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In vitro antioxidant activity of polysaccharides extracted from *Bryopsis plumosa*

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

Polysaccharides extracted from *Bryopsis plumosa* (Hudson).C. Ag. are a group of sulfated hetero polysaccharides, and their antioxidant activities were investigated employing various established in vitro systems. Available data obtained with in vitro models suggested that among the three samples, B3 (extraction with sodium hydroxide) showed significant inhibitory effects on superoxide radical and DPPH with IC_{50} values of 9.2 μg mL^{-1} and 1.7 mg mL^{-1}; its reducing power was also the strongest among the three samples. These in vitro results clearly establish the possibility that polysaccharides extracted from *B. plumosa* could be effectively employed as ingredient in health or functional food, to all deviate oxidative stress. However, comprehensive studies need to be conducted in experimental animal models.

1. Introduction

A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially superoxide and hydroxyl radical) as mediators of inflammation, shock, and ischemia/reperfusion injury. Furthermore, the radicals also play a role in the process of ageing and carcinogenesis (Cuzzocrea, Riley, Caputi, & Selvemini, 2001). In order to reduce damage to the human body and prolong the storage stability of food, synthetic antioxidants are used for industrial processing. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. In recent years, there has been increasing interest in finding natural antioxidants since the synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are suspected of being responsible for liver damage and carcinogenesis (Grice, 1988; Witschi, 1986).

In the search of new antioxidants, exploration of aquatic habitats has led to the discovery that marine plants and invertebrates also contain antioxidants. Marine algae are now being considered to be a rich source of antioxidants (Nagai & Yukimoto, 2003). Cell walls from marine algae characteristically contain sulfated polysaccharides, which are not found in land plants and which may have specific functions in ionic regulation (Kloareg & Quatrano, 1998). In recent years, algal polysaccharides were reported to be useful candidates in the search for an effective non-toxic substance and have been demonstrated to play an important role as free radical scavengers in vitro and antioxidants for the prevention of oxidative damage in living organisms (Ruperez, Ahrazem, & Leal, 2002; Xue et al., 2001; Zhang et al., 2003, 2004). Ruperez et al. (2002) found that sulfated polysaccharides from *Fucus vesiculosus* showed antioxidant activity by the ferric reducing antioxidant power assay; sulfated polysaccharides from *Laminaria japonica and Ecklonia kurome* were also demonstrated to have free radical scavenging activities (Xue et al., 2001).

Bryopsis plumosa (Hudson).C. Ag. belongs to Bryopsidaceae of Chlorophyceae, mainly living in warm sea, clustering round rocks of tideland. During the last years, many studies have been made on algae, and most of the investigations focused on the red algae and brown algae. However, studying on green alga is still limited. Green alga distribute widely in the sea. In previous studies, People focused on species such as Ulva lactuca (Beer, Sand-Jensen, Vindbaek Madsen, & Nielsen, 1991). *B. plumosa* is newly exploited in recent years, and it is widely distributed in Chinese sea area. Polysaccharides are important composition of marine algae (Jiang, Liu, Tang, Ceng, & Wang, 2008), but by now no study on antioxidant activity of polysaccharides from *B. plumosa* was reported.

The aim of this study is to evaluate their in vitro antioxidant activity and characterize the relationship between chemical characteristic and antioxidant activity.

2. Experimental

2.1. Materials

Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Sigma Chemicals Co. All other chemicals and reagents, unless specified otherwise, were not purified, dried or pretreated.

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

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B. plumosa, cultured in Zhanqiao, Qingdao, China, was collected in September 2008. The fresh seaweed was soon washed; sun dried and kept in plastic bags at room temperature for use.

2.2. Chemical analytical methods

Total sugar content was determined according to the method of Dubois, Gillis, Hamilton, Rebers, and Smith (1956) and using galactans or glucose as standard, respectively. Sulfate content was analyzed by the barium chloride-gelatin method of Kawai, Seno, and Anno (1969) Uronic acid was estimated in a modified carbazole method using D-glucuronic acid as standard (Bitter & Muir, 1962). Infrared spectra were recorded from polysaccharide powders in KBr pellet on a Nicolet-360 FTIR spectrometer. Protein content was measured following Kjeldahl method; Ash content was determined according to burning with high temperature. 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.

2.3. Neutral sugar analysis (Honda, Akao, Suzuki, & Okuda, 1989)

2.3.1. Hydrolysis of polysaccharide

Polysaccharide sample (15.0–20.0 mg) was dissolved in 2 M trifluoroacetic acid (2.0 mL) in a 10 mL ampoule. The ampoule was sealed in a nitrogen atmosphere and incubated for 4 h at 110 °C. Following incubation, the ampoule was cooled to room temperature. The reaction mixture was then neutralized to pH 7 with 2 M sodium hydroxide, and we added 2.0 mL of the internal standard solution. The mixture was shaken well, diluted to 10 mL and filtered. The filtrate was retained for PMP (1-phenyl-3-methyl-5-PyraZolone) determination.

