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Regioselective syntheses of sulfated porphyrans from *Porphyra haitanensis* and their antioxidant and anticoagulant activities *in vitro*

Zhongshan Zhang [a,b], Quanbin Zhang [a,*], Jing Wang [a,b], Houfang Song [a,b], Hong Zhang [a], Xizhen Niu [a]

[a] *Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China*
[b] *Graduate University of the Chinese Academy of Sciences, Beijing 100049, China*

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ABSTRACT

Porphyran extracted from *Porphyra haitanensis* is a sulfated polysaccharide, which possesses excellent antioxidant activities. In this study, we prepared porphyran and alkali-treated porphyran and their derivatives, sulfated porphyran. And then we evaluated their antioxidant and anticoagulant activities *in vitro* and characterized the relationship between activities and chemical characteristics. The activities were strongly dependant on the degree of sulfation and the position of sulfate. In this study, the antioxidant activities mainly depend on degree of substitution, and the anticoagulant activities mainly depend on the position of sulfate. Further studies are needed to improve our understanding of antioxidant and anticoagulant activities mechanism.

1. Introduction

Sulfated polysaccharides extracted from algae such as agarose, starch, k-carrageenan and porphyran are widely distributed in nature (Takano et al., 1996). They may act as structural components, storage materials and as protective substances. Besides such naturally occurring polysaccharide sulfated, artificially sulfated polysaccharides are also synthesized in order for similar interests and applications because of their various chemical and biological functions (Toshihiko, Amornrut, & Linhart, 2003). And moreover, it was reported that when introduced the sulfate groups, the polysaccharide might show stronger or weaker bioactivities (Chaidedgumjorn et al., 2002; Danalev, Vezenkov, & Grigorova, 2005; Huang, Du, Yang, & Fan, 2003; Yang, Du, Huang, Wan, & Wen, 2005). Therefore, chemical modifications such as partial, per-*O*-sulfonation or desulfonation are expected to alter the function of sulfated polysaccharides.

Porphyra (Rhodephyta), commonly known as nori or laver, is an important food source in many parts of the world (Zhang et al., 2003). It is also used as a drug in traditional Chinese medicine. Porphyran, sulfated polysaccharide that comprises hot-water soluble portion of cell wall, is one of the main components of *Porphyra haitanensis* (Zhao et al., 2006). Structurally, it has a linear backbone of alternating 3-linked β-D-galactosyl units (G* or G) and 4-linked α-L-galactosyl 6-sulfate (A*) or 3,6-anhydro-α-L-galactosyl units (A). In previous study, the content of ester sulfate in porphyran extracted from *Porphyra haitanensis* was 16–19% and it showed generic antioxidant activity (Zhao et al., 2006).

There are six free hydroxyl groups in disaccharide unit of porphyran, including two primary hydroxyls. These hydroxyl groups and the sulfate groups at themselves play an important role as antioxidants for prevention of oxidative damage in living organisms. The activity of polysaccharide depends on several structural parameters such as degree of sulfation, the molecular weight, the position, type of sugar and glycosidic branching (Alban, Schauerte, & Franz, 2002). In order to study on the relationship between chemical modification of the porphyran and their biological activity, we made the regioselective sulfated modifications on the primary and secondary hydroxyl groups and prepared the regioselective oversulfated derivatives. And what's more, we evaluated their antioxidant and anticoagulant activities *in vitro* and characterized the relationship between activities and chemical and structural characteristics.

2. Materials and methods

2.1. Materials

Porphyran **(1)** was isolated from *Porphyra haitanesis*, cultured in the coast of Lianjiang County, Fujian, China (Nishide, Ohno, Anzai, & Uchida, 1988). Nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide-reduced (NADH), phenazine methosulfate (PMS), ethylene diamine tetra-acetic acid (EDTA), hydrogen peroxide (H_2O_2), 4-dimethylaminopyridine (DMAP), 4,4′-dimethoxytrityl chloride (DMT-Cl) and ferrozine were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

* Corresponding author. Tel.: +86 532 82898708; fax: +86 532 82898703.
E-mail address: qbzhang@ms.qdio.ac.cn (Q. Zhang).

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2.2. Analytical methods

Sulfate content was determined by barium chloride–gelatin method (Kawai, Seno, & Anno, 1969). Total sugar content was determined by phenol–sulfuric acid method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956) using D-galactose as standard. 3,6-Anhydrogalactose content was determined as described previously (Yaphe & Arsenault, 1965).

FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 360 spectrophotometer.

Molecular weights of all the samples were determined by HP-GPC on a Waters 515 GPC system at 35 °C, where 0.7% Na_2SO_4 solution was used as mobile phase with a flow rate of 0.5 mL/min. TSK G3000 column (300 mm × 7.8 mm) and 2140 refractive index detector was used. A series of different molecular weight dextrans purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China) were used as standard.

^{13}C NMR spectra (125.7 MHz) were recorded on a Bruker AMX-500 NMR spectrometer at ambient temperature. The samples were dissolved in D_2O.

2.3. Alkali modification of porphyran

A solution of polysaccharide (1.6 g) in 250 mL distilled water was treated with $NaBH_4$ (0.2 g) at room temperature for 48 h. And then NaOH (10.0 g) and $NaBH_4$ (3.0 g) were added into and the mixture was heated to 80 °C for 2 h (Brasch, Chang, Chuah, & Melton, 1981). The reactive solution was neutralized, dialyzed, and freeze-dried to give the alkali porphyran **(2)**.

2.4. Sulfation of porphyran

2.4.1. Fully sulfated modification of porphyran

Chemical sulfation to obtain fully sulfated porphyran was carried out under mild conditions with adducts of sulfur trioxide (SO_3) in aprotic solvents (Wang, Liu, Zhang, Zhang, & Qi, 2009). The sulfation reagent, SO_3-DMF, was obtained by dropping of chlorosulfonic acid ($HClSO_3$, 50 mL) into *N*,*N*-dimethylformamide (DMF, 300 mL) under cooling in an ice-water bath. Porphyran (2.0 g) was added to formamide (FA, 80 mL), and then the mixture was stirred at 50 °C for 30 min in order to disperse into solvent. Then SO_3-DMF (15 mL) reagent was added. After 3 h, the mixture was precipitated, dialyzed and lyophilized to give the fully sulfated porphyran **(3)**.

2.4.2. Regioselective modification of porphyran

A solution of porphyran (2.0 g), DMT-Cl (6.0 g) and DMAP (0.12 g), used as a catalyst, in 40 mL FA were stirred for 7 h at 75 °C. Then, trifluoroacetic anhydride was added dropwise, and the homogeneous solution was stirred at 0 °C for 3 h and then at RT for 1 h. The white solid obtained by pouting the mixture into ethanol was washed with chloroform and ethanol and then dried to give the intermediate product **(4)**. To a solution of the intermediate product **4** in water was added acetic acid solution (80%) and stirred for 2 h, and then ethanol (75%) was dropped in the mixture to remove the DMT group. The product above was sulfated with the procedures of 2.4.1. The sulfated product neutralized in aqueous solution was stirred for 24 h at 60 °C to remove the trifluoroacetic group. Then the product was dialyzed and lyophilized to give the 6-*O*-sulfated porphyran **(5)**.

And also, the compound **2** was protected on the primary hydroxyl groups with DMT-Cl by repeating the procedures above to obtain the synthetic intermediate **(6)** which, as following, was sulfated with $HClSO_3$ as the procedures carried above. After being treated with acetic acid (80%, RT, 2 h), the product was dialyzed and lyophilized to give the 2,2′,4-*O*-sulfated porphyran **(7)**.

2.5. Antioxidant activity assays

2.5.1. Superoxide anion-scavenging activity

The superoxide radical scavenging ability of all different derivatives was assessed by the method of Nishimiki, Rao, and Yagi (1972). In this experiment, superoxide anion radicals were generated in 4.5 mL Tris–HCl buffer solution (16 mM, pH 8.0) containing NBT (300 μM, 0.5 mL), NADH (468 μM, 0.5 mL) and the sample solution (0.5–50 μg/mL). The reaction was started by adding PMS (60 μM, 0.5 mL) solution to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion-scavenging activity. The capability of scavenging the superoxide anion radicals was calculated using the following equation:

$$\text{Scavenging effect}(\%) = \left(1 - A_{\text{sample } 560}/A_{\text{control } 560}\right) \times 100$$

2.5.2. Reducing power

The reducing power was determined as described previously by Yen and Chen (1995). Briefly, different concentration of samples (0.47–6.0 mg/mL, 1.0 mL) in phosphate buffer (0.2 M, pH 6.6) was mixed with potassium ferricyanide (1%, 1.0 mL), and then the mixture was incubated at 50 °C for 20 min. Afterwards, trichloroacetic acid (10%, 2.0 mL) was added to the mixture to terminate the reaction. Then the solution was mixed with ferric chloride (0.1%, 1.2 mL) and the absorbance was measured at 700 nm. Increased absorbance of reaction mixture indicated increased reducing power.

