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ORIGINAL ARTICLE

Structural characteristics and anti-complement activities of polysaccharides from *Sargassum hemiphyllum*

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Abstract

Three polysaccharides (SH-1, SH-2 and SH-3) were purified from a brown macroalgea, *Sargassum hemiphyllum*. The autohydrolysis products from each polysaccharide were separated to three fractions (S fractions as oligomers, L fractions as low molecular weight polysaccharides and H fractions as high molecular weight polysaccharides). Mass spectroscopy of S fractions (SH-1-S, SH-2-S and SH-3-S) showed that these three polymers all contained short stretches of sulfated fucose. The structures of L fractions (SH-1-L, SH-2-L and SH-3-L) were determined by nuclear magnetic resonance (NMR). SH-1-L was composed of two units, unit A (sulfated galactofucan) and unit B (sulfated xylo-glucuronomannan). Unit A contained a backbone of (1, 6-linked β-D-Gal) n_1, (1, 3-linked 4-sulfated α-L-Fuc) n_2, (1, 3-linked 2, 4-di-sulfated α-L-Fuc) n_3, (1, 4-linked α-L-Fuc) n_4 and (1, 3-linked β-D-Gal) n_5, accompanied by some branches, such as sulfated fuco-oligomers, sulfated galacto-oligomers or sulfated galacto-fuco-oligomers. And unit B consisted of alternating 1, 4-linked β-D-glucuronic acid (GlcA) and 1, 2-linked α-D-mannose (Man) with the Man residues randomly sulfated at C6 or branched with xylose (Xyl) at C3. Both SH-2-L and SH-3-L were composed of unit A and their difference was attributed to the ratio of n_1: n_2: n_3: n_4: n_5. Based on monosaccharide analysis, we hypothesize that both SH-1-H and SH-2-H contained unit A and unit B while SH-3-H had a structure similar to SH-3-L. An assessment of anti-complement activities showed that the sulfated galactofucan had higher activities than sulfated galacto-fuco-xylo-glucuronomannan. These results suggest that the sulfated galactofucans might be a good candidate for anti-complement drugs.

Keywords Sulfated galactofucan · Sulfated galacto-fuco-xylo-glucuronomannan · Fucoidan · Anticomplement activity · *Sargassum hemiphyllum*

Introduction

Sargassum hemiphyllum, a brown macroalgae, is widely distributed around the seashores in the Far East, including Korea, Japan and China [1]. It has been reported that *S. hemiphyllum* contains many bioactive compounds, such as sulfoglycolipids, fucoxanthins, phlorotannins and polyphenols [2, 3]. Studies on the water extraction of polysaccharides from *S. hemiphyllum* have been reported [1, 4–10]. Hwang and coworkers reported that polysaccharides from *S. hemiphyllum* displayed anti-

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inflammatory activities involving down-regulation of NF-kappa B in the nucleus [1, 4]. Long noncoding RNAs (IncRNAs) switched on oligo-fucoidan-induced apoptosis [5]. Water, ethanol and acetone extraction products can be used as pharmaceuticals and functional foods that can serve to reduce the dosages of synthetic diabetes drugs [3, 6]. Low molecular weight (LMW) polysaccharides can trigger osteogenic differentiation *in vitro* and have an anabolic effect on bone mineralization *in vivo*, suggesting that LMW polysaccharides might play a role in decreasing enhanced bone loss with increasing age [7]. Moreover, there are reports showing these polysaccharides exhibit antioxidant and neuroprotective activities [8, 9]. These polysaccharides have been suggested as cosmetic ingredient in cosmeceuticals owing to the skin whitening and anti-wrinkling activity [10]. Although there are many studies on the bioactivities of *S. hemiphyllum* polysaccharides (SH), a detailed characterization of the structures of these polysaccharides has not been reported.

The complement system, as a part of innate immunity, displays a wide array of functions, protecting against foreign organisms, removing immune complexes from the circulation, and cleaning up of cellular debris that accumulates over time [11, 12]. However, when complement regulatory proteins are overactive, the complement system will become disordered, leading to diseases, such as paroxysmal nocturnal hemoglobinuria, glomerulonephritis, heart disease and cancer [11, 13, 14]. Therefore, various complement inhibitors have been developed. According to the principle of anti-complement therapy, these inhibitors can be divided into five types: (1) C5 convertase inhibitor; (2) C1 convertase inhibitor; (3) replacements for deficient complement inhibitors; (4) augmenter of complement inhibition; and (5) multi-level inhibitors of the complement system [11]. Heparin, a multi-level inhibitor of the complement system, is a naturally occurring glycosaminoglycan [15]. Like heparin, many polysaccharides [16–21] also display anti-complement activity, indicating that polysaccharides might be the good candidates as the inhibitor of the complement system.

In the current study, we elucidated the structures of polysaccharides purified from *S. hemiphyllum (SH)*. Fractions of autohydrolysis products from these polysaccharides were subjected to mass spectrometry (MS) and NMR analysis. The anti-complement activities of SH and its derivatives were then evaluated. The results from this study should lead to the development of anti-complement therapeutics.

Materials and methods

Preparation and purification of polysaccharides

The brown seaweeds *S. hemiphyllum* were collected in Zhanjiang, China. Polysaccharides (SH) from *S. hemiphyllum* were prepared according to a modified method from previous studies [22]. SH (5 g) was purified by anion exchange chromatography on a DEAE-Bio Gel Agarose FF gel (6 cm × 40 cm) with elution by water (5 L), 0.5 M NaCl (5 L, SH-1), 1 M NaCl (5 L, SH-2) and 2 M NaCl (5 L, SH-3) at a flow rate of 10.0 mL/min to obtain different fractions. The polysaccharide fractions were desalted by dialysis, concentrated and precipitated by ethanol.

