Ex vivo Anti-Oxidation Activity of Polysaccharides from Red Alga Porphyra yezoensis

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Abstract: In this study, the ex vivo anti-oxidation activity of polysaccharide extracted from the red seaweed Porphyra yezoensis was studied. The scavenging efficiencies for free radical O$_2^-$, •OH and •DPFH, the inhibitory effects on mice erythrocytes hemolysis induced by H$_2$O$_2$, as well as on the lipid peroxidation in mice liver homogenate, were systematically measured and analyzed. Results suggested that Porphyra polysaccharide, in the experimental concentration range, possessed anti-oxidation activity. The scavenging efficiency for O$_2^-$ was found to be remarkably high and the maximum scavenging rate was 82.77%. The scavenging efficiency for •OH was even higher than O$_2^-$ with a maximum removing rate of 85.63% and the scavenging for •DPFH was 13.97%. The inhibitory effects on mice erythrocytes hemolysis and MDA formation in mice liver were significant and the maximum inhibition rates were 82.90 and 58.48%, respectively. The combined data indicated that the polysaccharide extracted from Porphyra had strong anti-oxidation activity.

Key words: Porphyra yezoensis, polysaccharide, anti-oxidation, free radical, DPPH, MDA

INTRODUCTION

Oxygen-derived free radicals are common byproducts in the metabolism process. However, these compounds are highly active and can cause apparent damages, commonly known as the oxidative damages, to the cell membranes and other cellular structures (Cuzzocrea et al., 2001). The free-radical damage has been reported to directly associate with diseases such as atherosclerosis, cataract formation, ageing and carcinogenesis (Cuzzocrea et al., 2001). To counteract the destructiveness of the free radicals, human body possesses a complex antioxidant defense system that utilizes various vitamins, minerals and other naturally producing substances (Cuzzocrea et al., 2001). It is possible, therefore, to prevent the radical damage by supplementing the diet with certain food, nutrients and herbs that have antioxidant activity.

In recent years, sulfated polysaccharides from the marine brown algae, Fucus vesiculosus, have been demonstrated to have antioxidant activities (Ruperez et al., 2002). Xue et al. (2001) also confirmed this activity by the antioxidant functional assay through ferric reduction. Sulfated polysaccharides from Laminaria japonica and Ecklonia kurome Okam have also been reported to possess free radical scavenging activities (Ruperez et al., 2002; Hu et al., 2001; Han et al., 2006). More recently, data from various research laboratories have suggested that the polysaccharide from the red alga, Porphyra haitanensis or P. yezoensis, had multiple biological functions, such as anti-coagulant, anti-senescence (Zhang et al., 2002), anti-fatigue (Guo et al., 2005), anti-cancer (Zhang et al., 2001), antivirus (Zhou and Chen, 1990). Effects, proliferation promotion effects for lymphocytes and sertoli cells (Xiao et al., 2003; Guo et al., 2006), immune-modulation and free radical scavenging (Zhou and Chen, 1989; Yashizawa et al., 1993).

The red alga, Porphyra, has been an important food source in the Asian diet, as well as a drug used in the traditional Chinese medicine. Previously, three polysaccharide fractions with different sulfate content from P. haitanensis have been identified to have anti-oxidation activity (Wang et al., 2004) and two polysaccharide fractions from P. yezoensis have been discovered to possess anti-fatigue, enhancement of immunity and anti-cancer properties (Guo et al., 2005, 2006; Zhang et al., 2007). In this study, the anti-oxidation activity of polysaccharides from Porphyra yezoensis was studied and the mechanism of the polysaccharide bioactivity from Porphyra yezoensis was proposed.

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MATERIALS AND METHODS

Materials: The polysaccharides, extracted from Porphyra yezoensis, were provided by the Lab of Seaweed Biotechnology, Shanghai Fisheries University, Shanghai, China. They were purified by DEAE-52 and SephadexG-200 columns following the protocol as previously described (Liu et al., 2005; Gu et al., 2007).

