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The antioxidant activities and neuroprotective effect of polysaccharides from the starfish *Asterias rollestoni*

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ABSTRACT

After the starfish was defatted with isopropyl alcohol and ethanol, crude polysaccharide was extracted by 0.15 mol/L HCl. Anion exchange chromatography was performed to fractionate the sample into two fractions, SF-1 and SF-2. Chemical analysis showed that the major component of SF-1 was a glucan consisting of a backbone of 1 → 3 linked β-D-glucopyranose residues, and it had a minor glucan component containing a backbone of 1 → 3 linked α-D-glucopyranose residues. SF-2 was a mannoglucan sulfate. SF-2 displayed the highest antioxidant activity among the polysaccharides. Moreover, SF-1 and SF-2 exhibited neuroprotective activities in a neurotoxicity model of Parkinson's disease (PD).

1. Introduction

Marine organisms are a rich source of natural products that have great pharmacological potential. There has been considerable interest in isolating natural antioxidants from algae, and such compounds have been shown to have diverse biological effects, including antioxidative, anti-coagulation, anti-tumor (Khan, Ameem, Naz, & Noureen, 2012), anti-viral (Tian, Zhao, Guo, & Yang, 2011), anti-infection, antithrombotic (Chen et al., 2012; Pomin & Mourão, 2012), anti-inflammatory (Mhadhebi et al., 2012; Pomin & Mourao, 2008) and anti-HIV (Beutler et al., 1993; Luk'yanov et al., 2007) properties. Although an enormous effort has been made to extract and monitor the bioactivities of polysaccharides derived from various seaweeds, little attention has been given to the exploration of marine animals. Recently, marine animals have been reported to contain many biologically active substances. In terms of antioxidant compounds, polysaccharides isolated from invertebrates have been demonstrated to be potential reactive oxygen species (ROS) scavengers. Because polysaccharides such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in processed foods and are thought to be less toxic than synthetic antioxidants, they have been investigated extensively (Song, Zhang, Zhang, & Wang, 2010).

Oxidative stress resulting from the imbalance of pro-oxidant/antioxidant homeostasis leads to the generation of toxic ROS (Tian et al., 2011). It also causes extensive damage to lipids, proteins and DNA. Accumulating lines of evidence have demonstrated that oxidative stress plays a crucial role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and PD (Hu et al., 2011; Reed, 2011; Tian et al., 2011).

Starfish have been a traditional Chinese medicine for thousands of years. In particular, they have been used to treat various stomach symptoms. In recent years, there have been more studies on the structure and pharmacodynamics of sterides than there have been on polysaccharides (Finamore, Minale, Riccio, Rinaldo, & Zollo, 1991; Minale, Pizza, Zollo, & Riccio, 1983). In this study, we report that the polysaccharides extracted from starfish exhibit antioxidant activities and neuroprotective activity. Thus, polysaccharides may be potential drugs for common neurodegenerative diseases such as AD and PD and may be potential candidates as antioxidants.

2. Materials and methods

2.1. Materials

The starfish was identified by Professor Zuhong Xu and purchased from Qingdao, China, and they were dried in an oven at 60 °C overnight.

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2.2. Extraction of heteropolysaccharides

The dried starfish were defatted with isopropyl and ethanol for 2 h. Then, they were dried at 60 °C. Then, the polysaccharides were extracted three times with 0.15 mol/L HCl for 2 h at 60 °C. The solution was neutralized with NaOH and dialyzed in water for 1 day and distilled water for 1 day, and then the dialysate was concentrated and ethanol precipitated. Finally, the precipitation was dissolved in water and deproteinized with 1% trypsin overnight at 37 °C. After deproteinization, the solution was concentrated and precipitated with ethanol. The precipitation was named after SF-c.

