Carbohydrate Polymers 79 (2010) 290–295

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Chemical modification and influence of function groups on the in vitro-antioxidant activities of porphyran from *Porphyra haitanensis*

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

ARTICLE INFO

Article history:
Received 6 July 2009
Received in revised form 23 July 2009
Accepted 3 August 2009
Available online 11 August 2009

Keywords:
Porphyran
Sulfation
Acetylation
Antioxidant activity
Function group

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 acetylated porphyran. Their antioxidant activities including scavenging effect on superoxide radical, 1,1-diphenyl-2-picrylhydrazyl radical and reducing power were investigated. The results of chemical analysis and FT-IR spectrums indicated that the modification was successful. And in addition, we found that certain derivative exhibited stronger antioxidant activity than raw material porphyran. The sulfated derivative with certain DS showed stronger antioxidant activity. The acetylated derivative showed the most excellent antioxidant activity in three assays we made, and so this derivative needs to be attended to.

1. Introduction

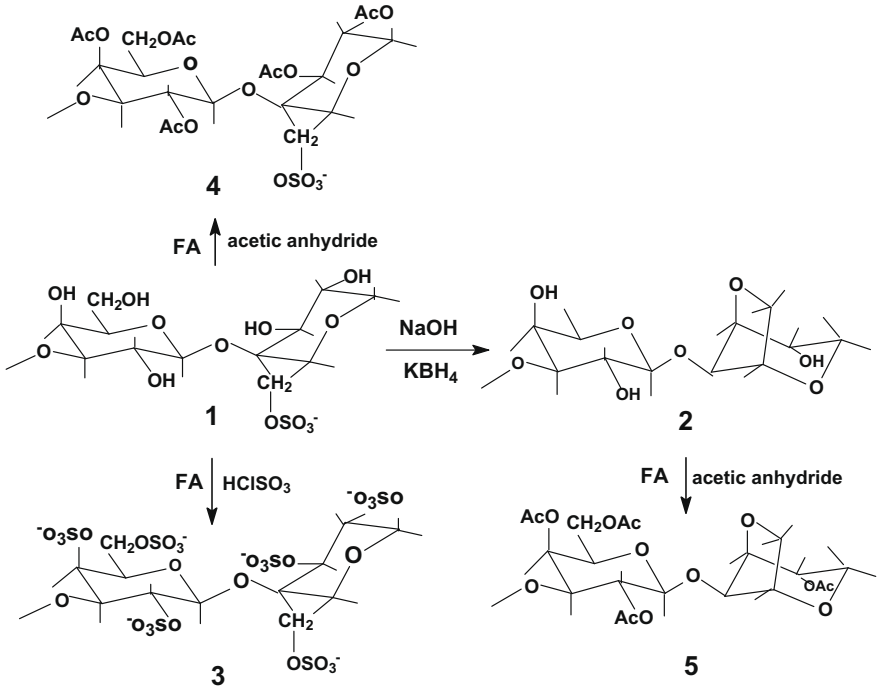
In aerobic organisms the energy needed to fuel biological functions is produced in the mitochondria via the electron transport chain. In addition to energy, reactive oxygen species (ROS) which have the potential to cause cellular damage are produced. ROS can damage DNA, RNA and proteins which theoretically contribute to the physiology of ageing (Patel, Cornwell, & Darley-USMAR, 1999). In order to reduce damage to the human body and prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. In general, the natural antioxidants mainly make up many compounds including phenolic, nitrogen compounds and carotenoids (Nandita & Rajini, 2004). In recent years, many marine resources have attracted attention in the search for bioactive compounds to develop new drugs and health foods (Nagai & Yukimoto, 2003). Qi et al. (2005) reported that polysaccharide extracted from *Ulva pertusa* showed excellent radical-scavenging activity against superoxidant and hydroxyl radical.

