Carbohydrate Polymers 97 (2013) 116–120

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

The neuroprotective activities of heteropolysaccharides extracted from *Saccharina japonica*

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ARTICLE INFO

Article history:
Received 6 October 2012
Received in revised form 14 April 2013
Accepted 15 April 2013
Available online 26 April 2013

Keywords:
Heteropolysaccharide
Neuroprotective activity
Saccharina japonica

ABSTRACT

Crude fucoidan extracted from *Saccharina japonica* was separated by anion-exchange chromatography. Then, the neuroprotective activities of the crude fucoidan (J) and its fractions (J0.4, J0.5, J1 and J2) were tested. J, J0.4 and J0.5 were shown to have neuroprotective effects. To simplify the structural features of the compounds, crude fucoidan was degraded to obtain low molecular weight fucoidan (DJ). DJ was further fractionated into DJ0.5, DJ1 and DJ2, and the neuroprotective activities of these fractions were determined. This analysis revealed that DJ and DJ0.5 retained the neuroprotective activity. However, the DJ0.5 fraction remained very complex. Thus, DJ0.5 was further separated into six fractions (F0.1, F0.2, F0.3, F0.4, F0.5 and F1). Finally, it was concluded that the anion-exchange fractions F0.1, F0.2 and F0.3 exhibited neuroprotective activities. These results suggest that heteropolysaccharides might contribute to the neuroprotective activity. Moreover, the neuroprotective heteropolysaccharide fractions contained relatively low fucose (less than 20%) and sulfate (25%), high UA content (more than 10%) and a high molar ratio of all other monosaccharides

1. Introduction

Fucoidans are members of a family of sulfated heteropolysaccharides that are extracted mainly from brown seaweed. The chemical compositions of fucoidans vary based on species, harvest time and the geographic location of the algae. To date, there are many studies on crude fucoidans. Hwang et al. (Hwang, Wu, Gau, Chien, & Hwang, 2010; Hwang et al., 2011) reported a water extracted fucoidan from *Sargassum hemiphyllum*, which showed antioxidant, immune-stimulating and anti-inflammatory activities. The fucoidans extracted from *Sargassum fusiforme* (*Hizikia fusiforme*) have also been widely studied. It was reported (Yoon et al., 2011) that an aqueous fucoidan extract from *S. fusiforme* had immunostimulatory effects in RAW 264.7 macrophages and whole spleen cells. In addition, Li, Zhao, and Wei (2008) reported that an aqueous fucoidan extract exhibited anticoagulant activity. Moreover, the fucoidan derived from *Undaria pinnatifida* (Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008) showed defensive effects against herpes simplex virus infection. In a previous study (Wang, Zhang, Zhang, & Li, 2008), we found that fucoidans extracted from *Saccharina japonica* (formerly known as *Laminaria japonica*) also had antioxidant activities. Because there are many species of brown seaweed, the fucoidans extracted from brown seaweed may include compounds other than those described in our previous study. There are many reviews describing fucoidan activities (Berteau & Mulloy, 2003; Holtkamp, Kelly, Ulber, & Lang, 2009; Jiao, Yu, Zhang, & Ewart, 2011; Li, Lu, Wei, & Zhao, 2008; Mestechkina & Shcherbukhin, 2010; Wijesekara, Pangestuti, & Kim, 2011; Wijesinghe & Jeon, 2012).

With the application of ion chromatography, an increasing number of fucoidans extracted from different species have been separated. To our limited knowledge, fucoidans derived from brown seaweed can be divided into two types: type 1 heteropolysaccharides include monosaccharides with low sulfate contents, such as mannose, glucuronic acid, fucose and galactose; type 2 heteropolysaccharides include sulfated galactofucan and fucan and other minor monosaccharides. There are more studies of type 2 than type 1 due to the complexity of the structural features of type 1. In terms of structure, it had been reported that type 2 (sulfated fucan) exists mainly as two structural types. One is built of 1,3-linked α-L-fucopyranose units as building blocks (Bilan et al., 2010), whereas the other comprises alternating

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http://dx.doi.org/10.1016/j.carbpol.2013.04.055

