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Structural characterization of natural ideal 6-*O*-sulfated agarose from red alga *Gloiopeltis furcata*

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ABSTRACT

A charge and size uniform polysaccharide GW2M was extracted with cold water from red alga *Gloiopeltis furcata* and purified by strong anion ion-exchange and gel permeation chromatography. Its chemical structure was identified by methylation, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC techniques. The experimental data showed that GW2M was composed of galactose (40.3%), 3,6-anhydro-galactose (34.1%) and sulfate (24.8%) with an average molecular mass of 20.6 kDa. The results proved GW2M was a linear repeating sequence of alternating (1 → 3)-linked 6-*O*-sulfated-β-D-galactose (G6S) and (1 → 4)-linked 3,6-anhydro-α-L-galactose (A) which made it to be an ideal 6-*O*-sulfated-agarose. The sequences of serial oligosaccharides prepared by mild acid and reductive acid hydrolysis from GW2M were confirmed using electrospray collision induced dissociation tandem mass spectrometry (ES-CID-MS/MS) technique.

1. Introduction

Galactans, commonly existed in red seaweeds, consist of alternating 3-linked β-D-galactopyranose (Gal, unit G) and 4-linked α-D or L-galactopyranose often occurring as its 3,6-anhydro form (anGal, unit A), which are classified either as carrageenan, if the 4-linked residue is D-configuration, or as agaran, if that is L-configuration (Falshaw, Furneaux, & Stevenson, 1998; Kolender & Matulewicz, 2002).

Gloiopeltis furcata, a red alga belonging to the genus *Gloiopeltis* of Endocladiaceae family, has been widely applied in traditional herbal medicine and food thickener in China and Japan (Schachat & Glicksman, 1959). The extracts from *G. furcata* have been reported with variety benefit properties, such as anti-inflammatory (Niu, Fan, & Han, 2003) and anti-tumor (Bae & Choi, 2007). In previous studies, the polysaccharide extracted from *G. furcata* was defined to be sulfated agaran by Hirase (1957), Izumi (1973), Ji and Gu (1987) and Takano, Iwane-Sakata, Hayashi, Hara, and Hirase (1998). The detailed structural feature of *G. furcata* derived polysaccharides, especially their oligosaccharides sequences are still unknown.

In this paper, we extracted and purified a sulfated polysaccharide from *G. furcata*, its fine structural characters of poly-/oligosaccharides were studied with NMR spectrometry and mass spectrometry.

2. Results and discussion

2.1. Extraction, purification and general analysis

The polysaccharide from *G. furcata* was extracted and isolated by our previous method (Yu et al., 2010). From the cold water extract of this alga, a polysaccharide mixture (GW) was obtained with 60% (w/w) yield. The polysaccharide mixture was separated on Q-Sepharose FF and Sepharose 6B FF columns, a size and charge uniform fraction GW2M was acquired with the yield 33.3%. The sulfated polysaccharide GW2M, whose molecular mass is 20.6 kDa, showed a symmetric peak on high performance gel permeation chromatography. General character analysis showed that GW2M contained 73.4% sugar and 24.8% sulfate with a small amount of crude protein (1.2%).

Monosaccharide composition analysis of polysaccharide is very important to structural determination. Owning to the instability of anGal, reductive hydrolysis is needed to avoid it being changed

Abbreviations: ES-MS, electrospray mass spectrometry; CID, collision-induced dissociation; GC–MS, gas chromatography–mass spectrometry; MMB, 4-methylmorpholine borane; TFA, trifluoroacetic acid; DP, degree of polymerization; Gal, galactose; anGal, 3,6-anhydrogalactose; A, 4-linked α-3,6-anhydrogalactose; G, 3-linked β-D-galactopyranose; G6S, 3-linked-6-*O*-sulfated-β-D-galactopyranose; RCA_{120}, Ricinus communis agglutinin 120.

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to 5-hydroxymethylfurfural (5-HMF) and other by-products under acid hydrolysis condition (Quemener & Lahaye, 1998; Yang et al., 2009). The gas chromatography (GC) analysis showed the molar ratio of anGal and Gal is 1:1.1 in GW2M. The IR spectrum (not shown) of GW2M gave some specific information of its functional groups. For example, the absorption at 1250 cm^{-1} and 820 cm^{-1} indicated the presence of 6-*O*-sulfate groups on Gal, and the absorption at 930 cm^{-1} proved the existences of anGal in GW2M.