Depolymerization of SH-1, SH-2 and SH-3 by autohydrolysis

The autohydrolysis was performed according to the modified method [23]. Briefly, polysaccharide samples (1.0 g) were converted to their H^+-form by using a cation-exchange column and left for 72 h at room temperature for autohydrolysis. The mixtures obtained were neutralized with 5% NH_4OH, then, concentrated and ethanol precipitated. Six fractions were obtained. The three supernatants were named as S-type fractions, SH-1-S, SH-2-S and SH-3-S, and the three precipitates were named as C-type fractions, such as SH-1-C, SH-2-C and SH-3-C. S-type fractions (SH-1-S, SH-2-S and SH-3-S) were desalted on a Sephadex G-10 column (4.5 × 40 cm) while C-type fractions (SH-1-C, SH-2-C and SH-3-C) were fractionated on a Bio-Gel P-10 Gel column (2.6 × 100 cm) eluted with 0.5 M NH_4HCO_3 into high molecular weight fractions (SH-1-H, SH-2-H and SH-3-H) and low molecular weight fractions (SH-1-L, SH-2-L and SH-3-L). The final products were desalted on a Sephadex G-10 column (4.5 × 40 cm).

Compositional analysis

The sulfate contents were determined by ion chromatography on a Shodex IC SI-52 4E column (4.0 × 250 mm) and eluted with 3.6 mM Na_2CO_3 at a flow rate of 0.8 mL/min at 45 °C [24]. The molar ratios of monosaccharides and fucose contents were determined as described by Zhang et al. [25]. Briefly, polysaccharides (10 mg/mL) were hydrolyzed by trifluoroacetic acid (2 M) under a nitrogen atmosphere for 4 h at 110 °C. The hydrolyzed mixture was neutralized to pH 7 with sodium hydroxide. The mixture was converted into its 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives and separated by high performance liquid chromatography (HPLC) on a YMC Pack ODS AQ column (4.6 × 250 mm). Uronic acid (UA) concentration was determined by the modified carbazole method [26]. Briefly, 6 mL sulfuric acid was carefully added into the sample or standard in ice. After shaking, the solution was heated in the boiling bath for 20 min and cooled to room temperature. Carbazole reagent (0.2 mL) was added and after 2 h absorbance was determined at 530 nm. The molecular weights of the polysaccharides were evaluated by gel permeation chromatography (GPC)-HPLC on TSK G3000 PWxl column (7 μm 7.8 × 300 mm) with elution in

0.05 M Na_2SO_4 at a flow rate of 0.5 mL/min at 40 °C with refractive index detection. Ten different molecular weight dextrans purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) were used as weight standards.

Infrared spectroscopy

IR spectra were determined on a Nicolet-360 FTIR spectrometer (36 scans, at a resolution of 6 cm^{-1}) between 400 and 4000 cm^{-1} using powders pressed into KBr pellets.

Desulfation and methylation

The desulfation of polysaccharides was performed according to the modified method of Nagasawa et al. [27–29]. Briefly, sample (10 mg) was dissolved in distilled water (1 mL) and mixed with cationic resin (H^+–Na^+) for 3 h. After filtration, the solution was neutralized with pyridine and lyophilized. The pyridine salt was dissolved in dimethylsulfoxide (DMSO): methanol: pyridine (9:1:0.1; v/v). The mixture was heated at 80 °C for 4 h, and the desulfated product was dialyzed and lyophilized to obtain desulfated sample.

The methylation of desulfated sample was performed based on the modified method of Hakomori [30]. Briefly, NaOH (80 mg) was dissolved in dimethylsulfoxide (1 mL) under nitrogen at 50 °C for 4 h until it turned to a dark-green solution. Then, desulfated sample (2 mg) and CH_3I (0.6 mL) were added to the mixture, and the reaction was stirred at room temperature for 2 h. The methylated sample was hydrolyzed with 2 M trifluoroacetic acid at 110 °C for 4 h and evaporated to obtain the hydrolysate. The hydrolysate was reduced with $NaBH_4$ and acetylated with acetic anhydride in pyridine. Finally, the product was analyzed using GC-MS.

MS analysis of oligosaccharides

Electrospray ionization (ESI)-MS was performed on a LTQ ORBITRAP XL (Thermo Scientific, Waltham, MA). The samples were dissolved in CH_3CN-H_2O (1:1, v/v). The solution was centrifuged, and the supernatant was analyzed. Mass spectra were acquired in the negative ion mode at a flow rate of 5 μL/min. The capillary voltage was set to − 3000 V, and the cone voltage was set at − 50 V. The source temperature was 80 °C, and the desolvation temperature was 150 °C. All spectra data were processed by Xcalibur.

NMR spectroscopy

Polysaccharides (50 mg) were deuterium oxide (99.9%) exchanged twice before dissolving in deuterium oxide (99.9%) containing 0.1 μL deuterated acetone. NMR and two-dimensional spectra were recorded on a Bruker AVANCE III 600 MHz (Billerica, MA) at 25 °C. The chemical shifts in the proton and the carbon spectra were adjusted using an internal standard (deuterated acetone, 2.05 and 29.92 ppm, respectively).

Anti-complement activities

The anti-complement activities of the polysaccharides were determined on the classical pathway using the methods described in previous studies [31]. For the classical pathway, various dilutions (100 μL) of polysaccharides were mixed with 1:10 diluted normal human serum (NHS, obtained from healthy adult donors) (100 μL), GVB^{2+} (veronal buffer saline [VBS] containing 0.1% gelatin, 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}) (200 μL) and sensitized erythrocytes (EA) (200 μL). Then, the mixtures were incubated at 37 °C for 30 min. The following assay controls were incubated under the same conditions: (1) 100% lysis: EA (200 μL) in water (400 μL); (2) sample control: sample (100 μL) in GVB^{2+} (500 μL); (3) complement: 1:10-diluted NHS (100 μL) and EA (200 μL) in GVB^{2+} (300 μL); and (4) blank: EA (200 μL) in GVB^{2+} (400 μL). After incubation, the mixture was centrifuged (5000 rpm × 10 min) and the erythrocyte lysis was determined at 405 nm. Decreased lysis in the presence of the tested polysaccharides indicated anti-complement activities. All of the samples were dissolved in GVB^{2+}. The percent inhibition was calculated using the following equation:

$$\text{inhibition of EA lysis}\ (\%) = \left(A_{\text{complement}} - \left[A_{\text{sample}} - A_{\text{sample control}}\right]\right)/A_{\text{complement}} \times 100.$$

The inhibition of the alternative complement pathway was determined using 150 μL of various dilutions of tested samples that were mixed with 150 μL of 1:10-diluted NHS and 200 μL of rabbit erythrocytes (ER). The mixture was then incubated for 30 min at 37 °C. Cell lysis was determined by the same method as described above for the classical pathway. Controls for 100% lysis, sample control, complement and blank were included. The percent inhibition was calculated using the following equation:

$$\text{inhibition of ER lysis}\ (\%) = \left(A_{\text{complement}} - \left[A_{\text{sample}} - A_{\text{sample control}}\right]\right)/A_{\text{complement}} \times 100.$$

Statistical analysis

All data are shown as the mean ± standard deviation (SD). Significant differences between experimental groups were determined by one-way ANOVA, and differences were

considered as statistically significant if $p < 0.05$. All calculations were performed using SPSS 16.0 Statistical Software.