2.3.2. Derivatization with PMP

We added 0.5 M methanolic solution of PMP (100 μL) and 0.3 M aqueous sodium hydroxide (100 μL) to the monosaccharide reference solution or a reducing polysaccharide solution (100 μL each). The mixture was incubated at 70 °C for 30 min. The reaction mixture was then cooled at 8 °C, and neutralized with 0.3 M hydrochloric acid. We then added 1 mL of chloroform to the solution. The mixture was shaken well and centrifuged at 5000 r min^{-1} for 10 min at 6–8 °C. The chloroform layer was discarded and the aqueous layer was extracted twice with chloroform. The final aqueous layer was analyzed directly by HPLC.

2.3.3. Chromatography

Chromatographic conditions were generally as follows: column, YMC-Pack ODS-AQ (250 × 4.6 mm, 5 μm); temperature, 25 °C; solvent A, 0.4% triethylamine in 20 mM ammonium acetate buffer solution (pH 6.30 with acetic acid)-acetonitrile (9:1); solvent B, 0.4% triethylamine in 20 mM ammonium acetate buffer solution (pH 6.30 with acetic acid)-acetonitrile (4:6); gradient, 10–14% in 9 min, 14–64% from 9 min to 30 min, 64% during the next 5 min at 1 mL min^{-1}. The eluate was monitored at 245 nm.

2.4. Preparation of natural B. plumosa

2.4.1. Extraction with water

The air-dried algal specimen was extracted at 115 °C for 3 h with 40 portions of water. The hot aqueous solution was separated from the algae residues by successive filtration through gauze and siliceous earth. The solution was dialyzed against tap water for 48 h and against distilled water for 24 h, and then the solution was concentrated under reduced pressure. The concentrated extract was lyophilized to give product (B1) as a white powder.

2.4.2. Extraction with sulfuric acid

Air-dried algal fronts were heated in 40 volumes of 1.25% sulfuric acid at 85 °C for 30 min. The hot aqueous solution was separated from the algae residues by successive filtration through gauze, neutralized with 1 M sodium hydroxide, and then filtrated through siliceous earth. The solution was dialyzed against tap water for 48 h and against distilled water for 24 h, and then the solution was concentrated under reduced pressure. The concentrated extract was lyophilized to give product (B2) as a white powder.

2.4.3. Extraction with sodium hydroxide

Air-dried algal fronts were heated in 40 volumes of 0.1 M sodium hydroxide at 85 °C for 4 h. The hot aqueous solution was separated from the algae residues by successive filtration through gauze, neutralized with 1 M hydrochloric acid, and then filtrated through siliceous earth. The solution was dialyzed against tap water for 48 h and against distilled water for 24 h, and then the solution was concentrated under reduced pressure. The concentrated extract was lyophilized to give product (B3) as a white powder.

2.5. Antioxidant activities

2.5.1. 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^{-1}). 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:

$$\text{Scavenging effect } (\%) = \left(1 - \frac{\text{Asample } 560}{\text{Acontrol } 560}\right) \times 100,$$

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

2.5.2. 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:

$$\text{Scavenging effect } (\%) = \left(1 - \frac{\text{Asample } 517}{\text{Acontrol } 517}\right) \times 100,$$

where Acontrol 517 is the absorbance of the control (50% ethanol solution, instead of sample).

2.5.3. 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^{-1}) 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. Chemical analysis

The FT-IR spectrum of the products was shown in Fig. 1. Infrared spectroscopy analysis indicated that all the three samples showed typical peaks of polysaccharide, including peaks at 3420, 2937, 1650, 1404, 1225, 1155, 1073 and 795 cm^{-1}. The peak at 1225–1265 cm^{-1} was assigned to the asymmetric stretching vibration of sulfate group and the signal at 817 cm^{-1} or so was indicative of sulfate group attached to primary hydroxyl group. The chemical constitutions of the polysaccharides were shown in Table 1. The result was consistent with infrared analysis. Neutral monosaccharide constitutions of the polysaccharides were analyzed by HPLC. For the sample B1, galactose was the main sugar unit, accounting for 43.0%. For the sample B2, glucose was the main unit, accounting for 90.3%. For the sample B3, galactose was the main sugar unit, accounting for 46.8%. The molar ratio of the monosaccharides was shown in Table 2.