2.2. Methylation analysis

The glycosidic linkage and the substitution position of the sulfate groups were elucidated by methylation analysis of the native GW2M and its desulfated product dsGW2M, and the comparative analysis between GW2M and dsGW2M provided key information for the accurate linkage position assignments. Samples were methylated according to the method described by Hakomori (1964), followed with acid hydrolysis, reduction, and acetylation. The partially methylated alditol acetates of GW2M and dsGW2M were detected by GC–MS and their data are listed in Table 1. The data indicated that GW2M was consisted of 1,4,5-Ac_3-2-Me-3,6-anGal and 1,3,5,6-Ac_4-2,4-Me_2-Gal with the approximate molar ratio of 1:1, indicated it to be a linear repeating sequence of alternating 3-linked galactopyranose and 4-linked 3,6-anhydrogalactopyranose. The methylation analysis of dsGW2M showed an increase of 1,3,5-Ac_3-2,4,6-Me_3-Gal together with a concomitant decrease of 1,3,5,6-Ac_4-2,4-Me_2-Gal (Table 1), which suggested that the sulfate groups were located at *O*-6 position of 1,3-galactose (G6S). This result was also confirmed by IR spectrum of GW2M, where an absorption band at 820 cm^{-1} assigned to the sulfate group at C6. A few 1,4,5-Ac_3-2,3,6-Me_3-Gal components found in dsGW2M indicated that its 3,6-anhydride was destroyed during the desulfation process. It was worth noting that results of methylated sugars suggested that $(1 \rightarrow 4)$ linked residues a little lower than $(1 \rightarrow 3)$ linked residues which was not very strictly agreed with the structure of red algal galactans generally consisting of equimolar $(1 \rightarrow 4)$ and $(1 \rightarrow 3)$ linked residues (Yang et al., 2011), and this phenomena was mainly due to the acid liability of 1,4-linked-3,6-anhydrogalactose. Collectively, GW2M, a sulfated galactan from *G. furcata*, was determined to consist of $(1 \rightarrow 4)$ linked-anGal and $(1 \rightarrow 3)$ linked-G6S.

2.3. The NMR spectroscopy analysis

Biological activities of polysaccharides have been reported to have a close relationship with their structure properties, including monosaccharide composition, linkage modes, molecular mass, sulfation level and pattern (Jiao, Yu, Zhang, & Ewart, 2011; Yang et al., 2011). In this study, the structural feature of GW2M was further identified by ^1H–^1H COSY, ^1H–^{13}C HMQC, ^1H–^{13}C HMBC and DEPT spectroscopy (Table 2). The anomeric resonance at 4.47 ppm in the first spin system gave easily identifiable correlations from H1 to H2–H6 of G6S in the ^1H–^1H COSY spectrum which demonstrated the presence of several spin systems (Fig. 1A). For example, the coupling constants between H-1 (δ 4.47 ppm) and H-2 (δ 3.48 ppm), H-2 and H-3 (δ 3.67 ppm), of this galactopyranose unit were observed. The anomeric proton at 5.00 ppm was the start signal of the next spin system corresponding to H-2 (δ 3.97 ppm) of anGal residue. In addition, the H-2 signal was correlated to H-3 at δ 4.44 ppm. The H-5 (δ 4.40 ppm) coupling with H-4 (δ 4.50 ppm) and H-6a (δ 3.86 ppm) were also identified. A relatively well-resolved ^{13}C NMR spectrum of GW2M would give useful information on the type of carbons present. As shown in Table 2, chemical shift values of the 12 peaks were identical to those for the signals from GW2M which was composed of an agarose 6-sulfate residue (Takano, Hayashi, Hara, & Hirase, 1995). Beta-configuration of the D-galactose residues was

