Food Chemistry 111 (2008) 428–432

Contents lists available at ScienceDirect

Food Chemistry

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

Purification, antitumor and antioxidant activities *in vitro* of polysaccharides from the brown seaweed *Sargassum pallidum*

Hong Ye [a], Keqi Wang [a], Chunhong Zhou [b], Jun Liu [a], Xiaoxiong Zeng [a,*]

[a] College of Food Science and Technology, Nanjing Agricultural University, Weigang, Nanjing 210095, China
[b] Jiangsu Environmental Monitoring Center, Nanjing 210036, China

ARTICLE INFO

Article history:
Received 11 November 2007
Received in revised form 10 January 2008
Accepted 7 April 2008

Keywords:
Antitumor activity
Antioxidant activity
Sargassum pallidum
Sulfated polysaccharides
Ultrafiltration

ABSTRACT

Supercritical CO_2 extraction, ultrasonic-aid extraction and membrane separation technology were applied to prepare *Sargassum pallidum* polysaccharides (SP). Three main fractions, SP-1, SP-2 and SP-3, were obtained by membranes of 1.0×10^{-4} mm pore size and normal molecular-weight cut-off of 50 kDa. The resulting three preparations were further purified by DEAE Cellulose-52 chromatography to afford seven polysaccharide fractions. Furthermore, the antitumor and antioxidant activities, *in vitro*, of the polysaccharide fractions were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and DPPH· (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging assay, respectively. SP-3-1 and SP-3-2 showed significantly higher antitumor activity against the HepG2 cells, A549 cells, and MGC-803 cells. SP-3 had the highest sulfate content (22.6%). These results indicate that the higher antitumor activity of SP-3-1 and SP-3-2 from SP-3 with lower molecular weights may be related to their molecular weights and sulfate contents. The antioxidant activities of SP-1, SP-2 and SP-3 were low at the tested concentration.

1. Introduction

Seaweeds have caused an emerging interest in the biomedical area, mainly due to their contents of bioactive substances which show great potential as anti-inflammatory, antimicrobial, antiviral, and anti-tumoral drugs (Blunden, 1993; Smit, 2004). Indeed, several species of algae have been found to be the sources of polysaccharides and glycoproteins with immune-stimulant, antitumoral, or antiviral activity (Abdel-Fattah, Hussein, & Salem, 1974; Nishino, Yokoyama, Dobashi, Fujihara, & Nagumo, 1989; Smit, 2004). Among these macromolecules, polysaccharides from the Sargassum genus have been reported to have antitumor activity (de Sousa et al., 2007; Michio, Noriko, Ichiro, & Terukazu, 1984). *Sargassum pallidum*, a brown seaweed extensively distributed in China Yellow Sea and East China Sea, is rich in vitamins, amino acids, dihomogammalinolenic acid, trace elements and polysaccharides (Khomenko & Ovodov, 1975; Zhukova & Svetashev, 1999). However, little information about its polysaccharides and biological activity is available compared with those of other Sargassum types, such as *S. stenophyllum and S. vulgare* (de Sousa et al., 2007; Duarte, Cardoso, Noseda, & Cerezo, 2001). Therefore, evaluation of the polysaccharide bioactivity of *S. pallidium* seems to be imperative for the utilization of this rich resource.

Most methods to extract polysaccharides, nowadays, are quite time-consuming and need a large amount of organic solvents to precipitate the polysaccharides, which will result in a problem of environmental pollution. Therefore, modern extraction and separation techniques, such as supercritical extraction, ultrasonic-aided extraction and membrane separation have recently been applied in polysaccharides preparation (Sheng et al., 2007; Ye, Wu, & Zhou, 2006a; Ye, Zhou, & Bian, 2006b). Membrane separation has traditionally been used for size-based separations with the advantages of high-throughput and being environmentally–friendly, and it is especially suitable for isolation of heat-sensitive substances (García Salgado, Quijano Nieto, & Bonilla Simón, 2006; van Reis & Zydney, 2001). However, most current studies on polysaccharides put emphasis on structure elucidation and structure–activity relationships. There is little information on membrane separation of polysaccharides. In this paper, advanced membrane separation technology and anion-exchange chromatography were applied to prepare *S. pallidum* polysaccharides, affording seven polysaccharide fractions. Furthermore, the antitumor and antioxidant activities, *in vitro*, of the polysaccharide fractions were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and DPPH· (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging assay.