1,3-linked α-L-fucopyranose and 1,4-linked α-L-fucopyranose units as building blocks (Bilan et al., 2010; Wang, Zhang, Zhang, Zhang, & Niu, 2010). In contrast, little structural information has been reported for type 1 molecules (Bilan et al., 2010; Li, Wei, Sun, & Xu, 2006; Sakai et al., 2003; Wang et al., 2012). In addition, in terms of biological activities, type 2 showed anticoagulant, antioxidant and anti-inflammatory activities (Croci, Cumashi, Ushakova, Preobrazhenskaya, & Piccoli, 2011; Wang, Zhang, Zhang, Song, & Li, 2010). However, there are no reports about the activities of type 1 heteropolysaccharides.

As the population ages, the prevalence of age-related diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) is increasing. PD, which affects 2% of the population over age of 60 years of age, is the second most common neurodegenerative disorder. A previous study (Luo et al., 2009) showed that fucoidan had protective effects against MPTP-induced neurotoxicity in a model of Parkinson's disease via its antioxidative activity. Recently, it was also reported by Gao et al. (2012) that crude fucoidan exhibited neuroprotective effects on H_2O_2-induced apoptosis in PC12 cells via the activation of the PI3K/Akt pathway.

Thus, in this study, we aimed to identify the fucoidan fraction that was essential for the neuroprotective activity. Fractions J, DJ and DJ0.5 were separated by anion-exchange chromatography and the neuroprotective activities of all fractions were determined. The results indicated that the heteropolysaccharides contributed to the neuroprotective activities.

2. Materials and methods

2.1. Materials

The brown algae *S. japonica* was collected in Rongcheng, Qingdao, China, in April 2012. The L-fucose, D-galactose, D-mannose, D-glucuronic acid, L-rhamnose monohydrate, D-xylose and D-glucose standards were purchased from Sigma–Aldrich. The 3-methyl-1-phenyl-2-pyrazolin-5-one (PMP) (99%) was obtained from Sigma–Aldrich.

2.2. Extraction and preparation of polysaccharides and degraded polysaccharides

Algae (100 g) were cut into pieces. Crude fucoidan was extracted from the algae with 0.1 M HCl (2 L) at room temperature for 3 h. The extract solution was filtered with Celite and concentrated. $MgCl_2$ (0.475 g) was added into the concentrated solution (100 mL). Then, ethanol (25 mL) was added into the above solution. After removing the algin, the supernatant fluid was dialyzed with running water for 1 day and distilled water for 1 day. Finally, the dialysate was concentrated, and the crude fucoidan (J) was obtained by ethanol precipitation.

J was separated by anion-exchange chromatography on a DEAE-Bio Gel agarose FF (12 cm × 70 cm) column with elution by 0.4 M NaCl (35 L) (J0.4), 0.5 M NaCl (35 L) (J0.5), 1 M NaCl (35 L) (J1) and 2 M NaCl (35 L) (J2), respectively. Then, all fractions were ultra-filtered, concentrated and precipitated with ethanol.

Another sample of crude fucoidan was degraded by hydrogen peroxide and ascorbic acid (Jin, Zhang, Wang, & Zhang, 2013). Briefly, 50 g crude fucoidan was dissolved in 10 L water, and 50 mM hydrogen peroxide and 50 mM ascorbic acid were added at room temperature for 2 h. The degraded fucoidans were ultra-filtered and precipitated with ethanol. Then, the degraded fucoidan (DJ) was also separated by anion-exchange chromatography on a DEAE-Bio Gel agarose FF (12 cm × 70 cm) column with elution by 0.5 M NaCl (35 L) (DJ0.5), 1 M NaCl (35 L) (DJ1) and 2 M NaCl (35 L) (DJ2), respectively.

Fraction DJ0.5 was further separated by anion-exchange chromatography on a DEAE-Bio Gel agarose FF (12 cm × 70 cm) column with elution in a series of 0.1 M (35 L) (F0.1), 0.2 M NaCl (35 L) (F0.2), 0.3 M NaCl (35 L) (F0.3), 0.4 M NaCl (35 L) (F0.4), 0.5 M NaCl (35 L) (F0.5) and 1 M NaCl (35 L) (F1).