2.6. Anticoagulant activity assays

The anticoagulant activities of all the samples were investigated by the classical coagulation assays APTT, PT, and TT using UFH as reference compound (Weißbach, 1990). APTT assay was carried out as follows: citrated normal chicken plasma (100 μL) was mixed with a solution of samples (50 μL) and APTT assay reagent (100 μL), and then the mixture was incubated for 5 min at 37 °C. Afterwards, 0.025 mol/L $CaCl_2$ (100 μL) was added and clotting time was recorded. For PT assay, citrated normal chicken plasma (100 μL) was mixed with a solution of samples (50 μL) and incubated for 3 min. Then PT assay reagent (200 μL), pre-incubated for 10 min at 37 °C, was added and clotting time was recorded. For TT assay, citrated normal chicken plasma (150 μL) was mixed with a solution of samples (50 μL) and incubated for 2 min. Then, TT assay reagent (200 μL), pre-incubated for 5 min at 37 °C, was added and clotting time was recorded. All the samples were dissolved in saline. The final concentration of heparin in these assays was between 1 and 3 μg/mL, and the final concentrations of other samples ranged from 1 to 20 μg/mL. In the control group, only the saline was used.

3. Results and discussion

3.1. Sulfation of porphyran

Limited solubility of biopolymers restricts the number and nature of reagents that could be used for their chemical modification. Porphyran is only soluble in formamide among the organic solvents. In order for a maximum conversion of porphyran to its derivatives, it is advisable to carry out the reaction in a homogeneous medium, requiring a suitable solvent systematic. Porphyran was subjected to sulfation with sulfur trioxide-formamide to both

increase its negative charge and decrease its sequence heterogeneity. When FA acted as the solvent in the reaction system, degree of sulfation in product increased obviously, which suggested that effects of solvent prominent in the sulfation.

Scheme 1 outlined the synthetic procedure for the 6-*O*-sulfated porphyran and 2,2′,4-*O*-sulfated porphyran. Firstly, the regioselective modification should be focused on discrimination between the primary and secondary hydroxyl groups. Regioselective ether protection of primary hydroxyl groups in the presence of secondary or tertiary ones can be easily accomplished by reaction with DMT-Cl (Chaudhary & Hernadez, 1979). Since trityl ethers are readily deprotected by heating in aqueous acetic acid or by treatment with stronger acids without heating, trityl protection, even in case of polysaccharide chemistry, seems to be one of the most efficient temporary protections as established in the synthetic studies on polysaccharide derivatives. 6-*O*-Tritylation of porphyran was carried out in FA with 3-fold excess DMT-Cl at 75 °C. The structural elucidation of the product was made unambiguously with the IR spectrum (Fig. 1). Characteristic absorptions at 690, 710 and 750 cm^{-1} due to monosubstituted phenyl groups were observed in the spectrum (Fig. 1).