2.3. Anion-exchange chromatography

SF-c was separated by anion-exchange chromatography on a DEAE-Bio Gel agarose FF (50 mm × 40 cm) column with water and a linear gradient solution of NaCl (0–2 mol/L) at a flow rate of 10 mL/min. It was detected by the phenol–sulfuric acid method. Finally, SF-c was divided into two fractions, which were named after SF-1 (eluent with water) and SF-2 (eluent with 0–2 mol/L NaCl).

2.4. Compositional analysis

The total sugar content of the polysaccharides was determined according to the phenol–sulfuric acid method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of the protein was evaluated by an established method (Sedmak & Grossberg, 1977). Moreover, the sulfated content was measured by ion chromatography on a Shodex IC SI-52 4E column (4.0 mm × 250 mm) in 3.6 mmol/L Na_2CO_3 at a flow rate of 0.8 mL/min at 45 °C. The molecular weights were determined by HPLC on a TSK G3000 PWxl column (7 μm 7.8 mm × 300 mm), and the contents were eluted in 0.05 mol/L Na_2SO_4 at a flow rate of 0.5 mL/min at 40 °C and detected by measuring the refractive index of the solution. Ten different molecular weight dextrans were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China) and were used as the molecular weight standards. The molar ratio of the monosaccharides was calculated as described by Zhang et al. (Zhang, Zhang, Wang, Shi, & Zhang, 2009). Briefly, samples (10 mg/mL) were hydrolyzed in 4 mol/L trifluoroacetic acid (TFA, 1 mL) at 110 °C in a sealed tube for 4 h. Then, they were neutralized by NaOH. The solution was then reacted with 1-phenyl-3-methyl-5-pyrazolone (PMP) to convert the monosaccharides into their PMP derivatives and separated by HPLC on a YMC Pack ODS AQ column (4.6 mm × 250 mm).

2.5. NMR spectroscopy

Polysaccharides (50 mg) were co-evaporated with deuterium oxide (99.9%) twice before they were dissolved in deuterium oxide (99.9%) containing 0.1 μL deuterated acetone. NMR spectra were recorded in a Bruker AVANCE III 600 MHz at 25 °C. The chemical shifts were adjusted to the internal standard (deuterated acetone, 2.05 and 29.92 ppm).

2.6. Antioxidant activities

2.6.1. Determination of superoxide radical scavenging activity (Wang, Zhang, Zhang, & Li, 2008)

The modified system used to determine the capacity to inhibit the photochemical reduction was carried out in a mixture containing 0.5 mL nitro blue tetrazolium (NBT) (0.3 mmol/L) in Tris-HCl, 0.5 mL nicotinamide adenine dinucleotide (NADH) (5 mmol/L) in Tris-HCl, and 3.0 mL of various concentrations of the samples. Then, the reaction mixture was recorded at 560 nm. The scavenging activity was calculated as follows: Scavenging activity (%) = $(1 - A_1/A_0) \times 100$, where A_0 was the absorbance of the negative control, and A_1 was the absorbance in the presence of samples.

2.6.2. Measurement of hydroxyl radical scavenging activity (Smirnoff & Cumbes, 1989)

The ability of the extracts to scavenge hydroxyl radicals was determined by an improved method established by Smirnoff and Cumbes. The reaction mixture contained 0.5 mL of 2 mmol/L EDTA-Fe, 1.0 mL of various concentrations of the sample in water, 1 mL of crocus in sodium phosphate buffer (PBS) (pH = 7.4) and 1.0 mL 3% H_2O_2. In the negative control, distilled water was used instead of sample, and PBS replaced the H_2O_2. Then, the reaction mixture was incubated at ambient temperature for 30 min. The absorbance was measured at 510 nm, and the scavenging activities of the extracts were calculated according to the equation: Scavenging activity (%) = $(A_1/A_0) \times 100$, where A_0 was the absorbance of negative control, and A_1 was the absorbance in the presence of samples.