Results and discussion

Polysaccharides (SH-1, SH-2 and SH-3) were obtained by anion exchange chromatography on a DEAE-Bio Gel Agarose FF gel (6 cm × 40 cm) with elution by 0.5 M, 1 and 2 M NaCl, respectively. Autohydrolysis reaction has been widely used for the structural determination of polysaccharides [23, 32–35]. Autohydrolysis reactions were performed to obtain the structures of SH-1, SH-2 and SH-3. Six fractions, including S-type fractions (SH-1-S, SH-2-S, SH-3-S) and C-type fractions (SH-1-C, SH-2-C and SH-3-C), were obtained after ethanol precipitation. MS was performed to analyze S-type fractions (SH-1-S, SH-2-S and SH-3-S). The results are presented in Fig. 1. C-type fractions (SH-1-C, SH-2-C and SH-3-C) were next further fractionated on a Bio-Gel P-10 Gel column, which yielded high molecular weight H-type fractions (SH-1-H, SH-2-H and SH-3-H) low molecular weight and L-type fractions (SH-1-L, SH-2-L and SH-3-L). The chemical compositions of these polysaccharide fractions are shown in Table 1.

The yields of the fractions SH-1-S, SH-2-S and SH-3-S were 5.25%, 3.25% and 4.15% (w/w), respectively. MS results in Fig. 1 showed that they contained mono-sulfated fucose (*m/z* 243 (-1), FS), mono-sulfated difucoside (m/z 389 (-1), F2S), di-sulfated fucose (m/z 161 (-2), FS2) and di-sulfated difucoside (m/z 234 (-2), F2S2) [22, 23]. Based on a previous study [22], the major components of SH-1-S, SH-2-S and SH-3-S were suggested to be the branches or the ends of SH-1, SH-2 and SH-3, respectively, while the structures of SH-1-C, SH-2-C and SH-3-C represented the polysaccharide backbones.

NMR spectra of the polysaccharide mixtures having high molecular weight can be complex and ambiguous. C-type fractions were sub-fractionated into H-type fractions having high molecular weight and into L-type fractions of low molecular weight. It is interesting to note that the L-type fractions mainly consisted of sulfated galactofucan, with the exception of SH-1-L, which was also accompanied with xylo-glucuronomannan. The H-type fractions were heteropolysaccharides, with the exception of SH-3-H, which was a sulfated galactofucan. In addition, it was found (see Table 1) that the molar ratios of Gal to Fuc were decreased in SH-1-L, SH-2-L and SH-3-L, while the molar ratios of Gal to Fuc in the H-type fractions was SH-2-H > SH-3-H > SH-1-H. These results could be explained by the different stability of linkages to Gal, Man and GlcA.

The SH-2 and SH-3 polysaccharides were mainly sulfated galactofucans. Gal residues in sulfated galactofucans are more stable towards autohydrolysis than Fuc residues [36]. This explains why the molar ratios of Gal to Fuc in SH-2-H and SH-3-H are larger than the Gal to Fuc ratio in SH-2-L and SH-3-L, respectively. Both SH-1-H and SH-1-L are heteropolysaccharides, and include xylo-glucuronomannans. Xylo-glucuronomannan is more stable than galactofucan, thus, galactofucan was more easily degraded in autohydrolysis, explaining why SH-1-L shows a larger molar ratio of Gal to Fuc.

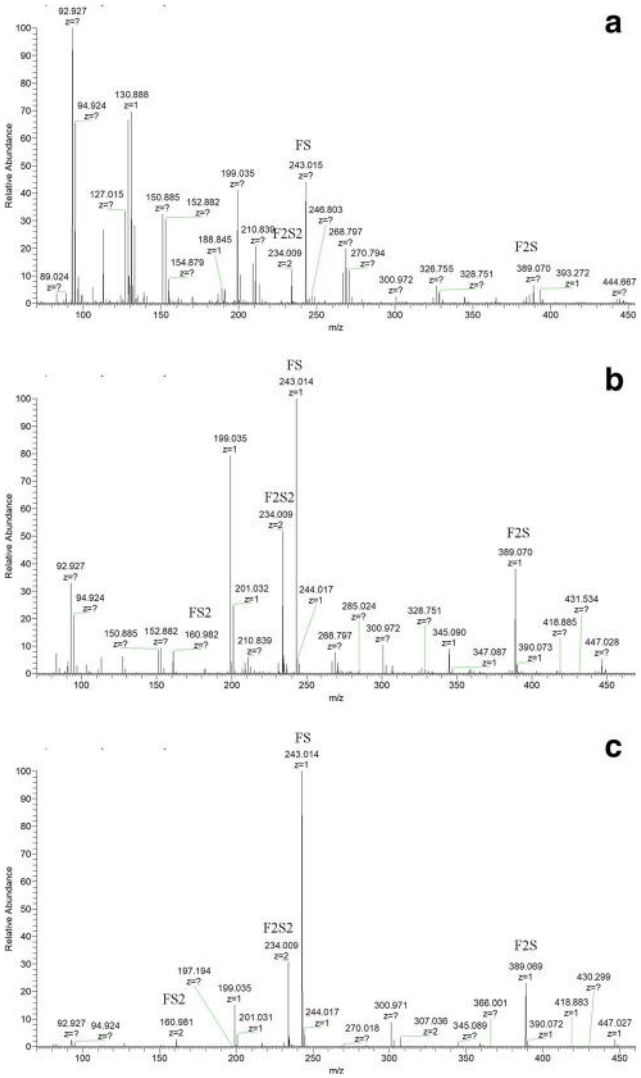

Fig. 1 Negative-ion mode ESI-MS spectra of SH-1-S (**A**), SH-2-S (**B**) and SH-3-S (**C**). (FS stands for mono-sulfated fucose, F2S stands for mono-sulfated difucoside, FS2 stands for di-sulfated fucose and F2S2 stands for di-sulfated difucoside.)

