Carbohydrate Polymers 79 (2010) 1124–1129

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

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ARTICLE INFO

Article history:
Received 6 September 2009
Received in revised form 16 October 2009
Accepted 22 October 2009
Available online 25 October 2009

Keywords:
Porphyran
Sulfation
Antioxidant activity
Anticoagulant activities

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.

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doi:10.1016/j.carbpol.2009.10.055

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