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In vitro antioxidant activities of acetylated, phosphorylated and benzoylated derivatives of porphyran extracted from *Porphyra haitanensis*

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

[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 red algae *Porphyra haitanensis* is a sulfated polysaccharide, which possesses excellent antioxidant activities. In this study, we prepared the acetylated, phosphorylated and benzoylated derivatives of porphyran. And then the antioxidant activities of all the samples were investigated including scavenging effects of superoxide and hydroxyl radicals and reducing power. The results of chemical analysis and FT-IR spectrum showed the modifications of porphyran were successful. And in addition, we found that certain derivative exhibited stronger antioxidant activity than raw material. And the mechanism of the structure–function relationship of these derivatives needs to be attended to.

1. Introduction

Reactive oxygen species (ROS), capable of causing damage to DNA, have been associated with carcinogenesis, coronary heart disease, and many other health problems related to advancing age (Cadenas & Davies, 2000; Uchida, 2000). In order to reduce damage to the human body and prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. Antioxidants are substances that restrain the oxidation of cellular oxidizable substrates. They exert their effects by scavenging ROS, activating a battery of detoxifying proteins, or preventing the generation of ROS (Kinsella, Frankel, German, & Kanner, 1993). Thus, it is essential to develop and utilize effective antioxidants which can protect the human body from free radicals and retard the progress of many chronic diseases (Nandita & Rajini, 2004). In general, the natural antioxidants mainly make up many compounds including phenolic, nitrogen compounds and carotenoids (Velioglu, Mazza, Gao, & Oomah, 1998). In recent years, many marine resources have attracted attention in the search for bioactive compounds to develop new drugs and health foods. In addition, marine algae are now being considered to be a rich source of antioxidants (Nagai & Yukimoto, 2003). Qi et al. (2005) reported that polysaccharide extracted from *Ulva pertusa* showed excellent radical scavenging activity against superoxidant and hydroxyl radical.

Polysaccharides and their derivatives have been found numerous applications in a variety of fields including food, chemical and pharmaceutical industries. 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). Xing et al. (2005) found that the sulfated derivatives of chitosan showed stronger scavenging activity on superoxide radical and reducing power. New derivatives showed interesting properties and biological activities, and it might be useful to understand the structure–function relationship of polysaccharide.

It was reported that polysaccharides extracted from algae possessed scavenging activities on reactive oxygen species. Porphyrans, the sulfated polysaccharides comprising the hot-water soluble portion of cell wall, are the main components of red algae, P. *haitanensis*. Porphyran is related to agarose in that it contains disaccharide units consisting of 3-linked β-D-galactosyl residues alternating with 4-linked 3, 6-anhydro-α-L-galactose, but differs in that some residues occur as the 6-sulfate (Gretz, McCandless, Aronson, & Sommerfeld, 1983). Zhao et al. (2006) reported porphyran showed strong antioxidant activities in both assay systems. In the present paper, we reported that the synthesis of a series of derivatives of porphyran obtained from *P. haitanensis* by means of acetylation, phosphorylation and benzoylation coupling and investigated their antioxidant activities. The relationship between

* Corresponding author. Address: Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China. Tel.: +86 532 82898708; fax: +86 532 82898703.
E-mail address: qbzhang@ms.qdio.ac.cn (Q. Zhang).

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the nature of functionalized groups and the chemically modified derivatives and their antioxidant activity were also discussed.

2. Materials and methods

2.1. Chemicals

Porphyran (P) was isolated from *P. haitanensis*, cultured in the coast of Lianjiang County, Fujian, China (Nishide, Ohno, Anzai, & Uchida, 1988). Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), ethylene diamine tetra-acetic acid (EDTA), ferrozine, nicotinamide adenine dinucleotide reduced (NADH), ferric chloride, trichloroacetic acid (TCA), and potassium ferricyanide were purchased from Sigma Chemicals Co. All other chemicals and reagents, unless otherwise specified, were of analytical grade. Dialysis membranes were produced by Spectrum Co., and molecular weight was cut off at 3600 Da.

Sulfate content was determined by barium chloride-gelatin method (check) (Kawai, Seno, & Anno, 1966). 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). The total phosphate content was determined by the ascorbic acid method (Lowry, Roberts, Leiner, Wu, & Farr, 1954). Infrared spectrums were measured by a Nicolet Magna-Avatar 360 with KBr disks.