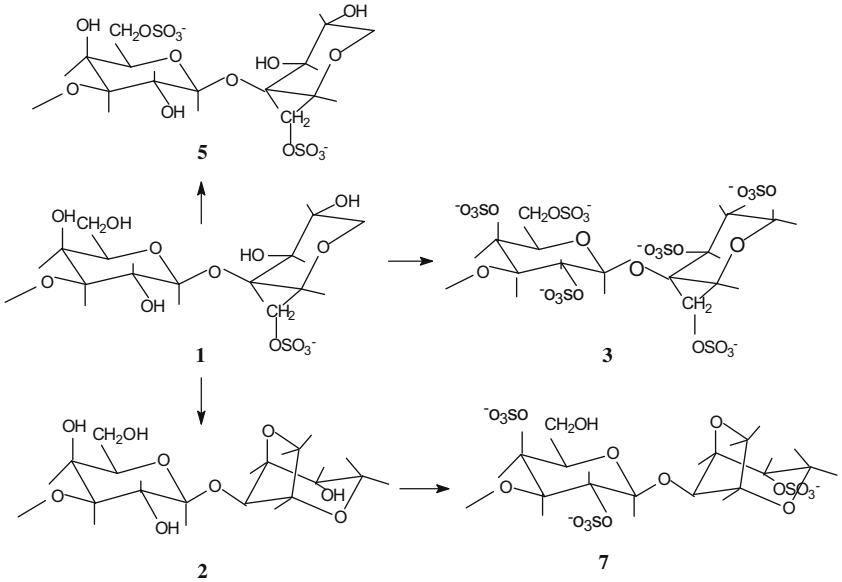
After completion of the reaction and blocking of the two primary hydroxyl groups, the tritylated reaction product was subjected to *in situ* trifluoroacetylation at the secondary hydroxyl groups. Since the trifluoroacetyl group is stable under glycosylation conditions and can be removed under extremely mild conditions, it was used as the protective agent in comparison with acetyl group which was hardly removed (Takatani, Matsuo, & Ito, 2003). In the IR spectrum of the product, characteristic absorptions at 1718 and 1183 cm^{-1} due to *O*-trifluoroacetyl groups appeared and a decrease in the absorption at 3475–3300 cm^{-1} due to hydroxyl groups was observed in the spectrum. Deprotection of trityl group was performed in a standard manner (Goncalves, Noseda, Duarte, & Grindley, 2006). Obviously, disappearance of absorptions due to monosubstituted phenyl groups was observed without appreciable change in absorptions due to 2,2′,4-*O*-trifluoroacetyl in the spectrum. After that, the free 6-*O* hydroxyl group was substituted with sulfate in FA. It is well known that trifluoroacetates are much more sensitive to hydrolysis than the corresponding acetates and it hydrolyzed completely at pH 7 under continuous stirring within 17 h (Winter & Scott, 1968). So it was feasible that the deprotection of trifluoroacetyl was carried out in our research.

In order to obtain the sulfated product at the secondary hydroxyl groups, we prepared the alkali-treated porphyran with no sulfate at 6′-*O*. Porphyran **(1)** contains disaccharide units consisting of 3-linked β-D-galactosyl residues alternating with 4-linked α-L-galactosyl 6-sulfate. When porphyran (SO_4^{2-}, 17.06%, 3,6-anhydro-L-galactose, 8.05%) was treated with alkali, we obtained a different polysaccharide **(2)** (SO_4^{2-}, 2.48%, 3,6-anhydro-L-galactose, 29.7%) which had a much stronger gelling tendency than porphyran (Table 1). IR spectroscopy of this polymer gave a sharp absorption band at 935 cm^{-1}(3,6-anhydro-L-galactose) and no absorption at 1250 cm^{-1} or 820 cm^{-1}(SO_4^{2-}) (Fig. 1). The following methods and procedures of sulfation were the same as the methods above made.

All the compounds were readily characterized as the desired regioselectively sulfated derivatives by their ^{13}C NMR spectra, which showed signals for carbon downshifted by the sulfation reaction. The ^{13}C NMR chemical shifts of the samples and their derivatives were summarized in Table 2. From the Table 2, it was found that a large low field chemical shifts of C-2, C-4, C-6 at G* residue and C-6 at A* of fully sulfated modification of porphyran **(3)** occurred. In all the case, sulfate groups were introduced predominantly to C-6. Although the degree of the sulfation of the second hydroxyl groups is far less than that of O-6, the difference in the reactivity of each hydroxyl group depended on the position, e.g., O-2 in the residue was more reactive than O-3 and O-4. For the 6-*O*-sulfated porphyran **(5)**, the second hydroxyl groups were not sulfated, whereas O-6 was moderately reactive. Similarly, in the derivative **7**, the second hydroxyl groups were sulfated, but the degree sulfation was low. In fact, the hydroxyl groups at C-6 that project from the polysaccharide chains are least sterically hindered and achieve the highest reactivity. The hydroxyl groups that were not sulfated are likely to have been sterically hindered, presumably due to the conformations of polysaccharide molecule. In the case of porphyran, O-2 and O-3 at the 4-linked L-Gal residue that are close to the axial O-1 and O-4, respectively, exhibited no reactivity, whereas O-2 at the 3-linked D-Gal residue close to equatorial O-1 exhibited the moderate reactivity.