Measurement of the superoxide radical scavenging activity: The method for the measurement of superoxide radical scavenging activity is similar to the protocol reported previously (Ponzi et al., 1978). Briefly, superoxide radicals were generated by the FMS-NADH-NBT system. The reaction solution (3 mL) contained 0.5M Tris-HCl (pH 8.0), 80 μM deoxy-coenzyme I (NADH), 50 μM Nitroblue Tetrazolium (NBT) and 16 μM Phenazin Methosulfate (PMS). Polysaccharides were added to the reaction buffer to the final concentrations of 2.5, 5, 10, 20, 40, 80 and 160 μg mL⁻¹ and absorbencies at 517 nm after 30 min reaction were recorded. For the control, NADH was substituted with Tris-HCl buffer.

Measurement of the hydroxyl radical scavenging activity: The measurement of hydroxyl radical scavenging activity was following the protocol described by Smirnoff and Cunmeal (1989). The reaction solution contained 150 mM sodium phosphate (pH 7.4), 0.15 mM FeSO₄, EDTA, 2 mM sodium salicylate and 6 mM H₂O₂. Different polysaccharides were added to the final concentrations of 0.5-400 μg mL⁻¹. For the control, H₂O₂ was replaced with sodium phosphate buffer (150 mM, pH 7.4). The mixtures were incubated at 37°C for 1 h and the absorbencies at 510 nm were recorded.

Measurement of the DPPH radical scavenging activity: Measurement of the DPPH radical scavenging activity was following the protocol described by Zhang et al. (2003). Free radicals were generated by 1,1-Diphenyl-Picryl-Hydrazil (DPPH). Briefly, DPPH was dissolved in minimal toluene and mixed with 50% ethanol to a final concentration of 127 μmol L⁻¹. The reaction system (2 mL) contained 1.9 mL DPPH and different concentrations (15-250 μg mL⁻¹) of polysaccharides. The absorbances of the mixtures were measured with a spectrophotometer at 525 nm after 20 min.

Measurement of the red cell hemolysis induced by H₂O₂ scavenging: Scavenging activities of polysaccharides on the rat erythrocytes hemolysis induced by H₂O₂ were measured according to the protocol described by Li et al. (2000). In brief, heparin-natrium was added to the rat blood as the anticoagulant and the erythrocytes were separated from the rat blood by centrifugation and resuspended in 1% physiological saline. The reaction solution (3 mL) contained 1 mL erythrocyte suspension, different concentrations (2.5-200 μg mL⁻¹) of polysaccharides and 1 mL 400 μM L⁻¹ H₂O₂. The mixture was incubated at 37°C for 1 h followed by addition of six volumes of physiological saline and centrifugation at 3000 rpm for 6 min. The absorbance of the supernatant was then measured with a spectrophotometer at 415 nm. For the control, no polysaccharide was added.

Measurement of the lipid peroxide inhibition activity in the rat liver homogenate: The rat liver homogenate was prepared following the protocol described by Li et al. (2000). Immediately after the mice were sacrificed, the liver tissues were separated and suspended in cold 0.5% physiological saline. The suspended solution was then centrifuged at 500 rpm at 4°C for 10 min to obtain the supernatant. To measure the inhibition effects of polysaccharides on the lipid peroxide in mice liver homogenate, the supernatant (1 mL) was incubated with 1 mL polysaccharide (2-280 μg mL⁻¹), 100 μL 6 mM FeSO₄ and 40 μL 20 mmol H₂O₂ at 37°C for 1 h. The reaction was stopped by 15% trichloroacetic acid (1 mL), followed by 0.7% 2-thiobarbituric acid (1 mL). After the protein precipitate was removed by centrifugation at 1000 rpm and 10 min, the absorbance at 532 nm was recorded. The color formation is due to the oxidation product of lipid peroxide, the malondialdehyde-thiobarbituric acid complex. For the control samples, FeSO₄ and H₂O₂ were omitted.