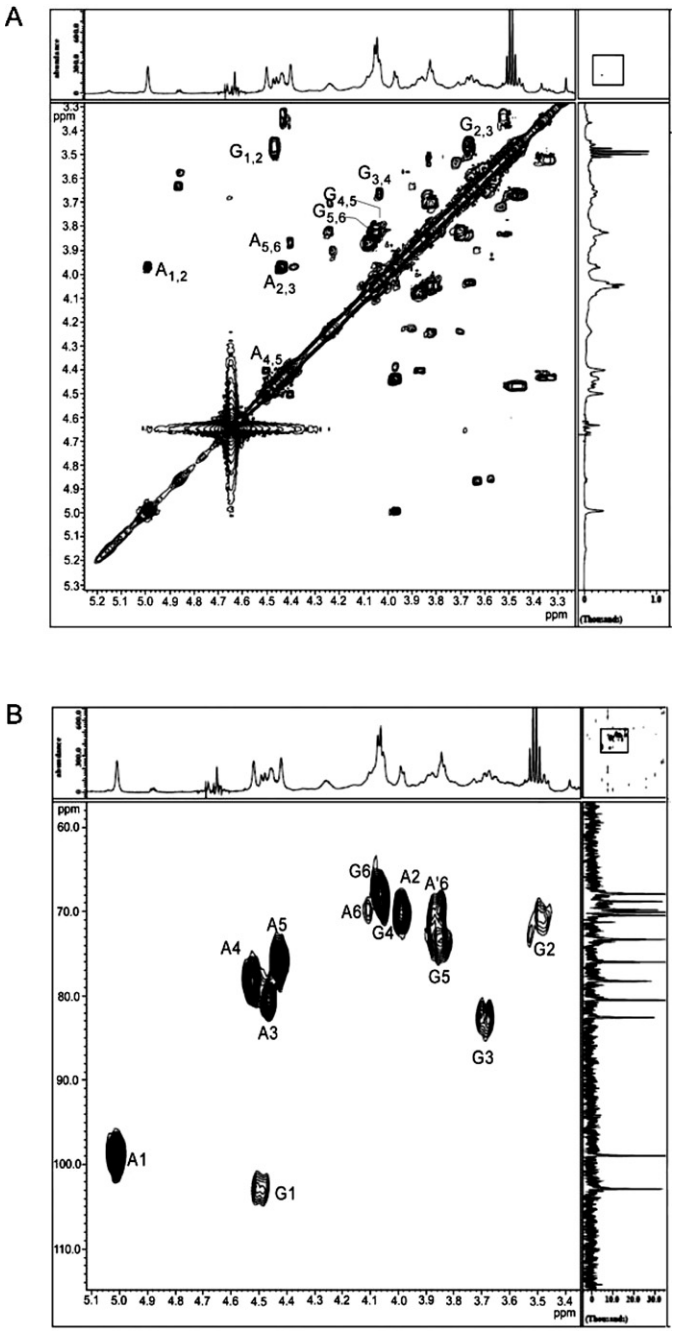

Fig. 1. 2D ^1H–^1H COSY (A) and ^1H–^{13}C HMQC (B) spectra of polysaccharide GW2M, recorded in D_2O at 25 °C. ^1H–^1H COSY: $A_{1,2}$ refers to a cross-peak between A H-1 and A H-2, etc.; $G_{1,2}$ refers to a cross-peak between G6S H-1 and G6S H-2, etc.; ^1H–^{13}C HMQC: A1 refers to a cross-peak between A H-1 and A C-1, etc.; G1 refers to a cross-peak between G6S H-1 and G6S C-1, etc.

indicated by the presence of anomeric signal at δ 102.0 ppm and C-6 at δ 67.0 ppm, which was agreed with a negative phased CH_2 signal at δ 67.0 ppm in DEPT-135 spectrum (not shown). In the case of anGal residue, the resonance signal at δ 98.1 ppm was assigned to its anomeric carbon. Additionally, C-6 absorption of this residue at δ 69.0 ppm was also identified in the DEPT-135 spectrum by a negative phased CH_2 signal at δ 69.0 ppm. The intensities of these two anomeric carbon peaks were consistent with the results of the sugar composition analysis. All the other carbon signals could be assigned unambiguously in the HMQC spectrum (Fig. 1B). The downfield chemical shift of C-6 (δ 67.0 ppm) in unit Gal clarified this residue carrying a sulfate group located at the *O*-6, which was consistent

Table 1
Composition of partially methylated monosaccharides produced by permethylation of GW2M and dsGW2M.

Linkages	Partially methylated alditol acetates	Mass fragments (*m*/*z*)	Molar ratio	
			GW2M	dsGW2M
→4)anGal(1→	1,4,5-Ac_3-2-Me-3,6-anGal	43,103,145	1.00	0.90
→3)Gal(1→	1,3,5-Ac_3-2,4,6-Me_3-Gal	101,117,129,161,233,277	–	0.95
→4)Gal(1→	1,4,5-Ac_3-2,3,6-Me_3-Gal	117,161,173,233	–	0.09
→3)G6S(1→	1,3,5,6-Ac_4-2,4-Me_2-Gal	87,117,129,189,233	1.10	0.11

Table 2
^1H and ^{13}C chemical shifts (in ppm) data of GW2M.

Compound	Nucleus	1	2	3	4	5	6a	6b
β-D-G6S	^1H	4.47	3.48	3.67	4.04	3.83	4.06	4.07
	^{13}C	102.0	69.6	81.7	68.0	72.4	67.0	
α-L-anGal	^1H[b]	5.00	3.97	4.44	4.50	4.40	3.86	4.08
	^{13}C	98.1	69.2	79.6	77.4	75.1	69.0	
Literature values[a]	^1H	4.56	3.63	3.75	4.12	3.71	3.80	3.75
β-D-Gal	^{13}C	102.3	70.1	82.2	68.6	75.2	61.3	
α-L-anGal	^1H	5.13	4.10	4.53	4.66	4.55	4.19	4.02
	^{13}C	98.2	69.7	80.0	77.4	75.5	69.3	

[a] Literature values were taken from references (Usov, Ivanova, & Shashkov, 1983; Welti, Rees, & Welsh, 1979).
[b] Recorded in D_2O at 25 °C.

with the IR data. Moreover, ^1H and ^{13}C assignment connections of GW2M were confirmed on the long-range heteronuclear ^1H–^{13}C chemical shift correlations (HMBC spectrum) (Table 3). For example, the connections of H-1 of anGal and C-3 of Gal, H-4 of anGal and C-1 of Gal, were observed, respectively, which supported the presence of (1 → 4)-linked-anGal and (1 → 3)-linked-G6S. All the above resonance signals were in agreement with the methylation analysis of GW2M. All together, the structural studies indicated that GW2M, obtained from *G. furcata*, was constructed with a regular repeating unit of $[\rightarrow 3G6S\,\beta 1 \rightarrow 4A\alpha 1\rightarrow]_n$.