3.2. Antioxidant activities

3.2.1. Superoxide radical assay

The superoxide radical is a highly toxic species that is generated by numerous biological and photochemical reactions (Banerjee, Dasgupta, & De, 2005). Fig. 2 shows that the inhibitory effect of all samples on superoxide radicals was marked and concentration related. Significant scavenging of superoxide radical was evident at all the tested concentrations of all products. Moreover, as shown in Fig. 2. IC_{50} of B1, B2, B3 was 0.011, 0.12, 0.0092 mg mL^{-1}, respectively. Xing et al. (2005) reported that scavenging activity of Vitamin C for superoxide radical was 68.2% at 2.0 mg mL^{-1}. Compared to this result, all samples had stronger scavenging activity for superoxide radical than Vitamin C. 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). These results clearly showed that the antioxidant activities of all samples were related to the abilities of scavenging superoxide radical.

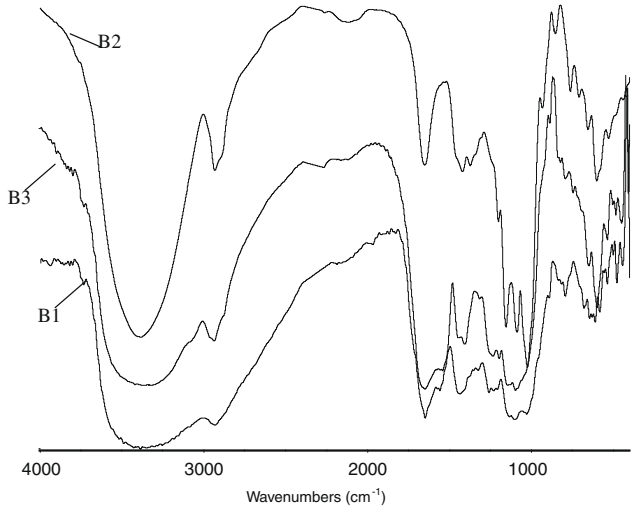

Fig. 1. FT-IR spectrums of the sample B1, B2, and B3.

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

Samples	Total sugar (%)	Sulfate (%)	Glucuronic acid (%)	Protein (%)	Ash (%)	Molecular weight (kDa)
B1	41.8	7.56	7.73	8.00	19.7	227
B2	87.2	6.71	3.73	0.556	23.5	5.54
B3	31.7	11.4	5.03	9.57	27.3	228

Table 2
Molar ratios of the neutral sugar components in polysaccharide samples from *Bryopsis plumose*.

Sugar	B1	B2	B3
Galactose	43.0	2.38	46.8
Arabinose	31.0	4.36	22.0
Glucose	4.62	90.3	9.85
Rhamnose	4.44	0.268	3.16
Mannose	7.13	0.838	5.16
Fucose	9.82	1.83	13.1

3.2.2. DPPH radical scavenging assay

DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al., 1998). Further it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (Chen & Ho, 1995). DPPH radical was scavenged by antioxidant through donation of hydrogen to form a stable DPPH molecule (Matthaus, 2002). Total DPPH scavenging effects of all samples at varying concentrations were measured and the results were depicted in Fig. 3. At the concentration below 1.25 mg mL^{-1}, the scavenging effect of B1 was weaker than B3. But when it was over the above concentration and below 2.0 mg mL^{-1}, the former showed stronger effect by the concentration. Then, when the concentration was above 2.0 mg mL^{-1}, the scavenging effect of B3 was still stronger than B1. Evidently, at all the tested concentrations B2 showed much weaker on DPPH radical scavenging effect than B1 and B3. How-

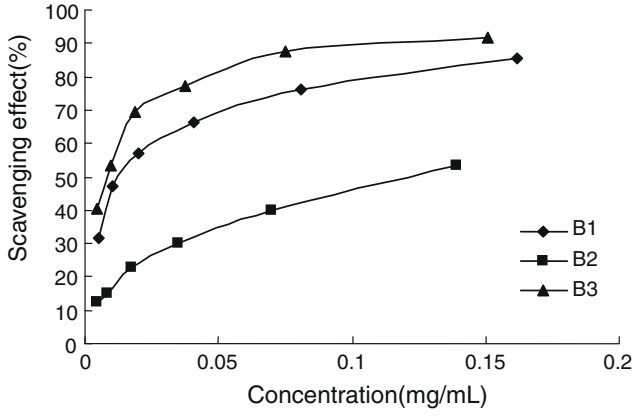

Fig. 2. Scavenging effects of the sample B1, B2, and B3 on superoxide radical. Values are means ± SD ($n = 3$).