3.2. Antioxidant activity assays

The superoxide radical ($\cdot O^-_2$) was a highly toxic species that was generated in a PMS/NADH system for being assayed in the



Scheme 1. The structure and synthesis of porphyran and its derivatives.

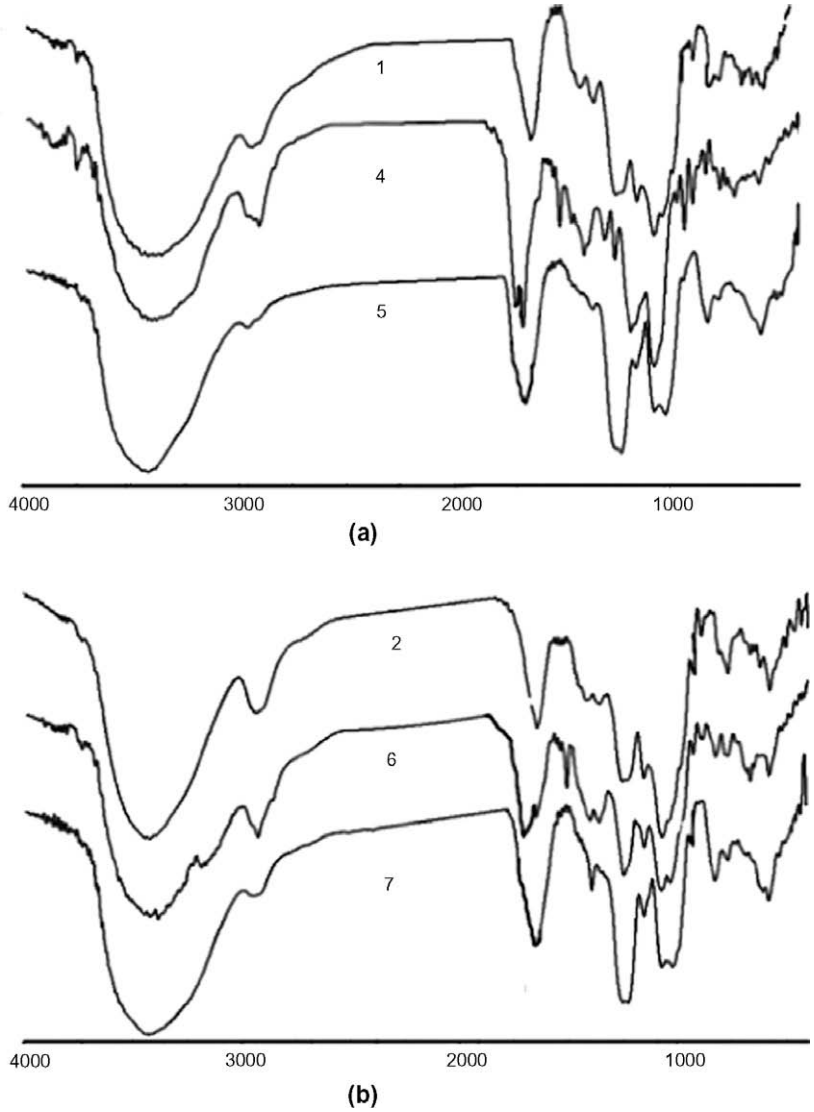


Fig. 1. FT-IR spectrums of the sample porphyran and its derivatives.

Table 1
The chemical composition and characterizations of all the samples.

Samples	Yield (%)	Total sugar (%)	Sulfate (%)	3,6-Anhydro-L-galactose (%)	MW Mn × 10^4
1	–	78.86	17.06	8.05	27.7
2	69.12	102.4	2.48	29.7	18.5
3	93.77	54.47	39.20	5.25	2.63
5	48.39	52.62	31.43	6.70	1.74
7	59.24	54.98	22.72	18.2	2.45

reduction of NBT (Banerjee, Dasgupta, & De, 2005). The scavenging ability of all tested samples on superoxide radicals were shown significant in a concentration-dependent fashion (Fig. 2). The inhibitory effect of all samples was marked and concentration related. As shown in Fig. 2, at the concentration below 0.01 mg/mL, the scavenging effect significantly increased with increasing concentration, and at the concentration higher than 0.02 mg/mL, the scavenging effect increased slowly and was up to smooth finally. As for the sulfated derivatives, the fully sulfated and 6-*O*-sulfated derivatives showed stronger scavenging activity than their raw material porphyran. However, the derivative 2,2′,4-*O*-sulfated porphyran showed weaker scavenging activity than other derivatives.