2.6.3. Measurement of DPPH radical scavenging activity (Shimada, Fujikawa, Yahara, & Nakamura, 1992)

A 0.1 mol/L DPPH ethanol solution (1 mL) was added to the sample solution (3 mL) in 50% ethanol. The negative control was a 0.1 mol/L DPPH ethanol solution (1 mL) in 3 mL 50% ethanol. The absorbance was measured at 517 nm after 20 min. The ability to scavenge the DPPH radical was calculated by using the following equation: Scavenging ability (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of negative control, and A_1 was the absorbance in the presence of samples.

2.6.4. Assessment of reducing power (Yen & Chen, 1995)

The reducing power of the extracts was determined according to the modified method by Yen and Chen. Briefly, different concentrations of samples (1.25 mL) were mixed with a 1.25 mL 1% (w/v) $K_3Fe(CN)_6$ solution, and they were incubated at 50 °C for 30 min. Afterwards, the reaction was inhibited with 10% (w/v) CCl_3COOH (2.5 mL). After 5 min, the mixture was combined with 0.1% (w/v) $FeCl_3$ (1.5 mL) for 30 min. Finally, the absorbance at 700 nm was analyzed.

2.7. Cell culture and MTT assay

To measure cell viability, a 3- (4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay was used. Cells were cultured in Dulbecco's modified Eagle's medium, containing 5% fetal bovine serum and penicillin-streptomycin (100 units/mL) in an atmosphere of 5% CO_2 at 37 °C. Then, cells were seeded in a 96-well plate at a density of 2×10^5 cells/well for 24 h. Subsequently, the cells were divided into the following three groups: (1) the control group in which cells were treated with serum-free medium for 24 h, (2) the 6-OHDA group in which cells were treated with 6-OHDA (100 μmol/L) in a serum-free medium for 24 h and (3) the experimental groups in which cells were treated with 6-OHDA (100 μM) and polysaccharides at different concentrations (1 and 0.1 mg/mL) in a serum-free medium for 24 h. After removal of the media, 10 μL of MTT (5 mg/mL suspended in 0.01 mol/L PBS) was added to each well. After 4 h incubation, the supernatants were removed, and dimethyl sulfoxide (DMSO) (200 μL) was added. The absorbance was measured at 490 nm. The following equation was used to calculate cell viability: Cell viability (%) = $(A_1 - A_0)/(A_c - A_0) \times 100$, where A_0 was the absorbance of the blank, A_1 was the absorbance in the presence of samples, and A_c was the absorbance of the control.

2.8. Statistical analysis

All data are shown as the mean ± standard deviation (SD). Significant differences between the experimental groups were determined by one way ANOVA, and the differences were considered to be statistically significant if $P < 0.05$. All computations were performed by statistical software (SPSS 16.0).

3. Results and discussion

3.1. Extraction and separation of the polysaccharides

After the starfish was defatted, the crude polysaccharide was extracted in 0.15 mol/L HCl and deproteinized in 1% trypsin. After purification by anion exchange chromatography, SF-1 and SF-2 were obtained. It was shown that SF-1 consisted of glucose while SF-2 was 85.31% sugar. By analyzing the monosaccharides, it was suggested that SF-1 was a glucan while SF-2 was most likely a mannoglucan sulfate (Table 1).