2.2. Acetylation of porphyran

The acetylation of porphyran was prepared by the method described previously in our laboratory (Wang, Liu, Zhang, Zhang, & Qi, 2009). Porphyran (2.0 g) was dispersed in 80 mL formamide (FA), and the mixture was stirred at 80 °C for 30 min, then added 50 mL acetic anhydride and 1% *N*-Bromosuccinimide (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. 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. The resultant was concentrated and lyophilized to give the product acetylated porphyran (AP).

2.3. Phosphorylation of porphyran

The phosphorylation of porphyran was prepared by the modified method of Inoue, Kawamoto, Nakajima, Kohno, and Kadoya (1983). Porphyran (2.0 g) was dissolved in formamide (100 mL), and tributylamine (10 mL) and polyphosphoric acid (5.0 g) were added to the clear solution. The mixture was stirred for 24 h at room temperature, and then poured into ethanol (600 mL). The resulting precipitate was collected by centrifugation, and dissolved in water (100 mL). The pH of the solution was adjusted to 10 with 2 M NaOH solution, and the solution was evaporated at 37 °C in vacuum, and dialyzed against distilled water for 24 h using 3600 Da Mw cutoff dialysis membranes. The resultant was concentrated and lyophilized to give phosphorylated porphyran (PP).

2.4. Benzoylation of porphyran

The benzoylation of porphyran was prepared by the modified method of Qi, Zhang, Zhao, and Hu (2006). Briefly, a mixture of porphyran (2.0 g) and formamide (80 mL) was heated at 80 °C for 30 min. Then, 4-dimethylaminopyridine (DMAP) (0.1 g), *p*-toluenesulfonyl chloride (*p*-TsCl) (7.0 g) and 1,2-benzenedicarboxylic anhydride (36.0 g) were added orderly. After reaction for 6 h at 60 °C, the mixture was terminated by pouring 50 mL of distilled water, cooled to room temperature, and precipitated with 85% ethanol. The precipitate was filtered off and washed three times with ethanol, and then dissolved in 100 mL distilled water. The solution was dialyzed against tap water for 48 h and then against distilled water for 24 h using dialysis membranes (3600 Da Mw cutoff). The product was then concentrated and lyophilized to give the product benzoylated porphyran (BP).

2.5. Antioxidant activity

2.5.1. Superoxide radical assay

The superoxide radical scavenging abilities of all samples were assessed by the modified method of Nishimiki, Rao, and Yagi (1972). In this experiment, superoxide anion radicals were generated in 4.5 mL Tris–HCl buffer (16 mM, pH 8.0) containing 0.5 mL of NBT (300 μM) solution, 0.5 mL of NADH (468 μM) solution and one sample (0.5–50.0 μg/mL). The reaction was started by adding 0.5 mL of 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 anion-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 $A_{\text{control } 560}$ is the absorbance of the control (Tris–HCl buffer, instead of sample).

2.5.2. Hydroxyl radical assay

The reaction mixture, containing all different derivatives (0.6–7.0 mg/mL), was incubated with EDTA–Fe^{2+} (2.0 mM), saffron (360 μg/mL), and H_2O_2 (3%) in potassium phosphate buffer (150 mM, pH 7.4), and was incubated for 30 min at 37 °C (Wang et al., 1994). The absorbance was read at 520 nm against a blank. Hydroxyl radical bleached the saffron, so decreased absorbance of the reaction mixture indicated a decrease in hydroxyl radical scavenging ability. The capability of scavenging hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the control (without samples) and A_1 is the absorbance of the mixture containing samples.

2.5.3. Reducing power assay

The reducing power was determined as described previously by Yen and Chen (1995). Briefly, 1.0 mL of 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 of potassium ferricyanide (1%, w/v), and was incubated at 50 °C for 20 min. Afterwards, 2.0 mL of 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. Chemical analysis

The chemical composition of all the samples was given in Table 1. The total sugar and sulfate contents of all the derivatives were lower than raw material because of the addition of other groups.

Table 1
Yield and chemical composition of the samples (% w/w of dry weight).

Sample	Yield	Total sugar	Sulfate	3,6-Anhydrogalactose
P	–	78.9	17.7	10.5
AP	85.7	74.4	14.1	9.7
PP	49.8	71.2	11.6	8.9
BP	39.7	68.9	9.3	6.3

The FT-IR spectrums of the products were shown in Fig. 1. Typical signals of porphyran at 3420, 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} or so was indicative of a sulfate group attached to a primary hydroxyl group (Brasch, Chang, Chuah, & Melton, 1981). Another weak peak at 933 cm^{-1} was due to the 3,6-anhydrogalactose unites in the polysaccharide. These results suggested that no major functional group transformations happened during the reaction.