Polysaccharide, made up of many monosaccharides joined together by glycosidic bonds, possess marked immunological properties ranging from nonspecific stimulation of host immune system, resulting in anti-tumor, anti-viral, and anti-infective effects, to antioxidant, anti-mutagenic or hematopoietic activity (Bohn & BeMiller, 1995; Kennedy, 1989; Kennedy & White, 1983). The high potential for exploiting these natural biopolymers with their broad range of structural, functional and physicochemical properties, in various applications has provided the stimulus for the search for new or modified polysaccharides (Geresh, Dawadi, & Arad (Malis), 2000). Recently, various physiological chemistry effects of polysaccharide from sea algae have been verified and the interest on sea algae inducing bioactive materials has been increased. Porphyran, the sulfated polysaccharides comprising the hot-water soluble portion of cell wall, is the main component of red algae, *Porphyra haitanensis*. It contains disaccharide units consisting of 3-linked β-D-galactosyl residues alternating with 4-linked α-L-galactose, and some residues occur as the 6-sulfate (Gretz, McCandless, Aronson, & Sommerfeld, 1983). Like the general polysaccharide, porphyran possesses excellent bioactivities. Zhao et al. (2006) reported porphyran showed strong antioxidant activities in two assay systems made.

Generally speaking, the antioxidant activity of polysaccharide depends on several structural parameters such as degree of substitution (DS), the molecular weight, type of sugar and functional groups (Melo, Feitosa, Freitas, & de Paula, 2002). Chemical modification of polysaccharides provided an opportunity to obtain new pharmacological agents with possible therapeutic uses (Franz & Alban, 1995). In the present study, porphyran and alkali-treated porphyran and their derivatives, sulfated porphyran and acetylated porphyran were prepared (Scheme 1). And then we evaluated their antioxidant activities in vitro and characterized the relationship between antioxidant activity and chemical characteristics.

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

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.08.004



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

2. Materials and methods

2.1. Materials

Porphyra haitanensis, cultured near the coast of Putian County, Fujian Province, China, was collected in December 2003. Natural polysaccharide porphyran (**1**) was isolated from hot-water soluble extracts of *P. haitanensis* as described previously (Nishide, Ohno, Anzai, & Uchida, 1988). In brief, the algae was first treated with dilute formaldehyde solution, and then extracted with hot water.

Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), 1,1-diphenyl-2-picry-hydrazyl (DPPH) and *N*-bromosuccinimide (NBS) were purchased from Sigma Chemical Co. Ascorbic acid, sodium citrate and other reagents were of analytical grade. Dialysis membranes were produced by Spectrum Co., and molecular weight was cut off at 3600 Da.

2.2. Analytical methods

Sulfate content was determined by barium chloride–gelatin method (Kawai, Seno, & Anno, 1969). The degree of substitution (DS) of sulfation was obtained as the following equation:

$$\mathrm{DS} = \frac{304 \times S\%}{96 - 102 \times S\%},$$ where $S\%$ is the content of sulfate group.

The acetyl content was determined by the modified hydroxylamine–ferric trichloride method (Notari & Munson, 1969). The degree of substitution (DS) of acetylation was obtained as the following equation:

$$\mathrm{DS} = \frac{304 \times W\%}{43 - 42 \times W\%},$$ where $W\%$ is the content of acetyl group.

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).

Infrared spectrums were measured by a Nicolet Magna-Avatar 360 with KBr disks.

Molecular weight of all samples was 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.

2.3. Preparation of the different derivatives of porphyran

2.3.1. Alkali treatment of porphyran

A solution of porphyran (1.6 g) in distilled water (250 mL) 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 and the mixture was heated to 80 °C for 2 h, neutralized, dialyzed and freeze–dried to give the alkali-treated porphyran (**2**) (Gretz et al., 1983).

2.3.2. Sulfation of porphyran

The sulfation reagent, SO_3–DMF, was obtained by dropping 50 mL chlorosulfonic acid ($HClSO_3$) into 300 mL *N*,*N*-dimethylformamide (DMF) under cooling in an ice-water bath. Porphyran (2.0 g) was added to FA (80 mL), and 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 cooled and precipitated with 75% ethanol. The precipitate which was dissolved in 100 mL distilled water was neutralized and dialyzed (Wang, Liu, Zhang, Zhang, & Qi, 2009). And then, the resultant was concentrated and lyophilized to give the product sulfated porphyran (**3**).