2.3. Compositional analysis

The sulfated components were separated by ion chromatography on a Shodex IC SI-52 4E column (4.0 mm × 250 mm) and eluted with 3.6 mM Na_2CO_3 at a flow rate of 0.8 mL min^{-1} at 45 °C. The molar ratio of monosaccharides and fucose content were determined as described by Zhang, Zhang, Wang, Shi, and Zhang (2009). Briefly, polysaccharides (10 mg mL^{-1}) were hydrolyzed by trifluoroacetic acid (2 M) under a nitrogen atmosphere for 4 h at 110 °C. Then, the hydrolyzed mixture was neutralized to pH 7 with sodium hydroxide. Later, the mixture was converted into its PMP derivatives and separated by HPLC chromatography on a YMC Pack ODS AQ column (4.6 mm × 250 mm). Uronic acid (UA) concentration was determined by a modified carbazole method (Bitter & Muir, 1962). The molecular weights of the polysaccharides were evaluated by GPC-HPLC on TSK G3000 PWxl column (7 μm 7.8 × 300 mm) with elution in 0.05 M Na_2SO_4 at a flow rate of 0.5 mL min^{-1} at 40 °C with refractive index detection. Ten different molecular weight dextrans purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China) were used as weight standards.

2.4. Cell culture and MTT assay on MES 23.5 cells

MES 23.5 cells were maintained in Dulbecco's modified Eagle's medium (Gibco Industries, Inc. (Auckland, New Zealand)), supplemented with 5% fetal bovine serum (Gibco Industries, Inc.), 100 units/mL penicillin-streptomycin (Gibco Industries, Inc. (Auckland, New Zealand)) in an atmosphere of 5% CO_2 at 37 °C. Then, MES 23.5 cells (200 μL) were seeded in a 96-well plate at a density of 2×10^5 cells/well for 24 h prior to experimentation. Subsequently, the cells were divided into three groups: (1) Control group: cells were treated with serum-free medium for 24 h. (2) 6-OHDA group: cells were treated with 6-hydroxydopamine (6-OHDA) (100 μM) (Sigma–Aldrich (St. Louis, MO, USA)) in a serum-free medium for 24 h; (3) experimental groups: cells were treated with 6-OHDA (100 μM) and various polysaccharides at two concentrations (1 mg mL^{-1} and 0.1 mg mL^{-1}) in a serum-free medium for 24 h. After the removal of medium from the wells, 10 μL of MTT (5 mg mL^{-1} suspended in 0.01 M PBS) was added to each well. After 4 h of incubation in an atmosphere of 5% CO_2 at 37 °C, dimethyl sulfoxide (DMSO) (200 μL) was added and the absorbance was measured at 490 nm. The following equation was used to calculate cell vitality: Cell vitality (%) = $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100$. A_{blank} means the absorbance of 0.01 M PBS.

2.5. Cell culture and MTT assay on SH-Y5Y cells

Experiments to test 6-OHDA-induced neurotoxicities on SH-Y5Y cells were carried out by The National Center for Drug Screening (Shanghai, China). Briefly, digested SH-Y5Y cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum (Gibco Industries, Inc.). Then, SH-Y5Y cells (100 μL) were seeded in a 96-well plate at a density of 2.5×10^5 cells/well in an atmosphere of 5% CO_2 at 37 °C for 24 h. Then, the old medium was removed, and new medium (100 μL) containing 10 μL of different concentrations (0.5 mg mL^{-1} and 0.05 mg mL^{-1}) of fractions (experimental groups) and solvents (control group and 30 μM 6-OHDA group) was applied. After 2 h, 0.3 mM 6-OHDA (10 μL) was added to the cells in

Table 1
Chemical compositions of the crude fucoidan and its fractions studied.