2.4. Preparation and sequence analysis of oligosaccharides

Detailed investigation of oligosaccharide fragments, including their preparation and sequence determination, is important for structure–function relationship study. According to previous studies, polysaccharides containing anGal from red algae, such as agarose and κ-carrageenan, can be easily hydrolyzed by mild acid to get series oligosaccharides (Yang et al., 2009; Yu et al., 2002). In this paper, we also got odd-numbered and even-numbered oligosaccharides from native polysaccharide GW2M by mild acid hydrolysis and reductive hydrolysis, respectively.

After mild acid hydrolysis, the hydrolysate was filtered, concentrated, desalted, and then separated on a Superdex peptide column (Fig. 2A). Five pooled fractions, A1–A5, were obtained, and analyzed by negative-ion ES-MS. The presence of sulfates in each fraction gave rise to multiple charged ions dominated in MS spectra, from which the molecular mass and degree of polymerization (DP) of oligosaccharides were determined (Table 4). For example, fraction A2 showed the triply charged ion at *m*/*z* 343.0 with a molecular ion ((Pham-Huy, He, & Pham-Huyc, 2008)$^-$) at *m*/*z* 1031.1, suggesting A2 was a pentasaccharide that contained three Gal residues, two

Table 3
Long-range ^1H–^{13}C couplings found in the HMBC spectrum for the signals of the GW2M.

Residue	^1H (δ, ppm)	^{13}C (δ, ppm)	Connectives
A H-1	5.00	81.7	G6S C-3
		69.2	A C-2
		69.0	A C-6
A H-2	3.97	79.6	A C-3
A H-3	4.44	77.4	A C-4
A H-4	4.50	102.0	G6S C-1
		69.2	A C-2
		79.6	A C-3
		75.1	A C-5
		69.0	A C-6
A H-5	4.40	98.1	A C-1
		79.6	A C-3
G6S H-2	3.48	102.0	G6S C-1
		81.7	G6S C-3
G6S H-3	3.67	69.6	G6S C-2
G6S H-4	4.04	72.4	G6S C-5
G6S H-5	3.83	68.0	G6S C-4
G6S H-6	4.06	72.4	G6S C-5

G6S: 1,3-linked-6-sulfated-β-D-galactose; A: 1,4-linked-3,6-anhydro-α-L-galactose.

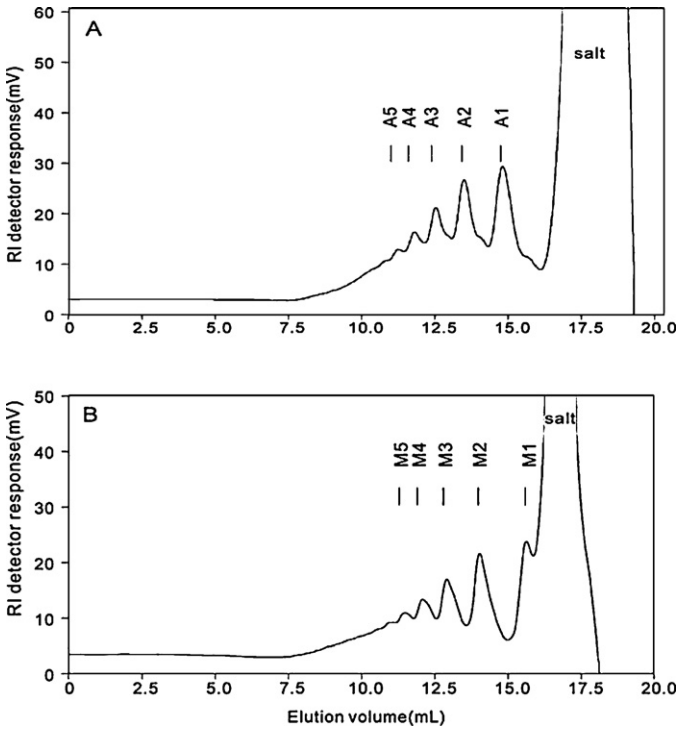

Fig. 2. Gel filtration chromatography of oligosaccharides. (A) Odd-numbered oligosaccharides resulting from mild acid hydrolysis. (B) Even-numbered oligosaccharide alditols resulting from reductive hydrolysis.

Table 4
Negative-ion ES-MS of mild acid and reductive hydrolysis products from GW2M.