2.3.3. Acetylation of porphyran and alkali-treated porphyran

Compound **1** (2.0 g) was, respectively, dispersed in FA (80 mL), and the mixture was stirred at 80 °C for 30 min, then added acetic anhydride (50 mL) and 1% NBS (50 mg NBS dissolved in 50 mL acetic anhydride). After reaction at 80 °C for 6 h, added 20 mL distilled

water to terminate reaction, cooled to room temperature and precipitated with 75% ethanol for 12 h. The precipitate was filtered off and washed three times with ethanol, and then was dissolved in 100 mL distilled water. The solution was neutralized with 1 M NaOH solution and dialyzed against tap water for 48 h and distilled water for 24 h using 3600 Da Mw cutoff dialysis membranes (Wang et al., 2009). The resultant was concentrated and lyophilized to give the product acetylated porphyran (**4**). The sample **2** was handled with the same method to give the acetylated alkali-treated porphyran (**5**).

2.4. Antioxidant activities

2.4.1. Superoxide radical assay

The superoxide radical scavenging abilities of all the samples were 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 0.5 mL NBT (300 μM) solution, 0.5 mL NADH (468 μM) solution and one sample (0.5–50.0 μg/mL). The reaction was started by adding 0.5 mL PMS (60 μM) 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 radical-scavenging activity. The capability of scavenging the superoxide anion radicals was calculated using the following equation:

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

where $A_{\text{sample}_{560}}$ is the absorbance of the control (Tris–HCl buffer, instead of sample).

2.4.2. DPPH radical-scavenging assay

The abilities of all the samples on scavenging DPPH radical was studied employing the modified method described earlier by Yamaguchi, Takamura, Matoba, and Terao (1998). Briefly, 1 mL DPPH solution (0.1 mM, in 50% ethanol solution) was incubated with varying concentrations of the sample. The reaction mixture was shaken well and incubated for 20 min at room temperature and the absorbance of the resulting solution was read at 517 nm against a blank. The radical-scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \left(1 - \frac{A_{\text{sample}_{517}}}{A_{\text{control}_{517}}}\right) \times 100,$$

where $A_{\text{sample}_{517}}$ is the absorbance of the control (50% ethanol solution, instead of sample).

2.4.3. Reducing power assay

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

3. Results and discussion

3.1. Derivation 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 (FA) among the organic solvents. In order to give a maximum conversion of porphyran to its derivative, it is advisable to carry out the reaction in a homogeneous medium, requiring a suitable solvent systematic. And then porphyran was subjected to persulfation with sulfur trioxide–formamide to both increase its negative charge and decrease its sequence heterogeneity. In the reaction system in which FA acted as the solvent, DS of product increased obviously, which suggested that effects of solvent prominent in the sulfation. And then porphyran was acetylated homogeneously in formamide with acetic anhydride in the presence of NBS as a catalyst. The products were characterized by chemical analysis and IR spectrum.

3.2. Chemical analysis

The chemical composition and characterizations of all the samples were given in Table 1. Relatively to raw material, the total sugars of the derivatives decreased after modified. The effects of alkali treatment under conditions recommended for the elimination of sulfate from galactose-6-sulfate with concomitant formation of 3,6-anhydrogalactose have been shown for porphyran (Rees, 1961). The sulfate and acetyl contents of the derivatives were higher than their raw material, which indicated the derivation was successful. And on the other hand, 3,6-anhydrogalactose content of sample **5** was remarkably lower than sample **2**. The reason was probably that some links with 3,6-anhydrogalactose in the molecular chain break down or that inner ether opened during the modified reaction and so the content of 3,6-anhydrogalactose decreased. In the sulfation and acetylation reaction process of polysaccharides, degradation was usually accompanied. Table 1 showed the molecular weights of all the samples. From that we can find the reactant degraded heavily because of the acid surroundings.