To elucidate the structure of SF-1, NMR spectra (Figs. 1 and 2) were determined. Based on the analysis of chemical composition of SF-1, it was shown that SF-1 was a glucan. Thus, it was speculated that the chemical shifts at 5.25 and 4.83 ppm were the anomeric hydrogens of the α and β glucopyranose residues, respectively. According to the peak areas in the ^{1}H NMR spectrum, it was suggested that the proportion of β and α glucan was 0.29: 1. In addition, the chemical shifts of H2-α, H3-α, H2-β and H3-β were confirmed by the positions of the correlation peaks of H1-α/H2-α, H2-α/H3-α, H1-β/H2-β and H2-β/H3-β in ^{1}H, which had ^{1}H-COSY spectrum at 5.25/3.51, 3.51/3.83, 4.83/3.43 and 3.43/3.83 ppm, respectively. Moreover, the chemical shifts at 62.91 and 61.00 ppm, which correspond to the negative peaks in the DEPTQ spectrum, are consistent with the C6 of α and β glucan. According to the correlation peaks in HSQC spectrum, the H6 of the α and β glucan were 3.54/3.43 and 3.73/3.63 ppm. The H5 of the αandβglucan were determined by the presence of correlation peaks H5/C6 at 3.63/62.91 and 3.29/61.00 ppm in the HMBC spectrum. Finally, the H4 was characterized by the correlation peaks H1-α/H2-α/H6-α/H3-α/H5-α/H4-α and H1-β/H6-β/H2-β/H5-β/H3-β/H4-β at 5.25/3.51/3.43/3.83/3.63/3.73 and 4.83/3.63/3.43/3.29/3.83/3.51 ppm, respectively, in the TOCSY spectrum. Then, the chemical shifts of the carbons were calculated by the HSQC spectrum. Finally, the linkage of SF-1 was proven by the correlation peaks H-1/C-3 at 5.25/73.36, 4.83/74.36 ppm in the HMBC spectrum.

Table 1
Chemical composition (%, dry weight) of SF-c, SF-1 and SF-2.

Sample	Total sugar (%)	Protein (%)	SO_4^{2-} (%)	Monosaccharide (molar ratio)		M_w (kDa)
				Mannose (Man)	Glucose (Glc)	
SF-c	83.29	0.09	12.11	0.17	1	–
SF-1	98.15	0.19	–	0	1	165.4
SF-2	85.31	0	13.85	0.27	1	151.1