Samples	Fuc (%)	U A (%)	Total sugar (%)	Sulfate (%)	Monosaccharides (molar ratio)						
					Man	Rha	Glc A	Glc	Gal	Xyl	Fuc
J	30.71	6.08	58.60	40.87	0.13	0.01	0.14	0.03	0.12	0.04	1
J0.4	12.04	9.97	74.78	21.75	0.29	0.17	0.33	0.17	0.37	0.23	1
J0.5	18.03	12.05	70.91	27.45	0.16	0.12	0.17	0.10	0.58	0.20	1
J1	37.81	1.48	54.16	45.12	0.03	0.01	0.03	0.02	0.10	0.04	1
J2	43.46	0.03	55.96	51.09	0	0	0	0	0.11	0.03	1

Table 2
Chemical compositions of the degraded fucoidan and its fractions studied.

Samples	Fuc (%)	U A (%)	Total sugar (%)	Sulfate (%)	Monosaccharides (molar ratio)						
					Man	Rha	Glc A	Glc	Gal	Xyl	Fuc
DJ	32.64	6.14	60.34	37.37	0.10	0.02	0.09	0.02	0.19	0.02	1
DJ0.5	27.04	7.44	65.80	31.49	0.13	0.03	0.11	0.04	0.25	0.08	1
DJ1	38.10	0.59	50.09	51.11	0.02	0	0.01	0	0.03	0	1
DJ2	41.67	0.35	49.77	56.35	0	0	0	0	0.11	0	1

the experimental groups and 6-OHDA group for another 24 h. Then, 10 μL MTT (5 mg mL^{-1}) was added, and the cells were incubated for an additional 3 h. The medium was discarded, 100 μl of DMSO was added to each well, and the plates were shaken to dissolve the formazan crystals. Finally, the absorbance was detected at 490 nm. The following equation was used to calculate cell vitality: Cell vitality (%) = $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100$. A_{blank} means the absorbance of 0.01 M PBS.

2.6. Statistical analysis

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

3. Results and discussion

Table 1 revealed the chemical compositions of crude fucoidan (J) and its fractions. The analysis showed that J consisted mainly of fucose and sulfates, with little UA and other monosaccharides. In addition, the analysis of its fractions indicated that the fucose and sulfate contents increased from J0.4 to J2 while the UA content decreased, suggesting that the separated fucoidans could be divided into two types: type 1 fucoidans (J0.4 and J0.5) contain little fucose and sulfate but rather contain other major monosaccharides (not fucoidan-related polysaccharides), and type 2 fucoidans (J1 and J2) contain substantial fucose and sulfate together with other minor monosaccharides (sulfated galactofucan). This phenomenon was also reported previously (Bilan et al., 2010; Croci et al., 2011; Wang, Zhang, Zhang, Zhang, et al. 2010). However, earlier studies of the structural features of fucoidans focused predominantly on the type 2 fucoidans because of the complicated compositions of monosaccharides. In addition, studies on the biological activities of fucoidans involved mainly crude fucoidans or type 2 fucoidans. In this study, we focused on the biological activities of type 1 fucoidans. Fig. 1a shows that J (1 mg mL^{-1}), J0.4 (1 mg mL^{-1} and 0.1 mg mL^{-1}) and J0.5 (1 mg mL^{-1}) had neuroprotective activities, while neither J1 nor J2 (Fig. 1b) had these activities at either of the concentrations used. It was concluded that the type 1 fucoidans (J0.4 and J0.5) might have stimulated these neuroprotective effects.

The protective blood-brain barrier prevents some chemicals, including highly charged particles and large molecules, from transferring from the blood to the brain. Therefore, we tried to degrade

