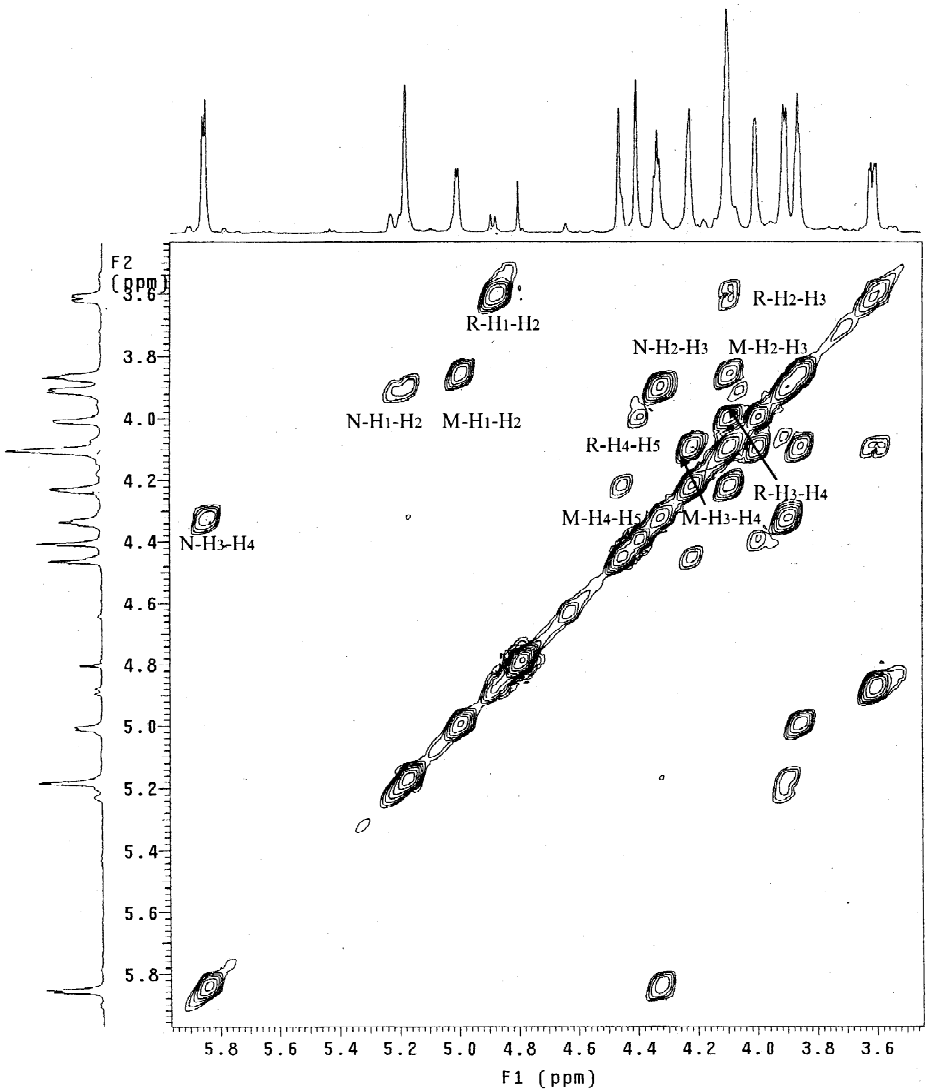

Fig. 4. H-H COSY spectrum of ΔGG. N, non-reducing end; M, middle residue; R, reducing end. The numbers are the positions in pyranosyluronic acids.

Table 5
^{13}C-NMR chemical shifts of ΔGG

	Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
Reducing end	93.42	70.14	69.23	80.58	73.601	175.61
Middle	101.03	64.73	68.92	79.78	67.173	175.61
Non-reducing end	100.60	66.99	62.69	107.98	144.76	169.34