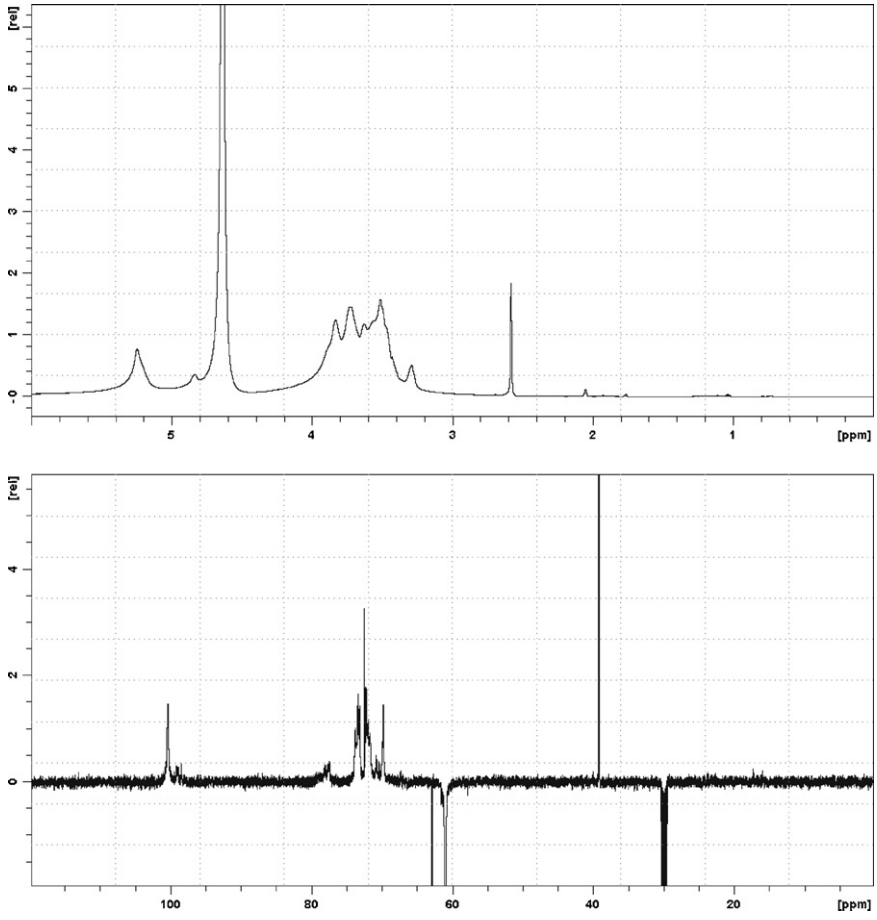

Fig. 1. The ^{1}H NMR and DEPTQ spectra of SF-1.