In the reducing power assay, the presence of reductant (antioxidant) in the tested samples would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}), and the yellow color of test solution changes into various shades of green and blue colors depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power of porphyran and its derivatives was depicted in Fig. 3. Higher absorbance value means stronger reducing power. Among the derivatives, only the reducing power of **5** was stronger than porphyran. To our surprise, the fully sulfated porphyran which had a higher DS showed much weaker than 6-*O*-sulfated porphyran. It indicated that DS was not the only factor influencing the bioactivity.

Sulfate group takes an important role in antioxidant activity. DS is an important influence factor. In general, the sample possessing higher DS showed stronger antioxidant activity such as three derivatives in the scavenging ability assays. However, in the reducing power assay, this was not applicable. The distribution of the sulfated group became an important parameter influencing reducing power. Obviously, sulfation at O-6 of porphyran seemed to increase reducing power. And what's more, sulfation at the secondary hydroxyl groups would decrease reducing power. Even

Table 2
^{13}C chemical shifts of all the samples.

	G*						A*						G						A					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
1	103.5	69.9	81.2	69.0	75.7	61.8	101.5	69.9	71.1	79.1	73.8	67.7	102.5	70.3	82.4	68.0	75.6	61.6	98.6	69.7	80.2	77.7	75.7	69.2
2													102.3	70.1	82.2	68.2	75.2	61.3	98.2	69.7	80.0	77.2	75.5	69.7
3	103.9	70.0	80.9	72.2	76.0	68.9	101.2	70.2	71.0	78.5	73.2	69.8	102.4	71.0	83.0	68.6	75.4	61.8	98.8	73.6	80.0	78.0	75.6	69.2
5	103.9	69.9	81.3	69.1	76.1	68.0	101.3	69.9	72.1	80.3	73.7	70.0	102.5	70.3	81.3	68.0	73.2	62.0	98.1	69.7	80.3	77.4	75.4	69.2
7													102.4	72.7	79.7	70.8	75.1	60.5	97.4	72.4	78.9	76.2	74.7	68.5

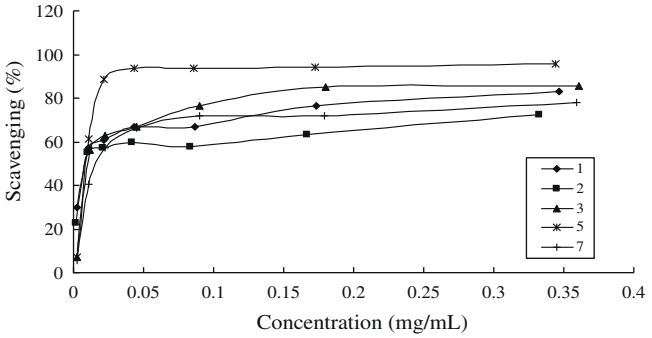


Fig. 2. Scavenging effects of the sample porphyran and its derivatives on superoxide radical. Values are means ± S.D. (n = 3).

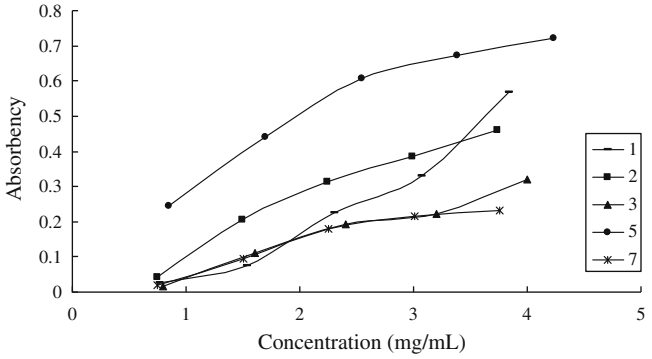


Fig. 3. Reducing power assay of the sample porphyran and its derivatives. Values are means ± S.D. (n = 3).

though sulfation of the fully sulfated derivative was both at primary and at secondary hydroxyl group sulfate at the secondary OH in the fully sulfated derivative could also influence the reducing power.