Fractions	Found ions (charge)	Calculated M_r (H form)	Assignment		Theoretical M_r (H form)
			DP[a]	Sequences	
A1	322.0 (−2)	646.0	3	G6S-A-G6S[b]	646.1
A2	343.0 (−3)	1032.0	5	G6S-A-G6S-A-G6S	1032.1
A3	353.5 (−4)	1418.0	7	G6S-A-G6S-A-G6S-A-G6S	1418.2
A4	359.8 (−5)	1804.0	9	G6S-A-G6S-A-G6S-A-G6S-A-G6S	1804.2
A5	364.0 (−6)	2190.0	11	G6S-A-G6S-A-G6S-A-G6S-A-G6S-A-G6S	2190.3
M1	405.0 (−1)	406.0	2	G6S-Aol	406.1
M2	395.0 (−2)	792.0	4	G6S-A-G6S-Aol	792.1
M3	391.7 (−3)	1178.1	6	G6S-A-G6S-A-G6S-Aol	1178.2
M4	390.0 (−4)	1564.0	8	G6S-A-G6S-A-G6S-A-G6S-Aol	1564.2
M5	389.1 (−5)	1950.5	10	G6S-A-G6S-A-G6S-A-G6S-A-G6S-Aol	1950.3

[a] DP: degree of polymerization.
[b] G6S: 1,3-linked-6-sulfated-β-D-galactose; A: 1,4-linked-3,6-anhydro-α-L-galactose; Aol: 4-linked-3,6-anhydro-galactitol.

anGal residues and three sulfate groups. All sulfate groups were linked to the Gal residues by the tandem mass spectra fragmentations analysis. Molecular mass identified for A3 was 1418.2, higher than that of A2, suggesting a heptasaccharide with an additional A-G6S biose unit. A regular increment of 386 mass was observed for the next two fractions, A4 and A5, which were determined as nanasaccharide and undecasaccharide (Table 4), respectively. The detailed sequences of odd-numbered oligosaccharides were corroborated by negative-ion ES-CID-MS/MS. For example, a single charged molecular ion ($[M-Na]^-$) at m/z 1075 of the fully sodiated forms as the precursors was chosen for further analysis as shown in Fig. 3C. The ion at m/z 955, $[M-Na-120]^-$, was a reducing terminal fragment as it shifted to m/z 958 in the spectrum of its alditol. The loss of 120 Da was frequently observed for a reducing terminal hexose as a loss of $C_4H_8O_4$ to give a ^{2,4}A ion (Chai et al., 2006; König & Leary, 1998). However, the cross-ring A-type cleavage ion could not be formed in this oligosaccharide after reduction due to its opening saccharide ring, which thus supported the sulfate loss in the form of $NaHSO_4$. In addition, the spectra feature of extensive B and C ions together with some prominent Y ions in the same