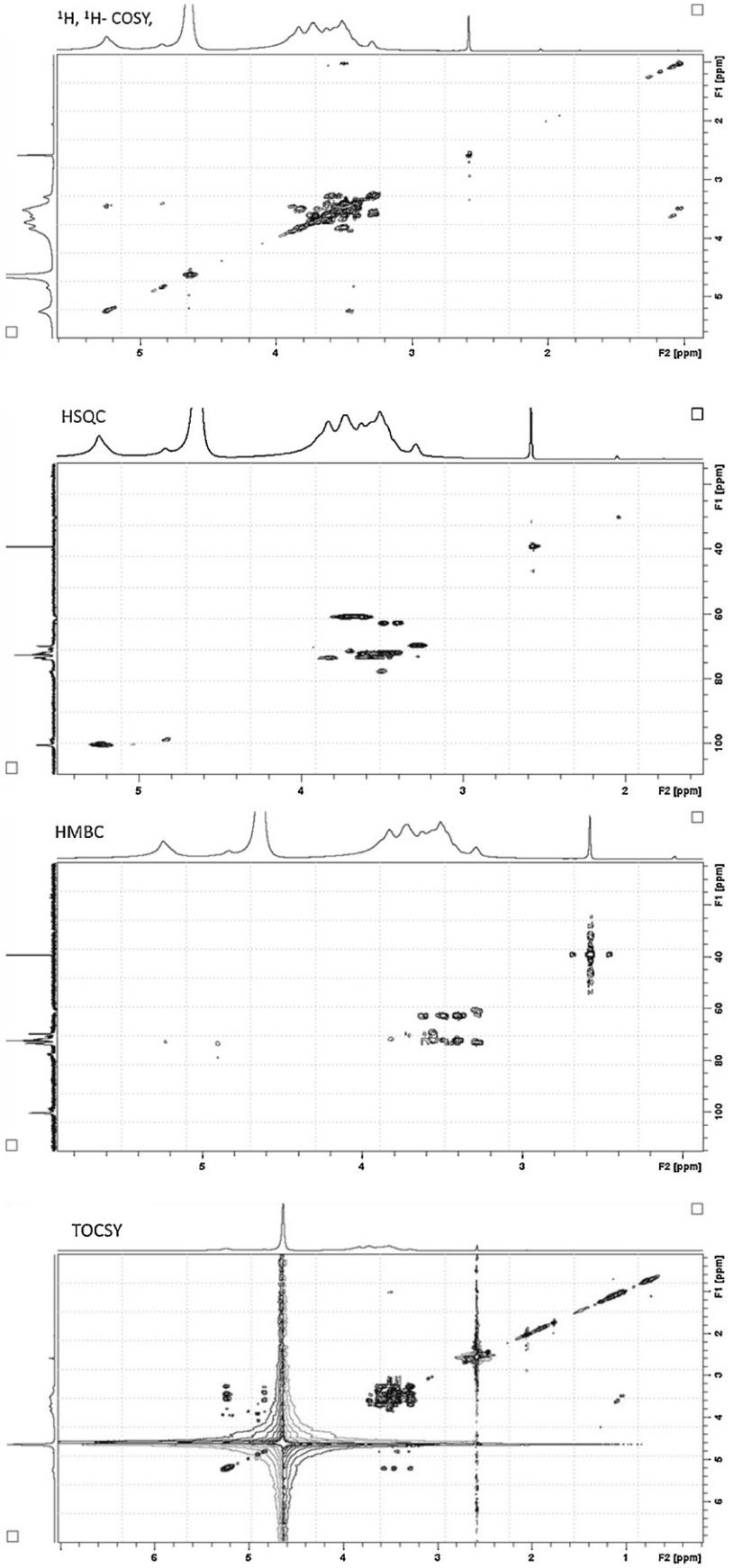

Fig. 2. The ^{1}H, ^{1}H-COSY, HSQC, HMBC, and TOCSY spectra (from top to bottom) of SF-1.

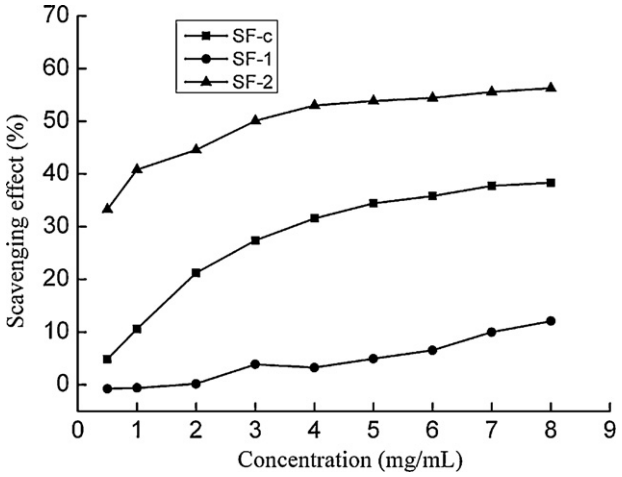

Fig. 3. The superoxide radical-scavenging effect of the polysaccharides extracted from starfish. Values are expressed as the mean ± SD from three measurements.

3.2. The ability to scavenging superoxide radicals

Among various ROS, superoxide radicals are one of the precursors to hydroxyl radicals and singlet oxygen, and they also produce other types of oxidizing agents. Therefore, it was of great importance to characterize the superoxide radical-scavenging potential of different antioxidants. As expected, the tested samples were able to scavenge the superoxide radicals in a concentration-dependent manner. Fig. 3 showed that the IC_{50} of SF-2 was 4 mg/mL, while the IC_{50} values of SF-c and SF-1 were higher than that of SF-2. SF-2 likely showed the greatest effect because of the highest contents of sulfate.

3.3. Scavenging activity of hydroxyl radical

Among the oxygen radicals, the hydroxyl radical is the most reactive radical and can induce oxidative damage to almost any biomolecule, which results in aging, as well as cancer and other diseases. Recently, many studies proposed that polysaccharides exhibit their antioxidant activities by providing electrons to stabilize radicals. In Fig. 4, a positive correlation between the concentration of the polysaccharide and the hydroxyl radical-scavenging activity is shown. Moreover, the scavenging activity of

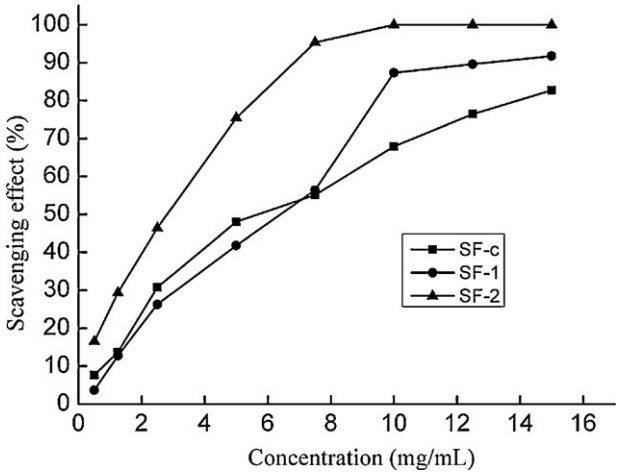

Fig. 4. The ability of the polysaccharides derived from starfish to scavenge hydroxyl radicals. Values are expressed as the mean ± SD from three measurements.