3.3. FT-IR spectrum

The FT-IR spectrum of the products was shown in Fig. 1. Typical signals of porphyran at 1646, 1419, 1225, 1155, 1073, 930 and 817 cm^{-1} were clear for all the samples. The signal at 1225 cm^{-1} was assigned to the asymmetric stretching vibration of sulfate group, and the signal at 817 cm^{-1} was indicative of a sulfate group attached to a primary hydroxyl group (Brasch, Chang, Chuah, & Melton, 1981). For the alkali-treated porphyran, IR spectroscopy gave a sharp absorption band at 930 cm^{-1} (3,6-anhydrogalactose) and a little absorption at 1225 and 817 cm^{-1}. FT-IR spectra of the sulfated porphyran showed two characteristic absorption bands at 1225 and 817 cm^{-1}. The IR spectra of acetylated porphyran prepared represented the characteristics of esterified products by displaying essential bands of ester compounds at 1730 cm^{-1} (C=O stretching vibration) and 1373 cm^{-1} (—C—O—C— st.).

3.4. Antioxidant activities

3.4.1. Superoxide radical assay

The superoxide radical ($\cdot O^{-2}$) was a highly toxic species that was generated in a PMS/NADH system for being assayed in the reduction of NBT (Banerjee, Dasgupta, & De, 2005). Fig. 2 depicted the inhibitory effect on the superoxide radical of all the samples. It indicated that all the samples exhibited varying degrees of antiox-

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

Samples	Yield (%)	Total sugar (%)	3,6-Anhydr-ogalactose (%)	Sulfate (%)	DS (sulfate)	Acetyl (%)	DS (acetyl)	Molecular weight Mn × 10^{-4}
1	–	78.86	8.05	17.05	0.67	–	–	27.7
2	69.12	102.4	29.7	2.48	0.08	–	–	18.5
3	93.77	54.47	5.25	39.20	2.13	–	–	2.63
4	93.61	74.39	11.12	14.08	0.52	5.88	0.44	2.53
5	87.23	85.25	10.86	2.14	0.07	6.30	0.47	2.55

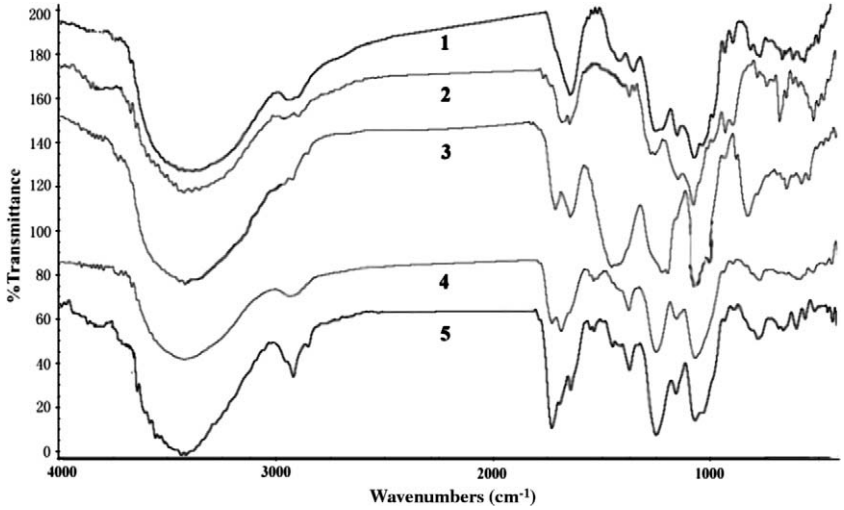


Fig. 1. FT-IR spectrums of the sample porphyran and its derivatives.

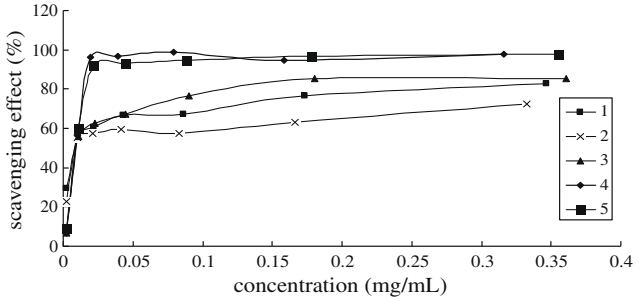


Fig. 2. Scavenging effects of the sample porphyran and its derivatives on superoxide radical. Values are means ± *SD* (*n* = 3).

idant activity. Especially, two acetylated products **4** and **5** showed significant scavenging effects. At the concentration of 22.0 μg/mL, the inhibitory effect was 96.3% and 91.9%, respectively. At the concentration below 45.1 μg/mL, sulfated porphyran **3** showed uniform scavenging activity against superoxide radicals with **1** and **2**. At the concentration over 50.0 μg/mL, scavenging activity of **3** was stronger than that of **1** and **2**.