Calculation of the scavenging rate: The percentage of scavenging activity was evaluated according to the following equation, where A stands for the absorbance of the free radical generation system, Ax stands for the absorbance of samples and Ao stands for the absorbance of the control.

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\text{Scavenging rate (\%)} = \frac{(A-Ax)}{(A-Ao)} \times 100\%
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Statistical analysis: Data were processed with the SPSS software (SPSS Inc., IL, USA) and analyzed with the student's t-test.

RESULTS AND DISCUSSION

Effects of the polysaccharide concentration on the scavenging rate for the superoxide radical (O²⁻): Superoxide radicals were generated by the NADH-PMS-NBT system. The superoxide radical scavenging rates
Effects of the polysaccharide concentration on the scavenging rate for hydroxyl free radicals: The effects of the polysaccharide concentration on the hydroxyl radical scavenging rate were summarized in Fig. 2. As shown, Porphyra polysaccharide also possessed hydroxyl radical scavenging activity. The scavenging efficiency reached 85.63% when the polysaccharide concentration was 400 g mL\(^{-1}\). Different polysaccharides were added in the reaction solution to the final concentrations of 0.5-400 g mL\(^{-1}\). For the control, \(\text{H}_2\text{O}_2\) was replaced with sodium phosphate buffer (150 mM, pH 7.4). The mixtures were incubated at 37°C for 1 h and the absorbencies at 510 nm were recorded.

Scaevenging effects of the polysaccharide on •DPPH radical: Figure 3 demonstrated the ability of Porphyra polysaccharide in quenching •DPPH radicals. Compared with the superoxide and the hydroxyl radicals, the scavenging activity of •DPPH radicals was much weaker. Only when the polysaccharide concentration was higher than 125 g mL\(^{-1}\), was the scavenging efficiency able to reach higher than 10% (Fig. 3). Free radicals were generated by 1,1-diphenyl-2-picryl-hydrazil (DPPH). DPPH was dissolved in minimal toluene and mixed with 50% ethanol to a final concentration of 127 mol L\(^{-1}\). The reaction system (2 mL) contained 1.9 mL DPPH and different concentrations (15-250 µg mL\(^{-1}\)) of polysaccharides. The absorbencies of the mixtures were measured with a spectrophotometer at 525 nm after 20 min.

Effects of the polysaccharide on the osmotic hemolysis of erythrocytes: The effects of the Porphyra polysaccharide on the hemolysis of the rat erythrocytes induced by \(\text{H}_2\text{O}_2\) were summarized in Fig. 4. As shown, at higher concentrations, Porphyra polysaccharide exhibited strong protective effects against hemolysis. The IC\(_{50}\) of Porphyra polysaccharide on hemolysis of erythrocytes induced \(\text{H}_2\text{O}_2\) was around 26 g mL\(^{-1}\). The scavenging efficiency of Porphyra polysaccharide on hemolysis of erythrocytes reached up to 82.90% at the polysaccharide concentration of 208 g mL\(^{-1}\). Heparin-natrium was added to the rat blood as the anticoagulant and the erythrocytes were separated from the rat blood by centrifugation and resuspended in 1% physiological saline. The reaction solution (3 mL) contained 1 mL erythrocyte suspension, different concentrations (2.5-200 µg mL\(^{-1}\)) of polysaccharides and 1 mL 400 µM L\(^{-1}\) \(\text{H}_2\text{O}_2\). The mixture was incubated at 37°C for 1 h followed by addition of six
Fig. 4: The inhibitory effects of Porphyra polysaccharide on the oxymatic hemolysis of red blood cells (Mean±SD, n = 6) p<0.01

Volumes of physiological saline and centrifugation at 3000 rpm for 6 min. The absorbance of the supernatant was then measured with a spectrophotometer at 415 nm. For the control, no polysaccharide was added.