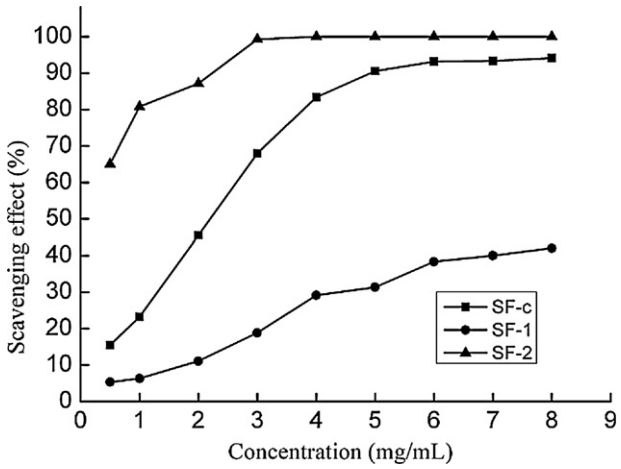

Fig. 5. The DPPH radical-scavenging effects of the polysaccharides extracted from starfish. Values are expressed as the mean ± SD from three measurements.

SF-2 was 100% at the concentration of 10 mg/mL. In summary, SF-2 might be a good candidate for an antioxidant.

3.4. Scavenging activity of DPPH radicals

DPPH radicals need an electron or hydroxyl radical to become a stable molecule. A DPPH radical assay had been widely used to evaluate the radical-scavenging ability of antioxidants. As shown in Fig. 5, SF-2 was the best at scavenging DPPH radicals, followed by SF-c, and SF-1 showed the lowest effect on scavenging DPPH radicals; this suggests that SF-2 might contribute to the scavenging of DPPH radicals. It was hypothesized that the charge of the sulfate group of SF-2 conferred its DPPH radical-scavenging ability. The IC_{50} of SF-c was 2 mg/mL. The IC_{50} values of SF-c and SF-2 were lower than the IC_{50} values of the polysaccharides DF1 (EC_{50} = 3.6 mg/mL) and DFPS (EC_{50} = 3.7 mg/mL) extracted from Laminaria japonica (Wang, Zhang, Zhang, Song, & Li, 2010). Therefore, it is possible that SF-2 can to help to prevent or postpone oxidative damage.

3.5. Reducing power

Fig. 6 described the reducing power of SF-c, SF-1 and SF-2. The reducing effect was expressed by the absorbance at 700 nm. The higher the absorbance is, the stronger the reducing power is.

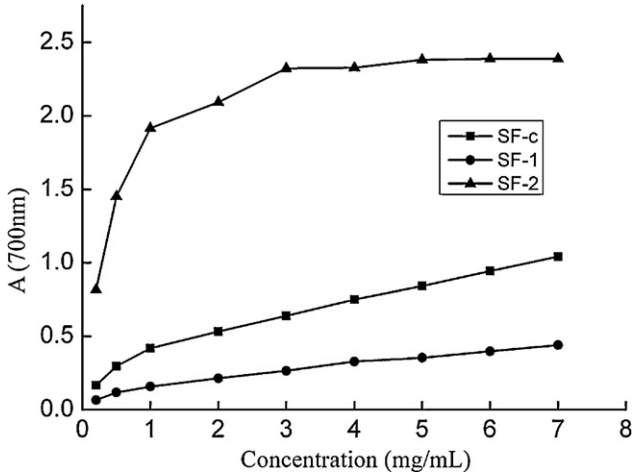

Fig. 6. The reducing power of the polysaccharides derived from starfish. Values are expressed as the mean ± SD from three measurements.