It was reported that addition of electron-withdrawing groups to the pyrrole enhanced antioxidant activity (Yanagimoto, Lee, Ochi, & Shibamoto, 2002). So the presence of the sulfated group could increase the activity of scavenging radicals. However, the electron density of the carbon atoms on a heterocyclic ring was not the only reason to determine the strength of the antioxidant. In this assay, acetylated products also showed strong scavenging activity. We supposed that presence of acetyl group could change the polarity of the compound, which has effect on antioxidant ability. Although superoxide was a relatively weak oxidant, it decomposed to form stronger, reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids. Furthermore, superoxides were also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Dahl & Richardson, 1978). Based on this, the antioxidant activities of all the samples were also related to their ability to scavenge superoxide radical.

3.4.2. DPPH radical-scavenging assay

Total DPPH scavenging effects of all samples at varying concentrations were measured and the results were depicted in Fig. 3. Except for the sulfated derivative, other samples showed stronger scavenging effect on DPPH. At the concentration below 1.25 mg/mL, the scavenging effects of **5** was weaker than **1** and **2**. But when it was over the above concentration, the former showed stronger effect by the concentration and the effect exceed the other acetylated derivative finally.

The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating abilities. DPPH was a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares, Dins, Cunha, &

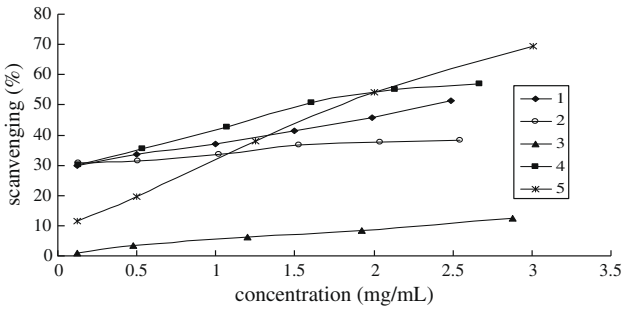


Fig. 3. Scavenging effects of the sample porphyran and its derivatives on DPPH radical. Values are means ± *SD* (*n* = 3).

Almeida, 1997). In our opinion, the presence of acetyl groups in the molecule could activate the hydrogen atom of the anomeric carbon. In the present study, the sulfated derivative showed weak scavenging activity on DPPH radicals, which may be attributable to its weak hydrogen-donating. At the concentration of 1.0 mg/mL, the scavenging effect of butylated hydroxyanisole (BHA) was only 12.2% (Mau, Chang, Huang, & Chen, 2004), which was lower than the acetylated derivatives, it was evident that these derivatives did show strong proton-donating abilities and could serve as free-radical inhibitors or scavengers, acting possibly as primary antioxidants.

3.4.3. Reducing power assay

The reducing power of all samples was shown in Fig. 4. As shown in the figure, the reducing power of the samples correlated well with increasing concentration. And the reducing powers of **4** and **5** were 0.71 and 1.01 at the concentration of 4.07 and 3.98 mg/mL, respectively, which showed stronger reducing powers. In contrast, the reducing power of sulfated derivative **3** was only 0.32 at 4.00 mg/mL because of the slow rate of increasing power with increasing concentration.

It has been previously reported that there was a direct correlation between antioxidant activities and reducing power of certain plant extracts (Duh, Du, & Yen, 1999). The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Nishide et al., 1988). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, acetylated derivatives with high donating-hydrogen abilities showed excellent reducing power probably. And for sulfated derivative, the introduction of the sulfate group lead to the diminution of hydroxyl groups, which resulted in the descent of the reducing power.