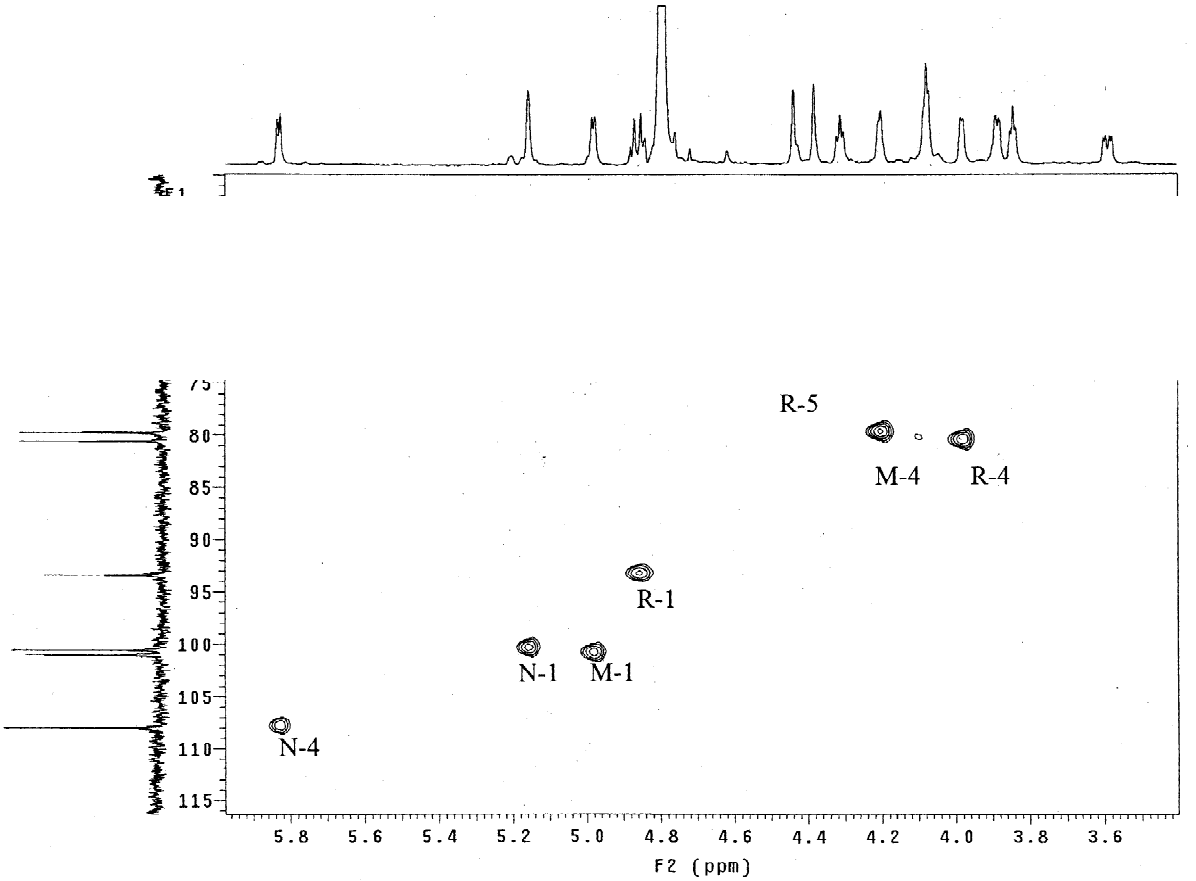

Fig. 5. HSQC spectrum of ΔGG. N, non-reducing end; M, middle residue; R, reducing end; the numbers are the positions in pyranosyluronic acids.

4. Conclusion

The main enzymatic hydrolysates of PolyG by supported alginate lyases are unsaturated diguluronate (ΔG) and triguluronate (ΔGG). The separation of these two oligoguluronates can be done on a Q-Sepharose FF anion-exchange column and the identification of purity obtained by HPAEC. The molecular masses of these two oligosaccharides are 396 and 594, respectively. The chemical shifts of ^{1}H NMR and ^{13}C NMR of these two oligosaccharides can be obtained easily by interpreting the ^{1}H NMR, ^{13}C NMR, H-H COSY and HSQC spectrograms together. Based on the data obtained, we propose a

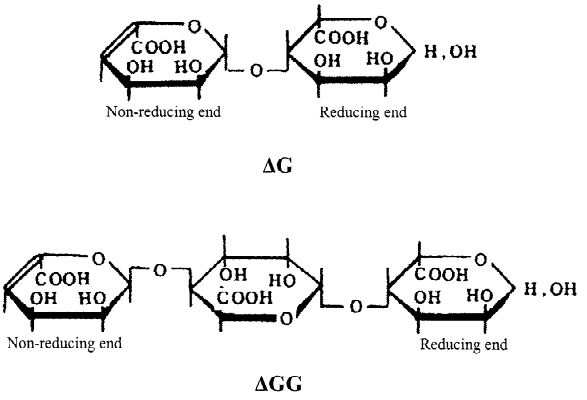

Fig. 6. Structures of ΔG and ΔGG.

rapid and reliable method for the investigation of the structure of oligoguluronate.

References

[1] A. Haug, B. Larsen, O. Smidsrod, Acta Chem. Scand. 20 (1966) 183.
[2] C. Boyen, Y. Bertheau, T. Barbeyron, B. Kloareg, Enzyme Microb. Technol. 12 (1990) 885.
[3] B.J. Brown, J. Preston, Carbohydr. Res. 211 (1991) 91.
[4] M. Kitamikado, C.H. Tseng, K. Yamaguchi, T. Nakamura, Appl. Environ. Microbiol. 58 (1992) 2474.
[5] Y. Yonemoto, H. Tanaka, Y. Yamashita, Y. Kitabaake, Y. Ishida, A. Kimura, K. Murata, J. Ferment. Bioeng. 75 (1993) 68.
[6] K. Murara, T. Inose, T. Hisano, S. Abe, Y. Yonemoto, T. Yanashita, M. Takagi, K. Sakaguchi, A. Kimura, T. Imanka, J. Ferment. Bioeng. 75 (1993) 427.
[7] P.J. Bolon, J.H. Prestegard, J. Am. Chem. Soc. 120 (1998) 9366.
[8] B.E. Bacon, R. Cherniak, K.J. Kwon-Chung, E.S. Jacobson, Carbohydr. Res. 283 (1996) 95.
[9] E. Vinogradov, K. Bock, Carbohydr. Res. 309 (1998) 57.
[10] K.N. Price, A. Tuinman, D.C. Baker, C. Chisena, R.L. Cysyk, Carbohydr. Res. 303 (1997) 303.
[11] A. Heyrand, C. Gey, C. Leonard, C. Rochas, S. Girond, B. Kloareg, Carbohydr. Res. 289 (1996) 11.
[12] J. Duus, C.H. Gotfredsen, K. Bock, Chem. Rev. 100 (2000) 4589.