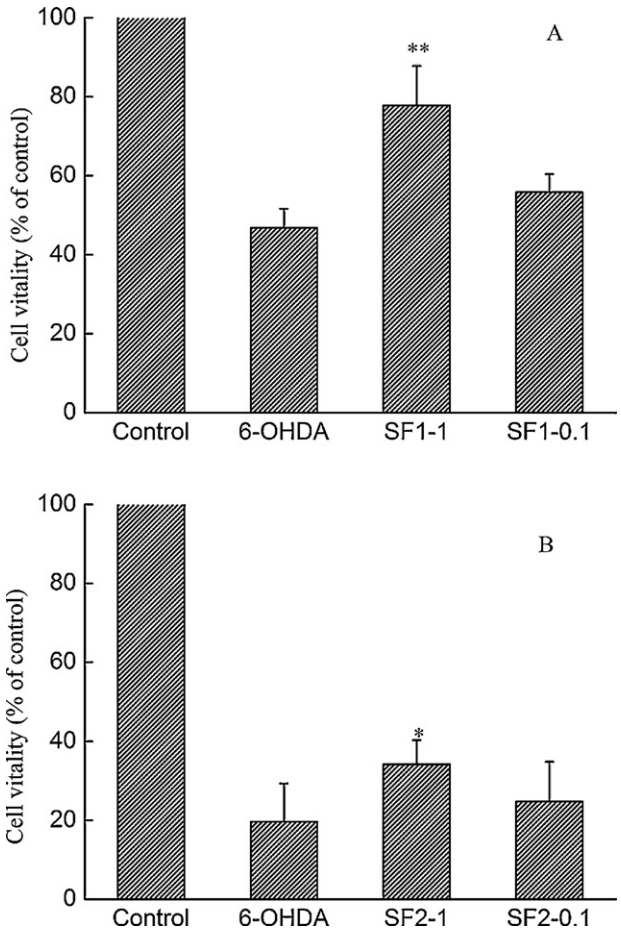

Fig. 7. Neuroprotective effects of the polysaccharides on 6-OHDA-induced neurotoxicity in MES 23.5 cells. The results are represented as the mean ± SD in terms of the absorbance at 490 nm ($n = 4$). A (SF-1); B (SF-2).

Moreover, the relative ability of the extracts to act as DPPH radical scavengers was similar to their reducing power, suggesting that the charge of sulfate group on SF-2 also plays a role in determining its reducing power.

3.6. Neuroprotective effects of the polysaccharides

Fig. 7 showed that SF-1 and SF-2 exhibit neuroprotective activity at the concentration of 1 mg/mL, while they do not have neuroprotective activity at the concentration of 0.1 mg/mL. In our previous report (Luo et al., 2009), it was hypothesized that fucoidan had neuroprotective activity in an MPTP-induced neurotoxicity model of PD because of its antioxidative activity. However, from the above analysis of the antioxidant activities of SF-c, SF-1 and SF-2, it was concluded that SF-2 showed the highest antioxidant activity while SF-1 displayed the lowest antioxidant activity. Thus, the antioxidant activities may not correlate with neuroprotective activity; the mechanism underlying the neuroprotective activity will be further studied.

4. Conclusions

In this study, three polysaccharides were extracted from starfish. Based on the chemical analysis of the monosaccharides, the polysaccharides could be divided into the following two types: glucan and sulfated mannoglucan. The polysaccharides also displayed numerous antioxidant activities, including hydroxyl radical scavenging, DPPH radical scavenging, superoxide radical scavenging and reducing power. They also exhibited neuroprotective activity in a 6-OHDA-induced cell model of PD, which produced oxidative stress and neurotoxicity associated with neurodegenerative disorders (Varcin et al., 2011). However, to the best of our knowledge, the effects on the disease and its underlying mechanisms have yet to be determined.

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