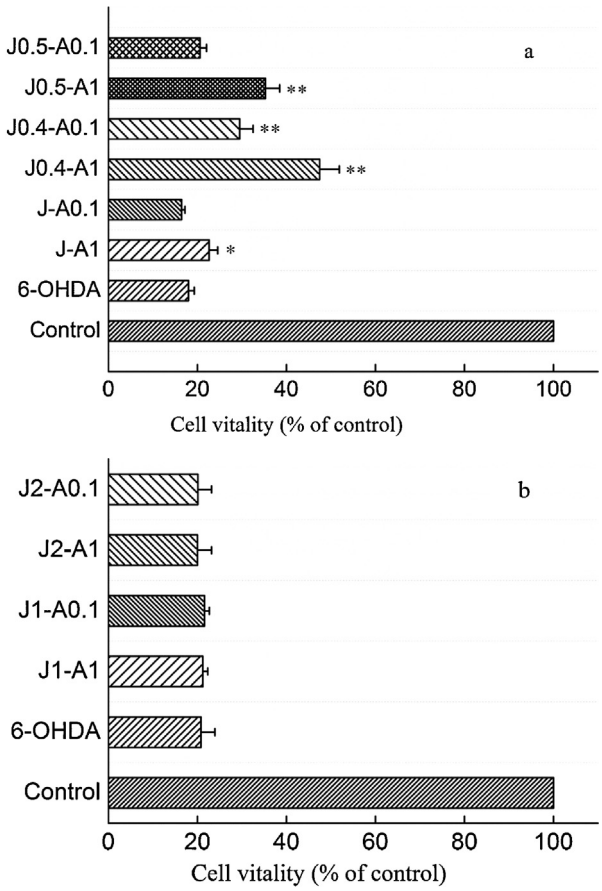

Fig. 1. The neuroprotective effects of crude fucoidan and its fractions on 6-OHDA-induced neurotoxicity in MES 23.5 cells. The results are the mean ± SD of absorbance measured at 490 nm ($n = 4$).

the crude fucoidans (J) into low molecular weight fucoidans (DJ) using hydrogen peroxide and ascorbic acid to further confirm the neuroprotective effects of type 1 fucoidans. Table 2 displays the chemical compositions of the degraded fucoidan and its separated polysaccharides. The chemical composition of DJ was similar to that of J. In addition, DJ0.5 exhibited higher contents of fucose and sulfate than did J0.4 and J0.5, while lower molar ratio of other monosaccharides than did J0.4 and J0.5. This was observed because monosaccharides are particularly susceptible to the degradation

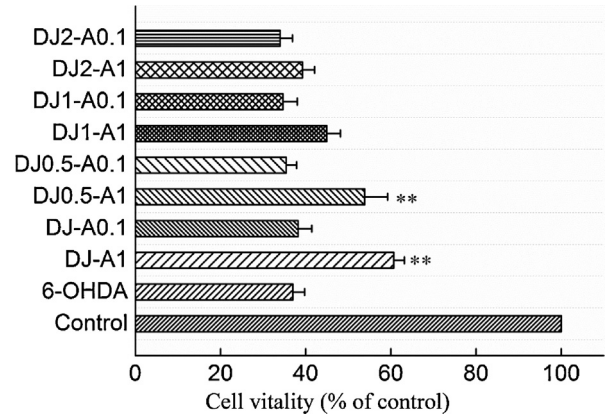

Fig. 2. The neuroprotective effects of degraded fucoidan and its fractions on 6-OHDA-induced neurotoxicity in MES 23.5 cells. The results are the mean ± SD of absorbance measured at 490 nm ($n = 4$).

process (sulfate groups were mainly substituted at fucose). Fig. 2 shows that DJ (1 mg mL^{-1}) exhibited the greatest neuroprotective effect. Moreover, DJ0.5 (1 mg mL^{-1}) also had neuroprotective activity. However, neither DJ1 nor DJ2 exhibited neuroprotective activities at either concentration used, similar to J1 and J2.

Though DJ and DJ0.5 retained their neuroprotective effects, DJ and DJ0.5 were still too complex for a thorough evaluation of their structural features. The chemical compositions of the fractions of DJ0.5 are shown in Table 3. The molecular weights of the DJ0.5 fractions were 5.83, 7.21, 5.69, 5.63, 6.25 and 7.52 kDa. As shown in Fig. 3a and b, F0.1 exhibited the highest neuroprotective activity, followed by F0.3 and F0.2, while F0.4, F0.5 and F1 did not exhibited the neuroprotective activity. Interestingly, F0.2 contained the highest UA content of the fractions and also had the largest molecular weight. Furthermore, F0.1, F0.2, and F0.3 contained less fucose (less than 20%) and sulfate (less than 25%) than did F0.4, F0.5 and F1. At the same time, F0.1, F0.2 and F0.3 had greater molar ratios of all other monosaccharides, i.e., more complicated structural features. These activities were confirmed by the results (in Fig. 4) obtained