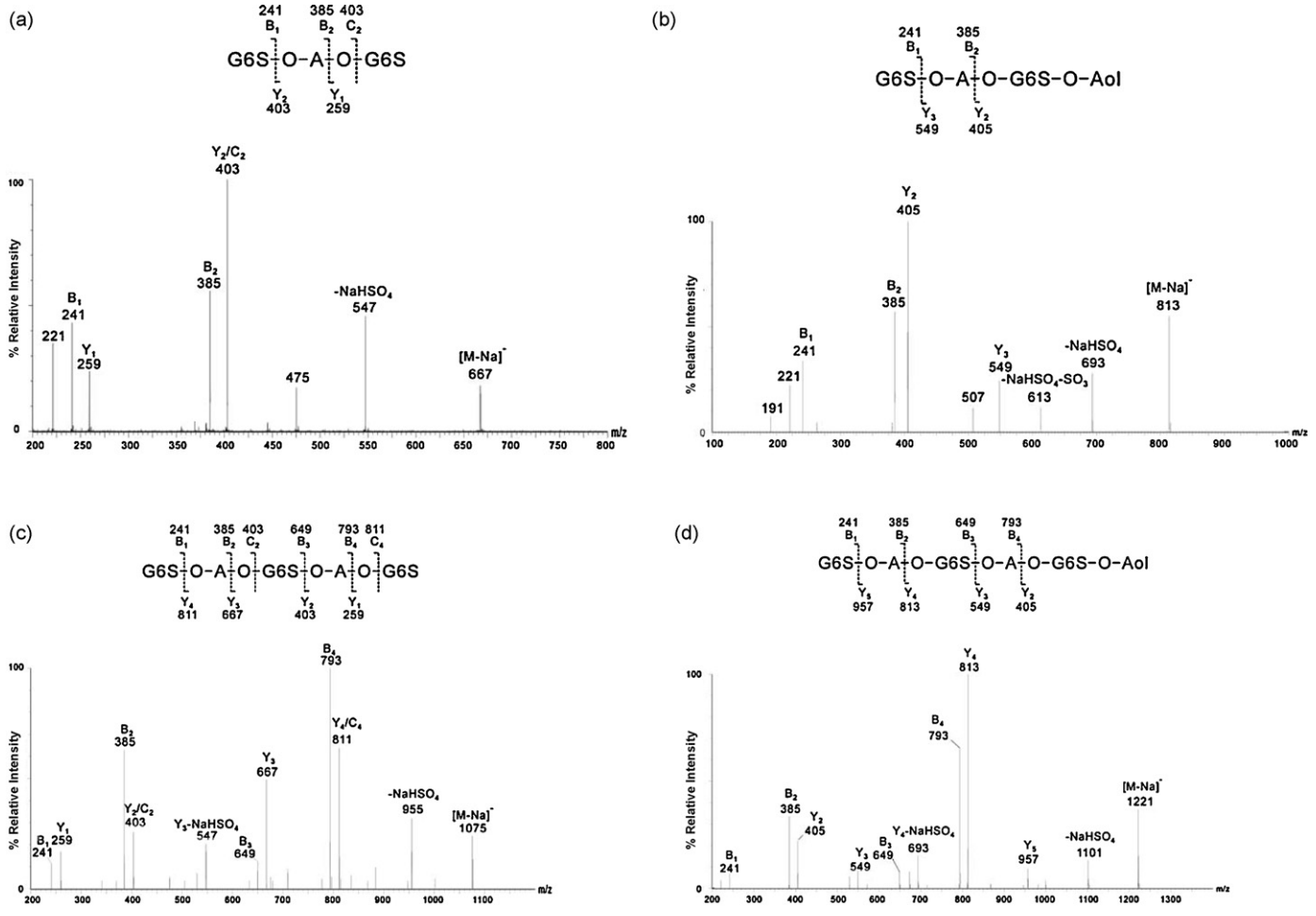

Fig. 3. Negative-ion ES-CID-MS/MS product-ion spectrum of acid and reductive hydrolysis oligosaccharides of polysaccharide GW2M. (A) GW2M-derived trisaccharide. (B) GW2M-derived tetrasaccharide. (C) GW2M-derived pentasaccharide alditol. (D) GW2M-derived hexasaccharide alditol.

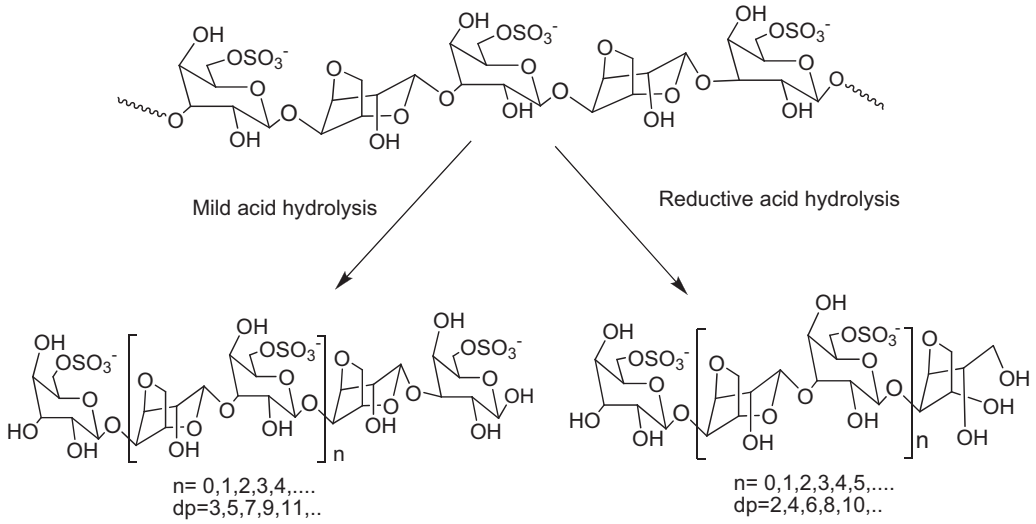

Fig. 4. Chemical structure of 6-*O*-sulfated-agarose from *G. furcata* and oligosaccharides prepared by mild and reductive acid hydrolysis.

spectrum could deduce the sequences of this oligosaccharide. The non-reducing terminal fragment ions were dominated by the B-/C-ion doublets at non-sulfated anGal residues, e.g. B_2 and C_2 (m/z 385 and 403), B_4 and C_4 (m/z 793 and 811), which differed with 18 mass units, a unique feature of the oligosaccharides with ideal biose compositions (Chai et al., 2006). However, intensities of B_1 and B_3 ions are extremely weak, thus there were not obvious corresponding C-ions. Y-type ions were prominently attributed to each fragment of sulfated Gal residues. The ions at m/z 259 and 667 had major contributions of the Y_1 and Y_3 ions, respectively (Fig. 3C). All these data confirmed the detailed sequences of the pentasaccharide were G6S-A-G6S-A-G6S.

Various methods were developed to prevent the degradation of the unstable anGal residue during acid hydrolysis of agaran (Quemener, Lahaye, & Metro, 1995). Conversions of monosaccharides to their alditols by reduction have been successfully used for this purpose (Gonçalves, Ducatti, Duarte, & Noseda, 2002; Stevenson & Furneaux, 1991). We use MMB as the reducing reagent to stabilize oligosaccharides with 3,6-anhydro-galctitol (Aol) at the reducing end. The oligosaccharide alditols from reductive hydrolysis fractionated by gel filtration chromatography, five fractions, M1–M5, were acquired (Fig. 2B). The molecular mass of M1–M5 determined by negative-ion ES-MS (Table 4) was unambiguously identified them as a series of even-numbered alditols. Negative-ion ES-CID-MS/MS of tetrasaccharide alditol M2, using its sodiated ion (m/z 813) as the precursor, indicated the predicted sequence of G6S-A-G6S-Aol, with a reduced terminal anGalol (Fig. 3B). The results indicated that acid labile anGal could be stabilized by reductive reaction. Therefore, the acid hydrolysis products, even-numbered oligosaccharides, can be preserved as alditols. The chemical structures of 6-*O*-sulfated-agarose (GW2M) from *G. furcata* and its oligosaccharides prepared by mild and reductive acid hydrolysis are shown in Fig. 4.