3.5. Analysis of structure–activity

Chemical modification of polysaccharide is an important part in obtaining news antioxidants. In general, the presence of sulfate group is an essential requirement for the antioxidant activity. DS of polysaccharide is also an important parameter influencing antioxidant activity. In this paper, the desulphurization of porphyran was executed via alkali treatment. The result was that the sample **2** with lower DS showed lower activities than raw material **1** in there assays. However, the DPPH radical-scavenging activity and reducing power also decreased for sulfated porphyran with high DS. This suggested that though the sulfate group was essential, the certain DS was also necessary for the activity. On the other hand, the acetyl group could significantly increase antioxidant activities. Two acetylated derivatives **4** and **5** showed more excellent antioxidant activities than other samples with no acetyl group. And especially, **4** and **5** contain the approximate content of acetyl group but **5** was with a little sulfate group. The result was that **5** showed parallel or higher activities in three activity assays. This indicated that acetyl group could much more increase antioxidant activities relatively to sulfate group.

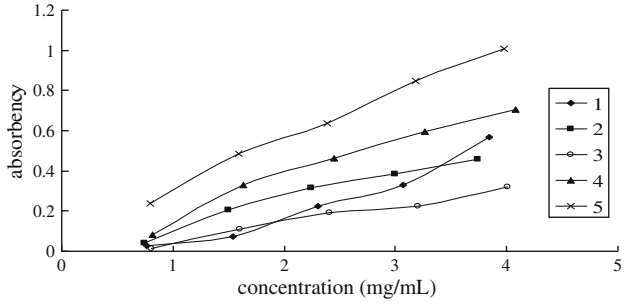


Fig. 4. Reducing power assay of the sample porphyran and its derivatives. Values are means ± *SD* ($n = 3$).

Besides DS, the molecular weight is another important parameter influencing antioxidant activities. Zhao et al. (2006) reported that the porphyran with different molecular weight showed different antioxidant activities. Higher antioxidant activities were found when the molecular weight decreased. In the present research, the decrease of molecular weight is due to the reaction of acidic surroundings. But on the lateral face, the acetylated derivative with lower molecular weight which showed higher bioactivity might be related. However, the bad activities of sulfated derivative **3** in the latter two activities assays indicated that sulfate group was a more influential parameter than molecular weight.

4. Conclusion

In the present study, porphyran and its derivatives, sulfated porphyran and acetylated porphyran were prepared. And then we evaluated their antioxidant activities in vitro and characterized the relationship between antioxidant activity and chemical characteristics. The activities were strongly dependant on the function group and DS. In this study, the antioxidant activities depend on the function group, degree of substitution and molecular weight. Function group and certain DS would be influential parameter on antioxidant activities. And further studies are needed to improve our understanding of antioxidant activities mechanism.

Acknowledgements

This work was supported by the Department of Science and Technology of Shandong Province (2007BS07004). We are grateful to Prof. Zuhong Xu and Prof. Zhien Li for their help in this experiment.

References

Banerjee, A., Dasgupta, N., & De, B. (2005). In vitro study of antioxidant activity of *Syzygium cumini* fruit. *Food Chemistry, 90*, 727–733.

Bohn, J. A., & BeMiller, J. N. (1995). (1 → 3)-β-D-Glucans as biological response modifiers: A review of structure–functional activity relationships. *Carbohydrate Polymers, 28*, 3–14.

Brasch, D. J., Chang, H. M., Chuah, C. T., & Melton, L. D. (1981). The galactan sulfate from the edible, red alga *Porphyran columbina*. *Carbohydrate Research, 97*, 113–125.

Dahl, M. K., & Richardson, T. (1978). Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and amino acid. *Journal of Dairy Science, 61*, 400–407.

Dubois, M., Gillis, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Duh, P. D., Du, P. C., & Yen, G. G. (1999). Action of methanolic extract of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. *Food and Chemical Toxicology, 37*, 1055–1061.

Franz, G., & Alban, S. (1995). Structure–activity relationship of antithrombotic polysaccharide derivatives. *International Journal of Biological Macromolecules, 17*, 311–314.

Geresh, S., Dawadi, R. P., & Arad (Malis), S. (2000). Chemical modifications of biopolymers: quaternization of the extracellular polysaccharide of the red microalgae *Porphyridium* sp.. *Carbohydrate Polymers, 43*, 75–80.