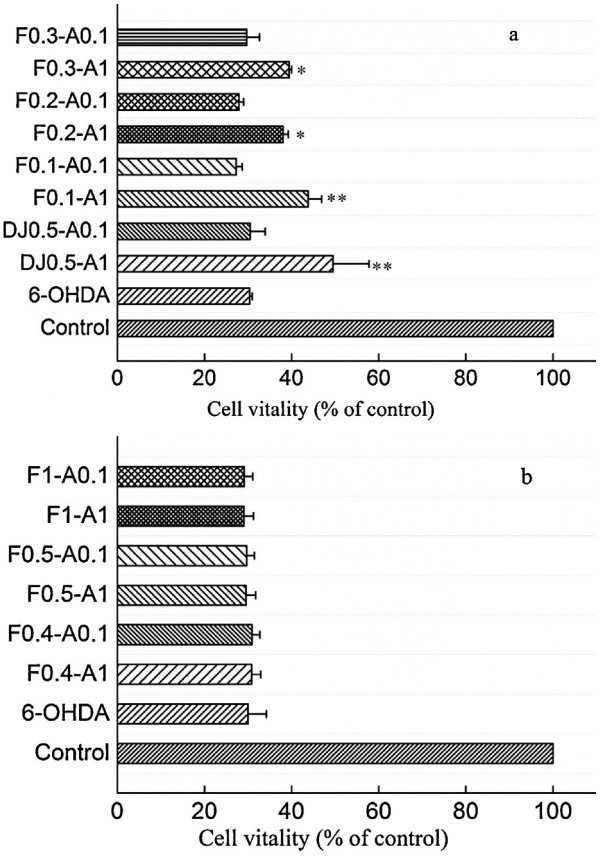

Fig. 3. The neuroprotective effects of DJ0.5 and its fractions on 6-OHDA-induced neurotoxicity in MES 23.5 cells. The results are the mean ± SD of absorbance measured at 490 nm ($n = 4$).

by The National Center for Drug Screening (Shanghai, China). However, the order of neuroprotective activities (in Fig. 4) was F0.2, F0.3 and F0.1. Thus, it was concluded that these heteropolysaccharides contributed to the neuroprotective activity. The structure of