The IR spectra of SH-1-H, SH-1-L, SH-2-H, SH-2-L, SH-3-H and SH-3-L are presented in Fig. 2. The band at approximately 1640 cm^{-1} was assigned to the bending vibration of water. The band at approximately 1450 cm^{-1} was assigned to the scissoring vibration of CH_2 and asymmetric bending vibration of CH_3 [37–39]. The very intense and broad band at approximately 1200 cm^{-1} was assigned to the asymmetric O = S = O stretching vibration of sulfate esters with some contribution from COH, CC and CO vibrations, while the

Table 1 Chemical compositions of the polysaccharides studied

Samples	Yields (%)	Fuc (%)	SO_4 (%)	UA (%)	Total Sugar (%)	Mw (kDa)	Monosaccharides (molar ratio)				
							Man	GlcA	Gal	Xyl	Fuc
SH-1	28.65	11.24	16.21	13.84	81.44	170.1	0.28	0.27	0.28	0.36	1
SH-1-H	76.2	13.92	14.17	18.65	86.07	141.4	0.52	0.31	0.30	0.34	1
SH-1-L	12.4	12.81	20.75	10.87	63.56	9.5	0.08	0.09	0.82	0.21	1
SH-2	19.48	31.60	23.33	4.87	65.42	116.8	-	0.03	0.27	0.03	1
SH-2-H	48.0	28.17	29.76	12.86	74.48	97.6	0.15	0.17	0.77	0.15	1
SH-2-L	48.1	42.44	28.19	-	60.18	19.1	-	-	0.28	-	1
SH-3	12.35	37.12	32.02	-	53.24	148.0	-	-	0.22	-	1
SH-3-H	20.1	30.14	39.70	-	51.44	114.2	-	-	0.43	-	1
SH-3-L	56.6	44.29	31.35	-	61.29	10.1	-	-	0.17	-	1

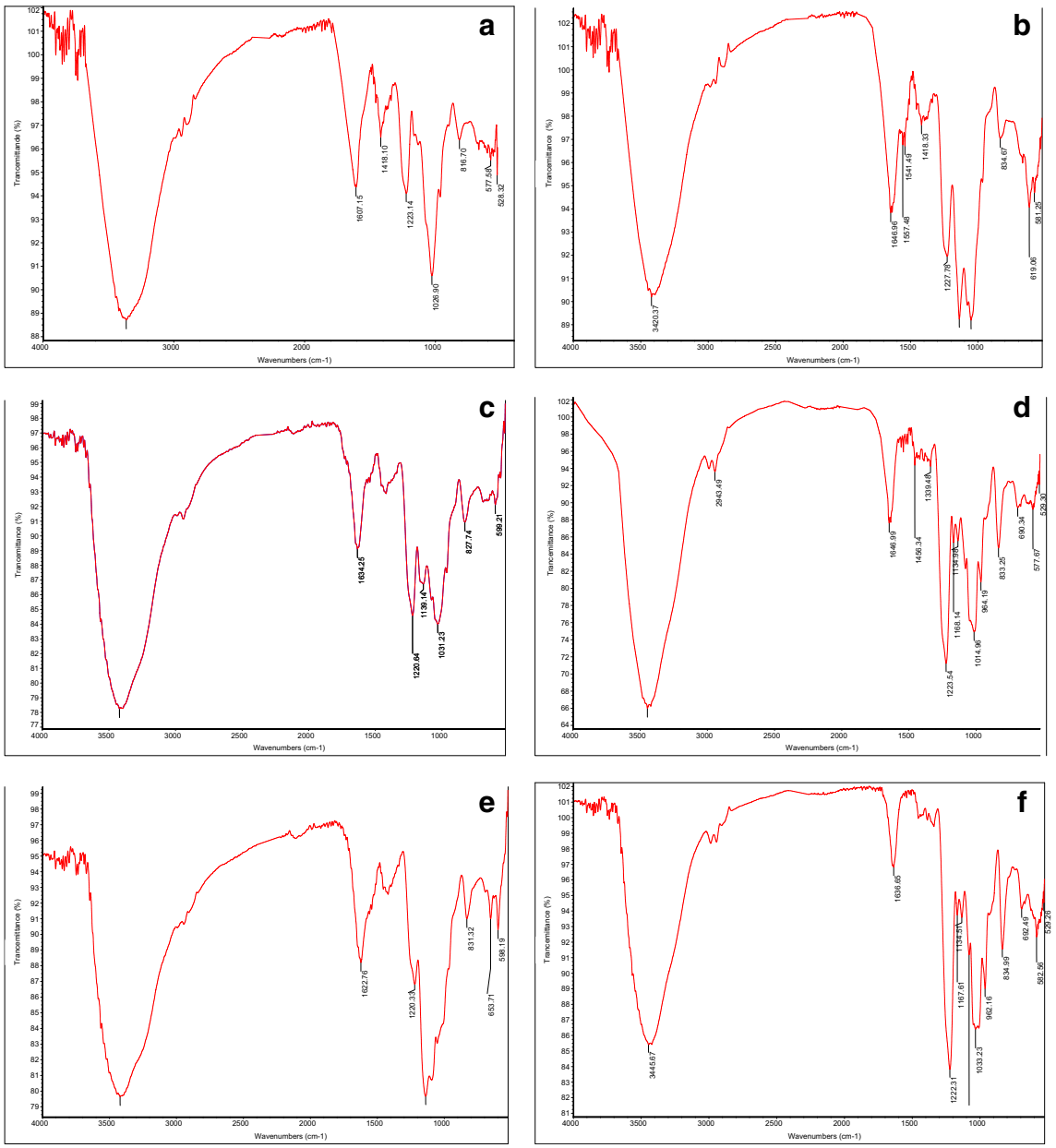

Fig. 2 .IR spectra of SH-1-H (**A**), SH-1-L (**B**), SH-2-H (**C**), SH-2-L (**D**), SH-3-H (**E**) and SH-3-L (**F**)