3.3. Anticoagulant activity assays

The anticoagulant activities of all the samples were determined by activated APTT, TT, and prothrombin time (PT) assays *in vitro* that characterize different stages of the coagulation process. The results were listed in Table 3 which showed that APTT, TT and PT of all the sulfated porphyran were prolonged. Prolongation of APTT and TT suggested inhibition of the intrinsic coagulation pathway and thrombin-mediated fibrin formation, respectively, and prolongation of PT indicated inhibition of extrinsic coagulation pathway (Yang, Du, Huang, Wan, & Li, 2002). In positive control, heparin could apparently prolong APTT, TT and PT. From these data, the anticoagulant activities of sulfated derivatives strongly depended on their degree of sulfation, the molecular and the distribution of the sulfated group.

From the Table 3, we found that the sample **3** could prolong much more blood clotting time than other two derivatives, which declared that high degree of sulfation could obviously improve anticoagulant activities. On the other hand, in our studies porphyran had higher degree than the alkali porphyran but both of them showed parallel anticoagulant activities. This indicated that though the sulfate group was essential, the certain degree was also necessary. We presumed that degree of sulfation must be above one certain value for anticoagulant activity. Besides the degree of sulfation, the distribution of the sulfated group was an important factor influencing anticoagulant activity. Porphyran has a linear

Table 3
Anticoagulant activities measured by APTT, TT and PT assay.

Samples	Concentration (μg/mL)	APTT	TT	PT
1	100	103.92 ± 3.34	58.21 ± 1.44	81.39 ± 2.59
	50	99.74 ± 1.36	57.17 ± 1.29	79.76 ± 1.35
	10	98.29 ± 0.86	52.70 ± 0.95	74.32 ± 2.75
2	100	99.07 ± 1.27	55.25 ± 1.53	77.32 ± 0.74
	50	98.25 ± 1.02	54.16 ± 1.47	76.67 ± 2.54
	10	98.10 ± 0.86	53.52 ± 1.49	69.80 ± 1.38
3	100	396.47 ± 12.47	311.70 ± 2.78	298.03 ± 4.61
	50	224.17 ± 10.87	136.88 ± 6.59	127.71 ± 4.74
	10	119.24 ± 9.24	58.35 ± 3.35	73.22 ± 3.09
5	100	222.69 ± 5.28	96.97 ± 3.00	109.79 ± 4.18
	50	153.43 ± 3.13	96.97 ± 2.59	87.96 ± 4.08
	10	114.36 ± 4.52	58.88 ± 2.24	71.61 ± 0.93
7	100	162.03 ± 8.78	66.62 ± 1.90	85.54 ± 3.18
	50	152.23 ± 7.11	60.54 ± 2.06	78.82 ± 1.82
	10	98.41 ± 1.47	55.92 ± 1.73	74.55 ± 4.92
Heparin	5	236.73 ± 11.45	114.16 ± 4.72	103.45 ± 10.9
	3	175.90 ± 7.25	90.34 ± 3.93	98.15 ± 6.45
Saline		97.87 ± 6.38	51.81 ± 5.42	61.29 ± 4.06

backbone of alternating 3-linked β-D-galactosyl units and 4-linked α-L-galactosyl 6-sulfate or 3,6-anhydro-α-L-galactosyl units. After alkali treatment, 6-*O*-desulfation in (1 → 4) linked residue was carried out, but anticoagulant activities little changed. And what's more, 6-*O*-sulfated derivative showed lower anticoagulant activity than 2,2′,4-*O*-sulfated derivative. It indicated that sulfate groups at the C-6 were not necessary for anticoagulant activity. Additionally, a dramatic increase in anticoagulant activity was observed on the fully sulfated derivative. It was considered that an increase in the overall molecular charge and sulfate groups at C-2, C-3 and C-4 were the dominating factors.

4. Conclusion

In the present study, porphyran and its different derivatives, sulfated porphyran were prepared. And then we evaluated their antioxidant and anticoagulant activities *in vitro* and characterized the relationship between activities and chemical characteristics. The activities were strongly dependant on the function group, degree of sulfation and the position of sulfate. In this study, the antioxidant activities depend on degree of substitution, and the anticoagulant activities mainly depend on the position of sulfate. Further studies are needed to improve our understanding of antioxidant activities mechanism.

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