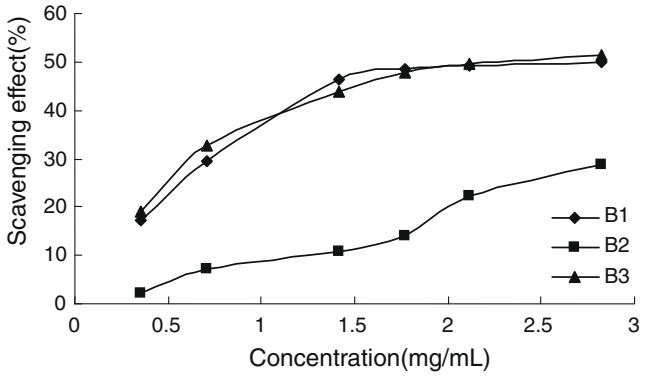

Fig. 3. Scavenging effects of the sample B1, B2, and B3 on DPPH radical. Values are means ± SD (n = 3).

ever, the mechanism of all products on DPPH radical need to be further researched.

3.2.3. Reducing power assay

The antioxidant activity has been reported to have a direct, positive correlation with the reducing power (Osman, Nasarudin, & Lee, 2004). 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 (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Xing et al., 2005). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power of all samples was shown in Fig. 4. Among the samples, the scavenging ability was found to be decreased in the order of B3 > B1 > B2. As shown in the figure, the reducing power of B1 and B3 correlated well with increasing concentration. Except for B2 the scavenging effect lowly increased with increasing their concentration. Our data on the reducing power of all samples, especially B1 and B3, suggested that it was likely to contribute toward the observed antioxidant effect.

3.2.4. Discussion

This study led to three sulfated polysaccharides samples from *B. plumosa*. B3 had highest sulfate content, protein content, molecular weight and lowest total sugar content, while B2 had lowest sulfate content, protein content, molecular weight and highest total sugar content. We presumed that these differences were caused by different extraction method.

In our experiment sulfated polysaccharide samples from *B. plumosa* exhibited significant antioxidant effects. This indicated that like sulfated polysaccharides from brown algae and red algae, sulfated polysaccharide from green algae are effective antioxidant.

The activity of polysaccharide depends on several structural parameters such as degree of sulfation, the molecular weight, protein content, type of sugar and glycosidic branching (Alban, Schauerte, & Franz, 2002). It shows that chemical property may have great influence on antioxidant activities, and has been proved in this research.

In the antioxidant assay, the scavenging activity and reducing power decreased in the order of B3 > B1 > B2, the same order of sulfate content in the samples. This demonstrated that the sulfate content affected their antioxidant activity, these results were in accordance with Zhang et al. (2003) found that phosphated and sulfated glucan exhibited greater antioxidant ability, indicating polyelectrolytes, such as glucan sulfate or phosphate, might have increased scavenging activity.

And what's more, the products with different molecular weight showed different antioxidant activities. Higher antioxidant activities were found when the molecular weight increased. These results were contrary to Zhao et al. (2006) investigated that in the antioxidant assay, the low molecular weight products are more effective than high molecular weight products. Therefore, we presumed that the antioxidant activity varying with molecular weight was different in different range. However, these results indicated that molecular weight plays an important role in antioxidation.

Furthermore, besides of sulfate content and molecular weight, protein content and monosaccharide constitution also influence their antioxidant activities. The antioxidant activities of products increased when the protein content increased. This result correlated to the findings of Liu, Ooi, and Chang (1997) that the content of proteinous substances in the polysaccharide molecules potentiate their free radical scavenging activity. But the exact correlation between the chemical characteristics and antioxidant activities of polysaccharide extracted from *B. plumosa* needs further investigation.

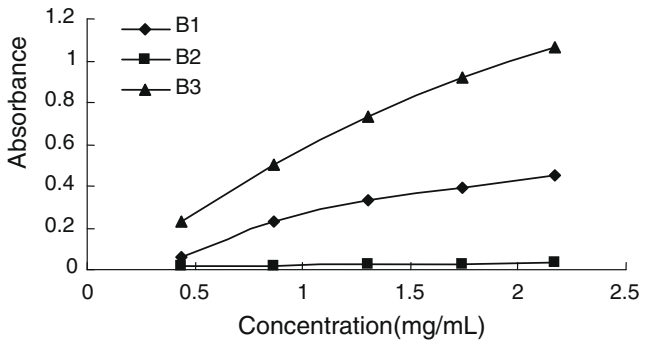

Fig. 4. Reducing power assay of the sample B1, B2, and B3. Values are means ± SD (n = 3).

4. Conclusion

The result of the present work indicated natural *B. plumosa* possessed antioxidant activities and free radical scavenging activities. Available data obtained with in vitro models suggested that among the three samples, B3 (extraction with sodium hydroxide) showed significant inhibitory effects on superoxide radical and DPPH with IC50 values of 9.2 μg mL^{-1} and 1.7 mg mL^{-1}; its reducing power was also the strongest among the three samples. These assays had important applications for the pharmaceutical and food industries. However, their in vivo antioxidant activity and the different antioxidant mechanisms need to be further researched.

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