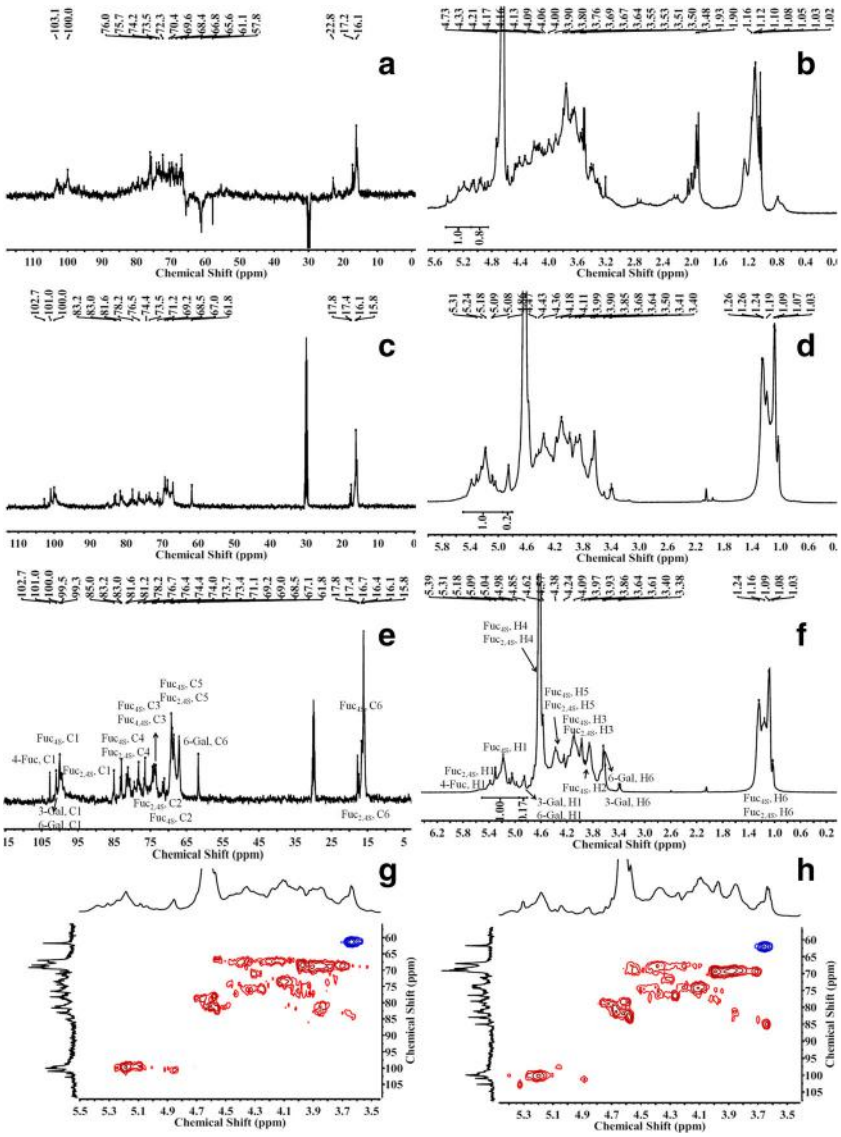

Fig. 3 The DEPTQ spectrum of SH-1-L (**A**), ^{1}H-NMR spectrum of SH-1-L (**B**), ^{13}C-NMR spectra and ^{1}H-NMR spectra of SH-2-L (**C** and **D**) and SH-3-L (**E** and **F**), HSQC spectra of SH-2-L (**G**) and SH-3-L (**H**). (6-Gal corresponds to 1, 6-linked β-D-Gal residues; Fuc_{4S} stands for 1, 3-linked 4-sulfated α-L-Fuc residues; $Fuc_{2,4S}$ stands for 1, 3-linked 2, 4-di-sulfated α-L-Fuc residues; 4-Fuc stands for 1, 4-linked α-L-Fuc residues; 3-Gal stands for 1, 3-linked β-D-Gal residues.)

Table 2 Methylation analysis of desulfated SH-3-L

Methylation products	Deduced position of substitution	Peak area percentage (%)
2,3,4-Fuc	Fuc (1→	6.0
2,4-Fuc	→3) Fuc (1→	24.8
2,3-Fuc	→4) Fuc (1→	9.5
3,4-Fuc	→2) Fuc (1→	5.4
2-Fuc	→3,4) Fuc (1→	7.0
3-Fuc	→2,4) Fuc (1→	4.3
4-Fuc	→2,3) Fuc (1→	8.0
2,3,4,6-Gal	Gal (1→	5.1
2,4,6-Gal	→3) Gal (1→	5.6
2,3,6-Gal	→4) Gal (1→	2.1
2,3,4,-Gal	→6) Gal (1→	12.8
2,3 -Gal	→4,6) Gal (1→	3.8
2,4-Gal	→3,6) Gal (1→	5.6

band at approximately 840 cm^{-1} was assigned to sulfate groups at the axial C-4 positions and the band at 820 cm^{-1} was assigned to sulfate groups at the equatorial C-2 or C-3 positions. The bands of L-type fractions (SH-1-L, SH-2-L and SH-3-L), at approximately 830 cm^{-1}, indicate that they are mainly sulfated at C4. These results are consistent with the previous results [23, 40], that show that sulfate groups substituted at the equatorial C2 and C3 of Fuc and Gal residues are easier to lose during autohydrolysis than sulfate groups at the axial C4 position. Most significantly, the bands of H-type fractions (SH-2-H and SH-3-H), at approximately 830 cm^{-1}, were identical with those in the L-type fractions; however, SH-1-H was at 816 cm^{-1}, which is explained by sulfation at C6 of Man and Gal.

NMR spectroscopy of SH-1-L, SH-2-L and SH-3-L was performed to elucidate their structural features (Fig. 3). It is interesting to note that SH-2-L and SH-3-L generally had