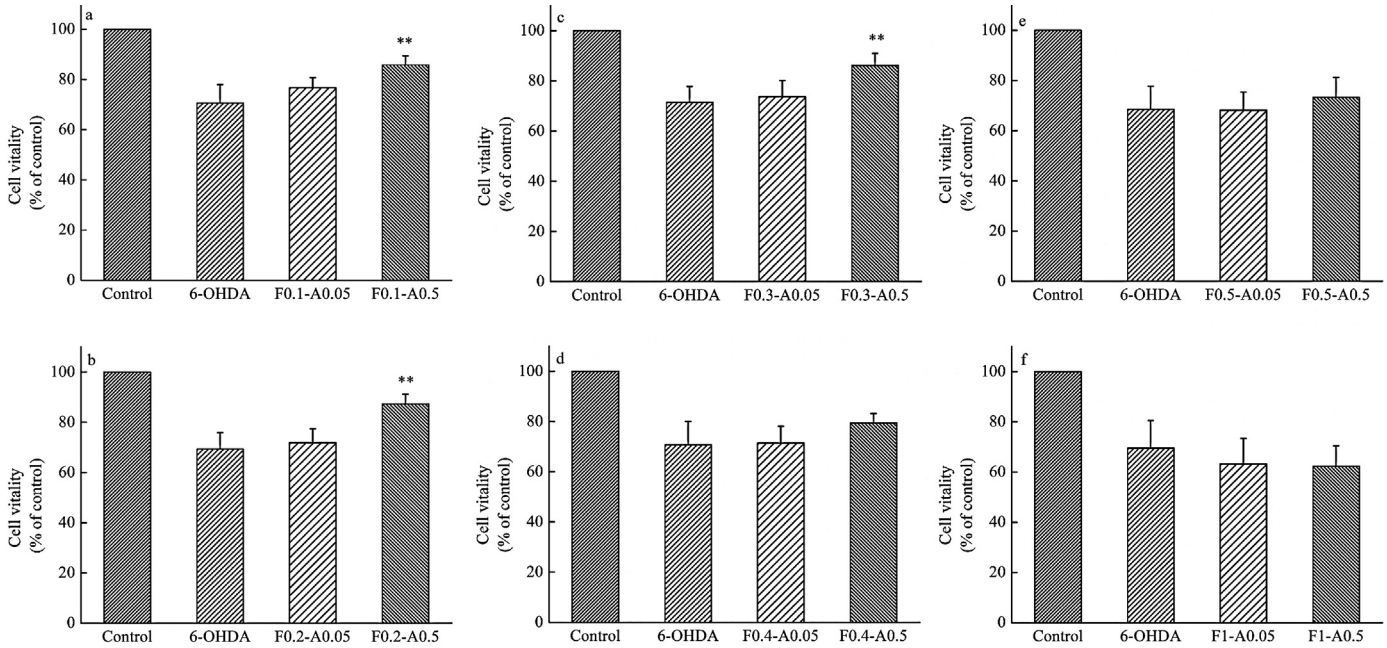

Fig. 4. The neuroprotective effects of DJ0.5 fractions on 6-OHDA-induced neurotoxicity in SH-SY5Y cells. The results are the mean ± SD of absorbance measured at 490 nm. ($n = 4$). (a) F0.1, (b) F0.2, (c) F0.3, (d) F0.4, (e) F0.5 and (f) F1.

Table 3
Chemical compositions of the DJ0.5 and its fractions studied.

Samples	Fuc (%)	U A (%)	Total sugar (%)	Sulfate (%)	Monosaccharides (molar ratio)						
					Man	Rha	Glc A	Glc	Gal	Xyl	Fuc
DJ0.5	27.04	7.44	65.80	31.49	0.13	0.03	0.11	0.04	0.25	0.08	1
F0.1	7.99	13.17	79.88	19.31	0.42	0.27	0.36	0.15	0.43	0.33	1
F0.2	12.85	17.24	80.19	17.86	0.24	0.17	0.40	0.14	0.46	0.19	1
F0.3	14.65	13.01	80.01	20.02	0.21	0.09	0.29	0.09	0.52	0.17	1
F0.4	20.06	6.98	75.35	34.36	0.11	0.04	0.12	0.04	0.29	0.09	1
F0.5	26.75	2.12	63.01	38.08	0.04	0.02	0.03	0.02	0.14	0.04	1
F1	44.66	0.69	55.99	41.62	0.02	0	0.01	0.02	0.10	0.05	1

DJ0.5, which is a sulfated heteropolysaccharide, was elucidated in a previous study (Jin, Wang, Ren, Song, & Zhang, 2012).

4. Conclusions

In this study, we explored fucoidans as potential drugs for treating increasingly common neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Due to the complexity of the fucoidans, a chromatography purification strategy was selected. The fucoidans were separated by anion-exchange chromatography on a DEAE-Bio Gel agarose FF column, and the neuroprotective activities of the resulting fractions were tested. Finally, it was found that the neuroprotective activity copurified with the heteropolysaccharides that contained relatively low fucose (less than 20%) and sulfate (25%), high UA (more than 10%) and high molar ratios of all other monosaccharides. Fractions with higher purity had weaker neuroprotective effects. To the best of our (admittedly limited) knowledge, the mechanisms underlying these neuroprotective activities might require multilateral cooperation between heteropolysaccharides. We will investigate these mechanisms further in future studies.

Acknowledgments

This study was supported by the Ocean Public Welfare Scientific Research Project, the State Oceanic Administration of the People's Republic of China (No. 201005024), the Doctorial Fund of Shandong Province (No. BS2011YY066), the Science and Technology Project of Shandong Province (No. 2011GHY11529), Science and Technology Program of applied basic research projects of Qingdao Municipal (No. 12-1-4-8-(3)-jch) and the Prospective Joint Research Projects of Jiangsu Province (No. BY2011189).

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