Effects of the polysaccharide on MDA levels: Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, is a convenient indicator for determining the extent of lipid peroxidation. According to our results, the Porphyra polysaccharide fractions from P. yezoensis significantly inhibited the microsomal lipid peroxidation (Fig. 5). The IC₅₀ of Porphyra polysaccharide on lipid peroxidation was about 299.07 μg mL⁻¹. The liver tissues were separated from mice and suspended in cold 0.5% physiological saline and then centrifuged at 3000 rpm at 4°C for 10 min to obtain the supernatant. The supernatant (1 mL) was incubated with 1 mL polysaccharide (2-280 μg mL⁻¹), 100 μL 6 mMol FeSO₄, and 40 μL 20 mMol H₂O₂ at 37°C for 1 h. The rate of reaction was stopped by 15% trichloroacetic acid (1 mL), followed by 0.7% 2-thiobarbituric acid (1 mL). After the protein precipitate was removed by centrifugation at 1000 rpm for 10 min, the absorbance at 532 nm was recorded. For the control samples, FeSO₄ and H₂O₂ were omitted.

Superoxide anion, hydroxyl and DPPH free radicals are hazardous to living organisms and they directly associate with many diseases, such as tumor and inflammation. Hence, it is of high importance for the living organisms to get rid of or balance these harmful free radicals (Xin and Liu, 2000). Researches in recent years indicated that polysaccharides from alga possess remarkable anti-oxidation activity. For example, Sargassum thunbergii polysaccharide scavenges Reactive Oxygen Species (ROS) effectively with an IC₅₀ of 0.5 mg, as measured by the Py-rogalb Huminol luminescent system (Zhang and Yu, 1997). Laminaria japonica fucoidan (IC₅₀ = 20.3 μg mL⁻¹) could scavenge superoxide anion and to a less extent, the hydroxyl radical and the DPPH radical (Zhang et al., 2003). For the higher plants, the superoxide anion scavenging ability is generally better. The highest reported values are from the peel extract of the red pomegranate and the extract of Quercus aliena acorn, with the IC₅₀ values being 4.01 μg mL⁻¹ and 4.92 μg mL⁻¹ (Jin et al., 2005; Guo et al., 2007). The Porphyra polysaccharides used in our investigation were found to have even stronger scavenging effect on the superoxide radical with an IC₅₀ value of 2.5-5.0 μg mL⁻¹ and scavenging rate of up to 82.77%. The removal efficiencies for •OH and DPPH were even better and the maximum removal rates were 85.63 and 13.97%, respectively.

The erythrocytes are in an oxygen-fully-loaded environment in vivo, with abundant polyunsaturated fatty acids around. The metal-chelating hemoglobin around the erythrocytes could easily catalyze lipid peroxidation, thus making erythrocytes susceptible to oxidation injury. Researches on erythrocytes structure and function have provided valuable information for preventing the cellular oxidation injury(Zhang and Yu, 1997; Yang et al., 1999). It has been reported that certain polysaccharide from the alga, e.g., Laminaria polysaccharide, possessed protecting roles on the erythrocytes oxidation hemolysis induced by H₂O₂ (Zhang et al., 2003; Luo et al., 2004). Present data suggested that Porphyra polysaccharide effectively inhibited oxymatic hemolysis of erythrocytes induced by H₂O₂. The IC₅₀ value was 25 μg mL⁻¹ and the inhibition rate reached up to 82.90% at the polysaccharide concentration of 200 μg mL⁻¹.

The MDA level increases when the immune system of the organism is down. The redundant lipid peroxides damage the cell membrane and accelerate aging and cell death. Therefore, the MDA level is an important indicator for lipid oxidation (Zhang et al., 2003; Luo et al., 2004; Han et al., 2006) According to present data, at high concentrations, Porphyra polysaccharide inhibited the
increase of MDA levels induced by Fe" and H2O2 in the homogenate of rat liver tissues, indicating that Porphyra polysaccharide could hinder the lipid peroxidation process in vitro.

ACKNOWLEDGMENT

This research was financially supported by National 863 Project and Shanghai Science Committee of China (Bio-Medicine Key Project #054319936, Pujiang Plan #05P114086) as well as Shanghai Leading Academic Discipline Project (#Y1101).

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