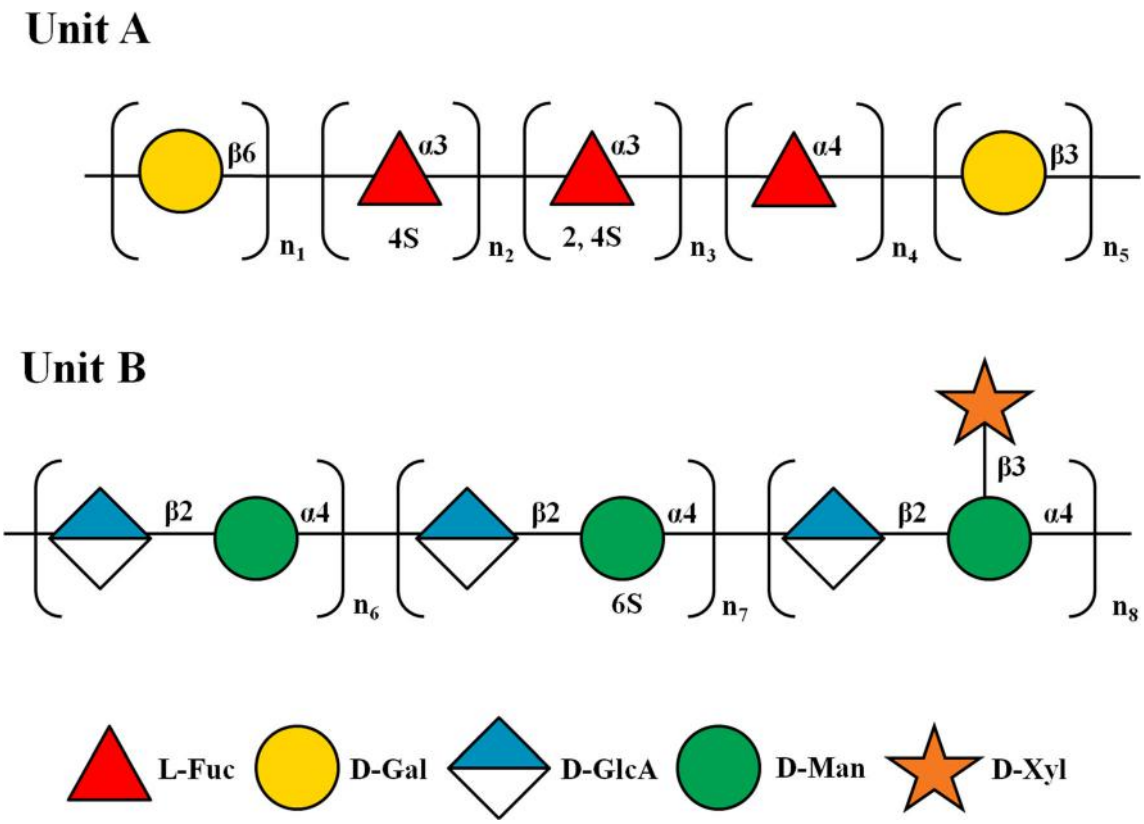

Fig. 4 The proposed structure feature of SH polysaccharides

similar ^{13}C-NMR and ^{1}H-NMR spectra. Combined with the above results (Table 1), the molar ratio of sulfate group to Fuc (calculated from the following formula: (sulfate content/96)/(fucose content/164)) for SH-2-L was 1.13 while it was 1.21 for SH-3-L. The molar ratio of Gal to Fuc of SH-3-L was 0.22 while the molar ratio of Gal to Fuc for SH-3-L was 0.17. SH-2-L and SH-3-L had similar IR spectra, indicating that they had similar functional groups. Thus, we conclude that SH-2-L and SH-3-L have similar structural features. Methylation was also performed to determine the glycosidic linkages of desulfated SH-3-L. The results in Table 2 show that there are thirteen linkage patterns of desulfated SH-3-L: Fuc (6.0%), 1,3-linked Fuc (24.8%), 1,4-linked Fuc (9.5%), 1,2-linked Fuc (5.4%), 1,3,4-linked Fuc (7.0%), 1,2,4-linked Fuc (4.3%), 1,2,3-linked Fuc (8.0%), Gal (5.1%), 1,3-linked Gal (5.6%), 1,4-linked Gal (2.1%), 1,6-linked Gal (12.8%), 1,4,6-linked Gal (3.8%) and 1,3,6-linked Gal (5.6%). Combined with the results of NMR, it was suggested that chemical shifts at 100.0 ppm (C-1) / 5.18 ppm (H-1), 67.1 ppm (C-2) / 3.97 ppm (H-2), 76.4 ppm (C-3) / 4.09 ppm (H-3), 78.23–83.25 ppm (C-4) / 4.62 ppm (H-4), 68.5 ppm (C-5) / 4.38 ppm (H-5) and 15.8–17.8 ppm (C-6) / 1.03–1.24 ppm (H-6) are characteristic of 1, 3-linked 4-sulfated α-L-fucan [35, 41–43]. Chemical shifts at 99.3–99.5 ppm (C-1) / 5.31 ppm (H-1) are characteristic of 1, 3-linked 2, 4-di-sulfated α-L-fucan [35, 41–43]. Chemical shifts at approximately 102.7 ppm (C-1) / 5.39 ppm (H-1) are characteristic of 1, 4-linked α-L-fucan with sulfation or branches [44, 45]. In addition, chemical shifts at approximately 100.96 ppm (C-1) / 4.84–5.08 ppm (H-1) were assigned to 1, 6-linked β-D-Gal or 1,3-linked β-D-Gal with sulfation or branches [34, 46–54]. Peak at 61.8 ppm / 3.61–3.64 ppm in HSQC spectra of SH-2-L and SH-3-L indicated the C-6/H-6 of 1, 6-linked β-D-Gal residues. The ratio of the Gal residue peak area to the Fuc residue peak area for SH-2-L (Fig. 3D) was 0.22, while this ratio for SH-3-L was 0.17, consistent with the molar ratios of Gal to Fuc presented in Table 1. Previous studies [22, 46–54] showed that galactofucan had a backbone of Gal and Fuc with main branches of sulfated fuco-oligomers, sulfated galacto-oligomers or sulfated galacto-fuco-oligomers. The signals of anomeric proton and carbon between backbone residues and branch residues were overlapped so that it was hard to determine the linkage and type of monosaccharides, like galactopyranose or fucopyranose. Further analysis is needed to dissect the branch structures of the sulfated galactofucan. Therefore, in this study, we confirmed that SH-2-L and SH-3-L had a backbone of (1, 6-linked β-D-Gal) n_1, (1, 3-linked 4-sulfated α-L-Fuc) n_2, (1, 3-linked 2, 4-di-sulfated α-L-Fuc) n_3, (1, 4-linked α-L-Fuc) n_4 and (1, 3-linked β-D-Gal) n_5. The difference of the backbone between SH-2-L and SH-3-L was attributed to the ratio of n_1: n_2: n_3: n_4: n_5. In addition, there might be some branches, such as sulfated fuco-oligomers, sulfated galacto-oligomers or sulfated galacto-fuco-oligomers.