Gretz, M. R., McCandless, E. L., Aronson, J. M., & Sommerfeld, M. R. (1983). The galactan sulphates of the conchocelis phases of *Porphyra leucosticta* and *Bangia atropurpurea*. *Journal of Experimental Botany, 34*, 705–711.

Kawai, Y., Seno, N., & Anno, K. (1969). A modified method for chondrosulfatase assay. *Analytical Biochemistry, 32*, 314–321.

Kennedy, J. F. (1989). *In carbohydrates*. Oxford: Oxford University Press.

Kennedy, J. F., & White, C. A. (1983). *In bio-active carbohydrates* (pp. 116–180). Chichester: Ellis Horwood.

Mau, J. L., Chang, C. N., Huang, S. J., & Chen, C. C. (2004). Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia, mushrooms. *Food Chemistry, 87*, 111–118.

Melo, M. R. S., Feitosa, J. P. A., Freitas, A. L. P., & de Paula, R. C. M. (2002). Isolation and characterization of soluble sulfated polysaccharide from the red seaweed. *Gracilaria cornea*. *Carbohydrate Polymers, 49*, 491–498.

Nishide, E., Ohno, M., Anzai, H., & Uchida, N. (1988). Extraction of porphyran from *Porphyra yezoensis* Ueda *F. narawaensis* Miura. *Nippon Suisan Gakkaishi, 54*, 2189–2194.

Nishimiki, M., Rao, N. A., & Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochemical and Biophysical Research Communications, 46*, 849–854.

Nagai, T., & Yukimoto, T. (2003). Preparation and functional properties of beverages made from sea algae. *Food Chemistry, 81*, 327–332.

Nandita, S., & Rajini, P. S. (2004). Free radical scavenging activity of an aqueous extract of potato peel. *Food Chemistry, 85*, 611–616.

Notari, R. E., & Munson, J. W. (1969). Hydroxamic acids. I. Factors affecting the stability of the hydroxamic acid–iron complex. *Journal of Pharmaceutical Sciences, 58*, 1060–1064.

Patel, R. P., Cornwell, T., & Darley-USMAR, V. M. (1999). The biochemistry of nitric oxide and peroxynitrite: implications for mitochondrial function. In E. Cadenas & L. Packer (Eds.), *Understanding the process of ageing: The roles of mitochondria, free radicals, and antioxidants* (pp. 39–40). New York: Academic Press.

Qi, H. M., Zhang, Q. B., Zhao, T. T., Chen, R., Zhang, H., Niu, X. Z., et al. (2005). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) in vitro. *International Journal of Biological Macromolecules, 37*, 195–199.

Rees, D. A. (1961). Estimation of the relative amounts of isomeric sulphate esters in some sulphated polysaccharides. *Journal of the Chemical Society, 85*, 5168–5171.

Soares, J. R., Dins, T. C. P., Cunha, A. P., & Almeida, L. M. (1997). Antioxidant activity of some extracts of *Thymus zygis*. *Free Radical Research, 26*, 469–478.

Wang, J., Liu, L., Zhang, Q. B., Zhang, Z. S., & Qi, H. M. (2009). Synthesized oversulphated, acetylated and benzoylated derivatives of fucoidan extracted from *Laminaria japonica* and their potential antioxidant activity in vitro. *Food Chemistry, 114*, 1285–1290.

Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Bioscience, Biotechnology, and Biochemistry, 62*, 1201–1204.

Yanagimoto, K., Lee, K. G., Ochi, H., & Shibamoto, T. (2002). Antioxidative activity of heterocyclic compounds found in coffee volatiles produced by Maillard reaction. *Journal of Agricultural and Food Chemistry, 50*, 5480–5484.

Yaphe, W., & Arsenault, G. P. (1965). Improved resorcinol reagent for the determination of fructose and 3,6-anhydro-galactose in polysaccharides. *Analytical Biochemistry, 13*, 143–148.

Yen, G. C., & Chen, H. Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry, 43*, 27–32.

Zhao, T. T., Zhang, Q. B., Qi, H. M., Zhang, H., Niu, X. Z., Xu, Z. H., et al. (2006). Degradation of porphyran from *Porphyra haitanensis* and the antioxidant activities of the degraded porphyrans with different molecular weight. *International Journal of Biological Macromolecules, 38*, 45–50.