For AP, a new brand of strong intensity at 1726 cm^{-1} in the AP was attributed to the C=O stretching vibration, which indicated that the acetyl group was successfully modified at raw material. And what's more, the signal at 1225 cm^{-1} which became stronger was also assertive evidence. In PP, the total phosphate content was 4.15% as we determined. For the FT-IR spectrum, the signal at 1268 cm^{-1} indicated the P=O stretching vibration, and the signal at 988 cm^{-1} was attributed to the P—O vibration. However, these signals were not evident because they overlapped with the signals of P. For BP, the peaks at 1724 cm^{-1} was assigned to the characteristic absorbance of C=O (aster) stretching vibration (Tosh, Saikia, & Dass, 2000) and the signals at 1561 and 1447 cm^{-1} were attributed to the C=C of phenyl group, which showed that BP were obtained.

3.2. Superoxide radical assay

The superoxide radical ($.O_2^-$) is 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 effects on the superoxide radical of all the samples. For these four samples, at the concentration below 5 μg/mL, the scavenging effects significantly increased with increasing concentration, and at the concentration over 5 μg/mL, the scavenging effects increased slowly. Compared with P, the IC_{50} values of AP, PP, and BP were 1.14, 4.06 and 3.54 μg/mL, respectively. However, at the concentration over 5 μg/mL, BP showed stronger effect and

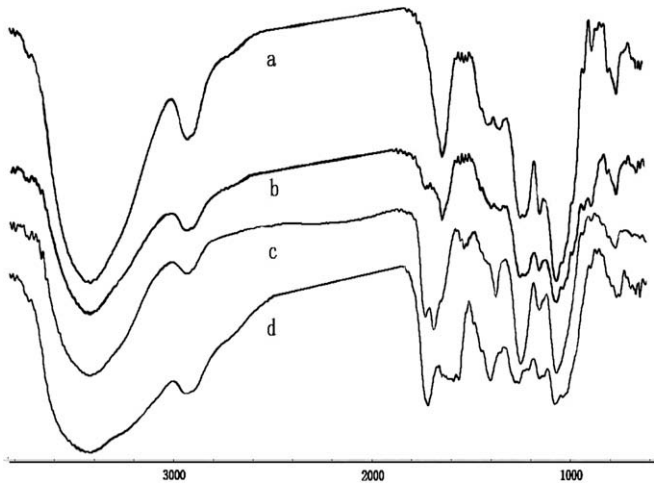

Fig. 1. FT-IR spectra of samples P (a), PP (b), AP (c) and BP (d).

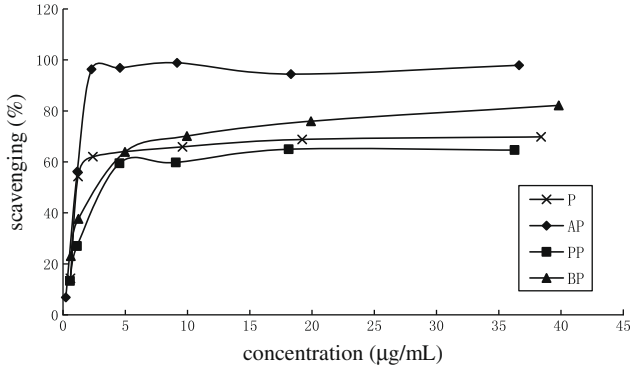

Fig. 2. Scavenging effects of P, AP, BP and PP on superoxide radical. Values are means ± SD ($n = 3$).

PP showed weaker effect than P. AP showed significant scavenging effect than P, PP, BP and the difference is obvious.

Yanagimoto, Lee, Ochi, and Shibamoto (2002) reported that addition of electron-withdrawing groups to the pyrrole enhanced antioxidant activity. So in this study the presence of acetyl and benzoyl groups could increase the activity of scavenging radicals. However, the electron density of carbon atoms on a heterocyclic ring may not determine the strength of antioxidant activity. Other properties of the compounds, such as polarity, may also be involved in their antioxidant activity. In this assay, PP showed weak scavenging activity. We supposed that presence of phosphate groups 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). The results above indicated that the antioxidant activities of all the samples were related to their abilities to scavenge superoxide.