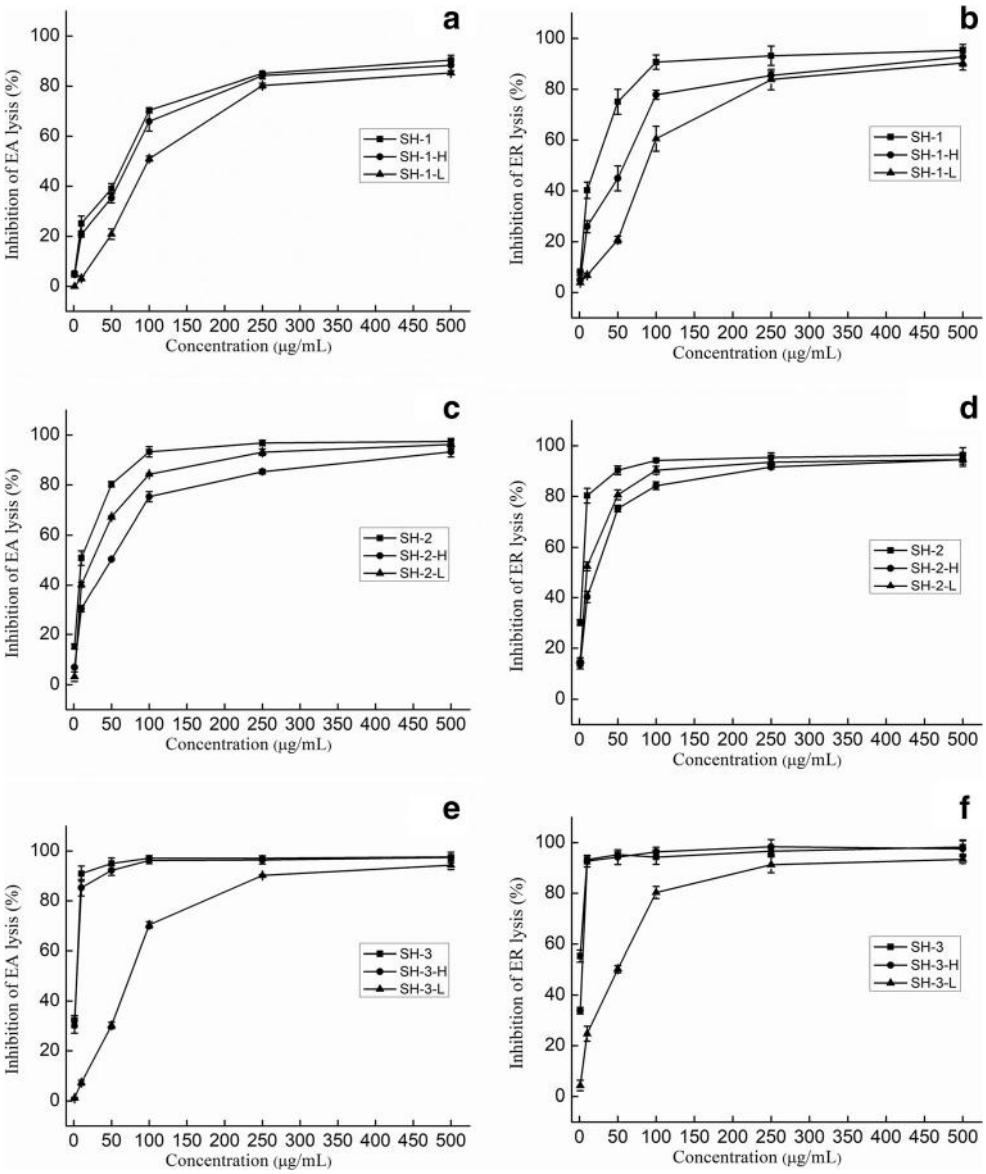

Fig. 5 Inhibition of the classical pathway-mediated haemolysis of EA (**A**, **C** and **E**) and alternative pathway-mediated hemolysis of ER (**B**, **D** and **F**) in 1:10-diluted NHS in the presence of increasing amounts of SH-1, SH-2, SH-3 and their derivatives. The results are expressed as percent inhibition of haemolysis. Data represent the means from three determinations +/- S.E.M

The NMR spectrum of SH-1-L was more complicated than the spectra of SH-2-L and SH-3-L. Although SH-1-L had smaller molecular weight, it contained Xyl, Man and GlcA besides Fuc and Gal. We propose that SH-1-L was a galacto-fuco-xylo-glucuronomannan, similar to SH-2-H, SH-1-H and SH-1. According to the previous studies [22, 51, 55], glucuronomannan might be the backbone of SH-1-L, which was confirmed by the the molar ratio of GlcA to Man (1.125 : 1).

There are remaining questions about the linkages to Xyl, Gal and Fuc. The ratio of Gal residues peak area (chemical shifts from 5.08 ppm to 4.84 ppm) to Fuc residues (chemical shifts from 5.44 ppm to 5.09 ppm, including 1, 2-linked α-D-Man residues), based on peak areas, was 0.84, which was similar to the molar ratio of Gal to Fuc (0.82). Therefore, we suggest that SH-1-L has a backbone of (1, 6-linked β-D-Gal) n_1, (1, 3-linked 4-sulfated α-L-Fuc) n_2, (1, 3-linked 2, 4-di-sulfated α-L-Fuc) n_3, (1, 4-linked α-L-Fuc) n_4 and (1, 3-linked β-D-Gal) n_5. Compared to SH-2-L and SH-3-L, we found that there were only two anomeric carbon signals at 100.0 ppm and 103.1 ppm. The anomeric carbon signal at 100.0 ppm might be overlapped or suppressed because the distorsionless enhancement by polarization transfer including the detection of quaternary nuclei (DEPTQ) spectrum making the structural assignment of SH-1-L ambiguous. The reversal peak at 65.6 ppm is attributed to the Gal or Man residues sulfated at C6. The molar ratio of Fuc to sulfate group was 2.77 (there are only two unsubstituted hydroxyl groups on Fuc residues), suggesting that sulfation was present on the Gal or Man residues. In a previous study [46, 49, 51–53, 56–59], it was reported that sulfated glucuronomannan consisted mainly of alternating 1, 4-linked β-D-GlcA and 1, 2-linked α-D-Man with the Man residues randomly sulfated at C6. Xylose always

accompanies a glucuronomannan [49]. Xyl residues are reportedly [49] substituted on the C3 of the α-D-Man residues. Therefore, we conclude that SH-1-L has two units (unit A, a sulfated galactofucan and unit B, a sulfated xylo-glucuronomannan). Unit A contains a backbone of (1, 6-linked β-D-Gal) n_1, (1, 3-linked 4-sulfated α-L-Fuc) n_2, (1, 3-linked 2, 4-di-sulfated α-L-Fuc) n_3, (1, 4-linked α-L-Fuc) n_4 and (1, 3-linked β-D-Gal) n_5, accompanying with branches and sulfation at Gal and Fuc residues. Unit B consists of alternating 1, 4-linked β-D-GlcA and 1, 2-linked α-D-Man with the Man residues randomly sulfated at C6 or branched with Xyl at C3.