3. Conclusion

From the red seaweed *G. furcata*, we purified a polysaccharide GW2M. The structural feature of GW2M was determined by 1D and 2D NMR techniques, and detailed sequences of a series of GW2M-derived oligosaccharides were confirmed by ES-CID-MS/MS analysis. GW2M is composed of a linear repeating unit of alternating (1 → 3)-linked 6-sulfated-β-D-galactose and (1 → 4)-linked 3,6-anhydro-α-L-galactose which makes it to be an ideal 6-*O*-sulfated-agarose. The ideal structure of GW2M and its oligosaccharides provided useful information for further structure–function relationship study.

Actually, two oligosaccharides, G6S-A-G6S and G6S-A-G6S-A-G6S, were converted into neoglycolipid (NGL) probes by reductive amination method and printed on nitrocellulose membranes for Ricinus communis agglutinin I (RCA_{120}) binding study in our group (Wang, Yu, Han, Wu, et al. 2011; Wang, Yu, Han, Yang, et al., 2011). RCA_{120}, which was widely used in affinity chromatography for separation of glycans (Tsao & Kim, 1981), has been recently applied to study cell surface glycoproteins which recognize oligosaccharides with non-reducing terminal β-D-galactose (Cabezas-Herrera, Moral-Naranjo, Campoy, & Vidal, 1994; Tateno et al., 2008). The GW2M-derived oligosaccharides, G6S-A-G6S and G6S-A-G6S-A-G6S, showed the strongest binding to RCA_{120} (Wang, Yu, Han, Wu, et al. 2011; Wang, Yu, Han, Yang, et al., 2011) which suggested the potential application of these oligosaccharides in marine carbohydrate research and human health.

4. Experiments

4.1. Extraction and purification of polysaccharide

Red seaweed *G. furcata* (Fujian, China) was purchased from Kunshan Yihong Seaweed Co. Ltd. (Jiangsu, China). Extraction of polysaccharides was carried out by the previously reported procedure (Yu et al., 2010). Briefly, the dried and grounded seaweed powder was extracted with 85% ethanol at 80 °C for 4 h (3 times) to remove lipids. The residue was then extracted with 20 volumes of cold water (3 times) and the extracting solutions were combined, concentrated and precipitated with 4 volumes of ethanol. The precipitated polysaccharide (GW) was dialyzed (MWCO 7 kDa) against distilled water and then freeze-dried.

For purification, GW (300 mg) was dissolved in 5 mL distilled water, and applied to Q-Sepharose Fast Flow column (XK 5.0/10 cm) on ÄKTA-Purifier system (Amersham Pharmacia Biotech, Sweden) by stepwise elution with water, 0.5 M, 0.8 M, 1.0 M, 1.2 M, 1.5 M and 2.0 M sodium chloride solutions at a flow rate of 120 mL/h. Fractions containing sugars were tested by phenol–sulfuric acid method, and combined, concentrated, dialyzed and subjected onto gel filtration using Sepharose 6B Fast Flow column (XK 2.6/90 cm). Elution was carried out with 0.2 M NaCl at a flow rate of 12 mL/h. The 2.0 M sodium chloride elution fraction (GW2M) from Q-Sepharose Fast Flow column was collected, dialyzed against distillated water and lyophilized (100 mg, yield 33.3%).

4.2. Purity and relative molecular mass analysis

The purity and relative molecular weight (M_r) of GW2M was determined on HPLC system (LC-20AD, Shimadzu Company, Japan) with a PL aquagel OH column (30 cm × 7.5 mm × 8 μm, Perkin Elmer Company, MA, USA) and eluted with 0.1 M Na_2SO_4 (pH 7.0) at a flow rate of 1.0 mL/min at 35 °C detected with a refractive index detector. The column was calibrated with Dextran standards (788 kDa, 404 kDa, 212 kDa, 112 kDa, 47.3 kDa, 22.8 kDa, 11.8 kDa, 5.9 kDa) purchased from Shodex Company (Tokyo, Japan). The M_r of GW2M was calculated as 20.6 kDa by the corrected regression equation $\log M_r = 14.8636–0.6837t_R$ (t_R, retention time; $r^2 = 0.998$).