3.3. Hydroxyl radical assay

The hydroxyl radical, known to be generated through the Fenton reaction in this system, was scavenged by polysaccharide samples. The scavenging effect of all samples was shown in Fig. 3. For all the samples, the effects of scavenging hydroxyl radicals were in a concentration-dependent manner. The IC_{50} values of P, PP, and BP were 6.55, 0.31, 1.04 mg/mL, respectively. But the IC_{50} value of AP could not be read. Among the four samples, PP and BP showed excellent scavenging effects and at the concentration of 5.02 mg/mL, the scavenging effects were 95%. As shown in Fig. 3, AP showed parallel scavenging effect with P, which suggested the modification of the acetyl group take little effect on scavenging action on the hydroxyl radicals.

For hydroxyl radical, there were two types of antioxidation mechanism; one suppresses the generation of the hydroxyl radical, and the other scavenges the hydroxyl radicals generated. In the former, the antioxidant activity may ligate to the metal ions which react with H_2O_2 to give the metal complexes. The metal complexes thus formed cannot further react with H_2O_2 to give hydroxyl radicals (Ueda, Saito, Shimazu, & Ozawa, 1996). Iron, a transition metal, is capable of generating free radicals from peroxides by the Fenton reaction and is implicated in many diseases. Fe^{2+} has also been shown to produce oxyradicals and lipid peroxidation, and reduction of Fe^{2+} concentrations in the Fenton reaction would protect against oxidative damage. In the present study, phosphate and

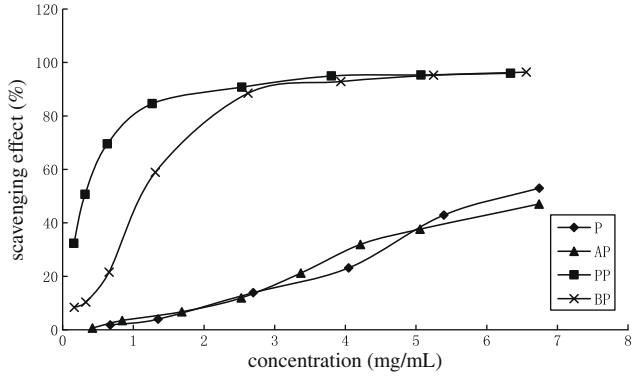

Fig. 3. Scavenging effects of P, AP, BP and PP on hydroxyl radical. Values are means ± SD (n = 3).

benzoyl groups had high nucleophilic characteristic and could chelate with metal ion, so the hydroxyl radical scavenging activities of phosphorylated and benzoylated derivatives were much stronger than P. And on the other hand, the quantities of hydroxyl groups reduced because of the introduction of the acetyl groups, which had weaker abilities on chelating with metal ion than hydroxyl group. So these two derivatives showed weaker hydroxyl radical scavenging activity. The mechanism of different derivatives on the hydroxyl radicals needs to be further investigation.

3.4. 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 concentrations except that of PP, which showed extremely weak reducing power even at higher concentration. However, the reducing powers of AP and BP were 1.13 and 0.94 at the concentration of 5.95 and 5.71 mg/mL, respectively. In contrast, the reducing power of P was only 0.42 at 6.17 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 reductant, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductant is also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, AP and BP with high donating-hydrogen ability showed excellent reducing power probably. And for PP, the introduction of the phosphate group lead to the diminution of hydroxyl groups, which resulted in the descent of the reducing power.

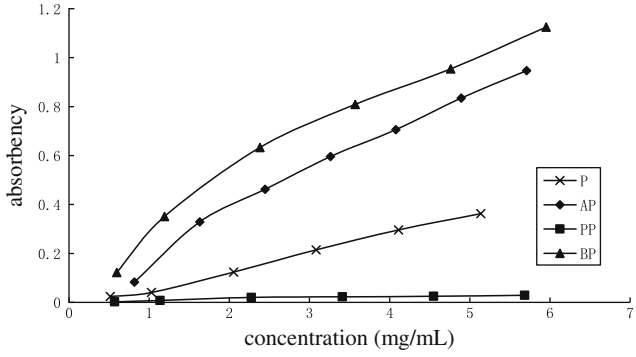

Fig. 4. Reducing power of P, AP, BP and PP. Values are means ± SD (n = 3).

4. Conclusion

The results of the present work indicated that all kinds of derivatives possessed antioxidant activities in certain assays. Of the four samples, AP, PP and BP had the strong radical scavenging effect in one or two systems of assay, respectively and they may have a use as a possible supplement in the food and pharmaceutical industries. The radical scavenging effect was stable at high temperatures so that these derivatives may be used as resources of medicine. However, factors effecting and attributing to radical scavenging effect need to be further studied.

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