SH-1-H, SH-2-H and SH-3-H had large molecular weight and their NMR spectra were complex and ambiguous. Thus, it is difficult to elucidate their exact structures. However, we can partially elucidate their structures from their corresponding low molecular weight fractions (SH-1-L, SH-2-L and SH-3-L). Comparing SH-3-H with SH-3-L, we found that SH-3-H had a backbone of (1, 6-linked β-D-Gal) n_1, (1, 3-linked 4-sulfated α-L-Fuc) n_2, (1, 3-linked 2, 4-di-sulfated α-L-Fuc) n_3, (1, 4-linked α-L-Fuc) n_4 and (1, 3-linked β-D-Gal) n_5, accompanied by some branches, such as sulfated fuco-oligomers, sulfated galacto-oligomers or sulfated galacto-fuco-oligomers. However, the ratio of Gal residues in SH-3-H was larger than SH-3-L. Comparing SH-2-H with SH-2-L, we found that SH-2-H might also have two units, similar to SH-1-L. We suggest that SH-2-L is a degraded form of SH-2-H. SH-1-H with SH-1-L both are sulfated galacto-fuco-xylo-glucuronomannans. Therefore, both of these have the two units, A and B. The differences can be attributed to the ratio of A and B. In summary, the structural features of SH are presented in Fig. 4.

The anti-complement activities of these polysaccharides were assessed in the classical and alternative pathway and the results are presented in Fig. 5. The complement groups displayed 98% ± 1% activation in the classical pathway and 80% ± 3% activation in the alternative pathway. The anti-complement activities of the polysaccharides showed concentration-dependent responses. However, all polysaccharides each reach a plateau at the different concentrations (Except SH-1-L in the classical pathway), indicating that the polysaccharides showed different anti-complement activities. The activities of SH-1 were similar to SH-1-H, which were both larger than SH-1-L. We attribute these results to their differences in molecular weight based on the chemical compositions analysis. We suggest that molecular weight is one important factor in anti-complement activity consistent with previous studies [60, 61]. These results also explain the activity of SH-3 and its derivatives. However, SH-2 and its derivatives show quite a different result. The activities are SH-2 > SH-2-L > SH-2-H and we suggest can be explained by their UA content. A previous study [62] showed that the higher UA contents resulted in lower activities. Therefore, we conclude that polysaccharides of the same molecular weight containing unit A had higher anti-complement activities than polysaccharides containing unit B, and that sulfated galactofucan showed higher anti-complementary activities than sulfated galacto-fuco-xylo-glucuronomannan. In addition, sulfation content also contributed the anti-complement activity.

Conclusions

Polysaccharides from brown algae were fractionated by anion exchange chromatography to obtain three fractions: (1) sulfated heteropolysaccharides; (2) low sulfated galactofucan; and (3) high sulfated galactofucan/fucan. After autohydrolysis, two fractions, SH-2-L and SH-3-L, contained a backbone of (1, 6-linked β-D-Gal) n_1, (1, 3-linked 4-sulfated α-L-Fuc) n_2, (1, 3-linked 2, 4-di-sulfated α-L-Fuc) n_3, (1, 4-linked α-L-Fuc) n_4 and (1, 3-linked β-D-Gal) n_5. The ratio (n_1: n_2: n_3: n_4: n_5) contributed to the difference between SH-2-L and SH-3-L. In addition, there might be some branches, such as sulfated fuco-oligomers, sulfated galacto-oligomers or sulfated galacto-fuco-oligomers. Comparing SH-3-H with SH-2-H, we showed that SH-3-H had higher sulfate content and a higher molar ratio of sulfate group to fucose (the molar ratio of sulfate group to fucose in SH-3-H was 2.25 while it was 1.80 in SH-2-H). In addition, SH-2-H contained unit B, consisting of alternating 1, 4-linked β-D-GlcA and 1, 2-linked α-D-Man with the Man residues randomly sulfated at C6 or branched with Xyl at C3. Unit B polysaccharides containing uronic acid had lower adsorption capacity than polysaccharides containing sulfate groups, like these found in unit A, containing a backbone of (1, 6-linked β-D-Gal) n_1, (1, 3-linked 4-sulfated α-L-Fuc) n_2, (1, 3-linked 2, 4-di-sulfated α-L-Fuc) n_3, (1, 4-linked α-L-Fuc) n_4 and (1, 3-linked β-D-Gal) n_5, accompanied by some branches, such as sulfated fuco-oligomers, sulfated galacto-oligomers or sulfated galacto-fuco-oligomers. Based on the anti-complementary activity analysis, polysaccharides containing unit A showed higher anti-complement activities than polysaccharides containing unit B. Specifically, sulfated galactofucan had higher activities than sulfated galacto-fuco-xylo-glucuronomannan, suggesting that sulfated galactofucan might be good candidates for anti-complement drugs.

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Author Contribution **Weihua Jin**: Experiments design, data analysis, MS preparation, project supervision; **Qiufu Fang**: experiment performance and data analysis; **Jiang Di**: method development, MS preparation; **Tongtong Li**: Experiments design and performance; **Bin Wei**: Experiments design and performance, MS preparation; **Jiadong Sun**: Experimental design and performance, MS preparation;; **Wenjing Zhang**: MS preparation; **Zhongshan Zhang**: MS preparation; **Fuming Zhang**: MS preparation and revision; **Robert J. Linhardt**: MS preparation and revision; **Hong Wang**: MS preparation and revision; **Weihong Zhong**: MS preparation and supervision.

Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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