4.3. Monosaccharide composition analysis

The monosaccharide alditols (2 mg) were prepared by two-step reductive hydrolysis method and further acetylated as previously described (Gonçalves et al., 2002). The obtained alditol acetates were analyzed by GC (Agilent HP5890 II, USA) using a fused silica capillary column DB-225 (30 m × 0.32 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA). Samples were detected with a flame-ionization detector (FID) at 250 °C, while the injector and oven temperatures were set at 250 °C and 210 °C, respectively. The composition and content of monosaccharides were determined by retention times and peak areas, in comparison with sugar standards (rhamnose, mannose, arabinose, fucose, xylose, galactose and glucose, Sigma Company, Shanghai, China).

4.4. General analysis

Total sugar content was determined by phenol–sulfuric acid method using galactose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of 3,6-anhydro-galactose was determined by resorcinol method using fructose as standard (Yaphe & Arsenault, 1965). Sulfate content was determined by $BaCl_2$–gelatin method (Dodgson & Price, 1962). The content of crude protein was determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

For IR analysis, the sample (1–2 mg) was dried in a P_2O_5 desiccator for 48 h, then mixed with 100 mg dried KBr and pressed under 7 kg/cm^2 to make a transparent film. The film was put into the FTIR spectrometer (Nicolet Nexus 470, Thermo Electron, USA) and scanned from 400 to 4000 cm^{-1}.

4.5. Desulfation and methylation analysis

Desulfation was operated as described (Kolender & Matulewicz, 2004) with some modifications. In brief, GW2M (20 mg) was first passed through an Amberlite IR 120 (H^+ form) column (20 mL) eluted with 40 mL water. The eluent was collected and adjusted to pH 9.0 with pyridine to get the GW2M-pyridine salt, then rotatory-evaporated and freeze-dried. The desulfated reaction (10 mg) was carried out in 2 mL of DMSO/methanol (volume ratio = 9:1) in a sealed Teflon tube at 80 °C for 10 h. Desulfated product was recovered by dialysis and then freeze-dried.

Methylation was performed as described previously (Hakomori, 1964). The methylated polysaccharide was hydrolyzed, reduced and acetylated as described above. Thereafter, partial methylated alditol acetates were analyzed by GC–MS that equipped with a DB-225MS (30 m × 0.32 mm × 0.25 μm) fused-silica capillary column under conditions reported by Yang et al. (2011). Mass spectra of the compounds were analyzed using Complex Carbohydrate Structural Database (CCSD) of Complex Carbohydrate Research Centre (http://www.ccrc.uga.edu/).

4.6. NMR spectroscopy

The sample (30 mg) was dissolved in 0.5 mL D_2O and freeze-dried twice to replace all exchangeable protons with deuterium. Both ^1H and ^1H–^1H 2D NMR were acquired at 25 °C using a JNM-ECP 600 MHz spectrometer and Delta software. The HOD signal served as a reference. Shift values were confirmed using acetone-d6 as an internal standard to give a signal at 2.22 ppm. In the ^1H–^1H COSY spectra, a 12-ppm spectral width was used in both dimensions. In F^2, 1024 complex points were collected while the resolution in the F^1 dimension was 1024 complex points using States Habercorn phase cycling. The ^{13}C spectrum was recorded on a JNM-ECP 600 MHz spectrometer; acetone-d6 served as an external reference. The ^1H–^{13}C HMQC and ^1H–^{13}C HMBC were run on a JNM-ECP 600 using a 1k × 1k matrix (80 scans per block, an acquisition time of 0.213 s).

4.7. Preparation and sequence analysis of oligosaccharides

4.7.1. Preparation and separation of oligosaccharides

GW2M was hydrolyzed with 0.1 M H_2SO_4 (10 mg/mL) at 60 °C for 2 h followed by neutralization with 2 M NaOH. The neutralized solution was mixed with the same volume of ethanol and centrifuged. The supernatant was collected, freeze-dried, and separated on a Superdex peptide column (1.0 cm × 30 cm) eluted with 0.1 M NH_4HCO_3 at a flow rate of 0.1 mL/min. Five oligosaccharide fractions (A1–A5) were collected, concentrated and freeze-dried.

At the meantime, GW2M was reductive hydrolyzed with 0.1 M H_2SO_4 (10 mg/mL) with 0.2 M MMB and five oligosaccharide alditols (M1–M5) were obtained.

4.7.2. ESI-MS and ES-CID-MS/MS analysis of oligosaccharides

In order to analyze the oligosaccharide sequences, deuterium reductive reaction was needed. Briefly, $NaBD_4$ reagent was added to the freeze-dried oligosaccharides, and the reductive reaction was carried out at 4 °C overnight as described (Yang et al., 2011). The reaction solution was then neutralized before passing through a mini cation-exchange column (AG50W-X8, H^+ form, Bio-Rad, USA). The by-product boric acid was removed by repeating co-evaporation with methanol under nitrogen gas.

ESI-MS and ES-CID-MS/MS analysis on Micromass Q-Tof Ultima instruments (Waters, Manchester, U.K.) was performed for all oligosaccharides sequence analysis (Yu et al., 2006) according to the procedure described by Yang et al. (2011).

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