* Corresponding author. Fax: +86 25 84396791.
E-mail address: zengxx@njau.edu.cn (X. Zeng).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.04.012

2. Materials and methods

2.1. Materials and reagents

Lyophilized *S. pallidum* was obtained from Weihai, Shandong province of China. Dimethyl sulfoxide (DMSO), MTT, and DPPH· were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade.

2.2. Extraction of S. pallidum polysaccharides

First, *S. pallidum was degreased* according to the procedure reported in our previous study. Briefly, *S. pallidum* powder (500 g) was put into the extractor vessel (1 l) with a Hua'an supercritical fluid extractor (Hua'an Co., Ltd., Jiangsu, China), and it was treated at 55 °C, 45 MPa, and a flow rate of 20 l/h of CO_2 for 4 h. The degreased *S. pallidum* powder was collected and dried for polysaccharides extraction.

Extraction of polysaccharides was carried out by the procedure reported in our previous study. The degreased *S. pallidum* powder was disrupted by ultrasonic waves with a JY98-cell-breaking apparatus prior to extraction. The resulting sample solution was incubated in a water bath at 90 °C for 5 h, and then centrifuged at 5000 rpm for 15 min to remove the pellet. The proteins in the supernatant were removed by adding trichloroacetic acid (TCA) and centrifuged at 5000 rpm for 15 min. The supernatant was adjusted to pH 7 and kept in the refrigerator for membrane separation.

2.3. Fractionation of S. pallidum polysaccharides by ultrafiltration

The extracted polysaccharides solution was pretreated through a membrane with the pore size of 4.5×10^{-4} mm to avoid fouling of the ultrafiltration membranes. The ultrafiltration was performed with a Millipore Ultrafiltration System equipped with different membranes with the area of 0.1 m^2 (Millipore, USA). The feed solution was pumped to the membrane surface (tangential flow) and the filtrate was collected while the retentate was directed back to the recycle tank. The circulation process lasted until the feed solution was concentrated to about one sixth of its original volume, which was later concentrated by rotatory evaporation, precipitated by ethanol and lyophilized. The filtrate after each ultrafiltration process was the feed solution for the next ultrafiltration with a different membrane. Membranes with different nominal molecular weight cut-offs (1.0×10^{-4} mm, 100 kDa, 50 kDa, 10 kDa, 3 kDa), in turn, were chosen to determine which part the main fraction of polysaccharides was in. Finally, the six fractions of polysaccharides were incorporated into three main crude polysaccharides, SP-1 (>1.0×10^{-4} mm), SP-2 (50 kDa–1.0×10^{-4} mm), and SP-3 (<50 kDa).

2.4. Purification of S. pallidum polysaccharides by anion-exchange chromatography

Crude polysaccharides obtained from ultrafiltration, were dissolved in 0.1 M NaCl (10 mg/ml) and 2 ml of solution were applied to a column of DEAE Cellulose-52 (2.6 × 30 cm), followed by stepwise elution with 0.1, 0.3, and 0.5 M sodium chloride solutions at a flow rate of 60 ml/h. Eluate (5 ml/tube) was collected automatically and the carbohydrates were determined by the phenol–sulfuric acid method, using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Finally, seven fractions of polysaccharides, SP-1-1, SP-1-2, SP-2-1, SP-2-2, SP-2-3, SP-3-1, and SP-3-2, were obtained, dialyzed with water, and lyophilized for further study.

2.5. Cell lines and culture

The human hepatoma cell line HepG2, human lung cancer cell line A549 and human gastric cancer cell line MGC-803 were provided by the Biology Preservation Center of China Pharmaceutical University and maintained with RPMI 1640 medium containing 10% fetal bovine serum and 100 ng/ml, each, of penicillin and streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.6. Growth inhibition assay

The inhibition effects of SP-1-1, SP-1-2, SP-2-1, SP-2-2, SP-2-3, SP-3-1 and SP-3-2 on the growths of HepG2 cells, A549 cells and MGC-803 cells were evaluated *in vitro* by MTT assay (Chen et al., 2006). Briefly, the HepG2 cells, A549 cells and MGC-803 cells (5×10^4 cells/ml) were incubated in 96-well plates containing 0.100 ml of the culture medium at 37 °C in a humidified atmosphere with 5% CO_2. Cells were permitted to adhere for 24 h, then washed with 0.100 ml of phosphate-buffered saline (PBS). PBS was prepared as follows: NaCl (8.00 g/l), KCl (0.20 g/l), $Na_2HPO_4 \cdot 2H_2O$ (1.44 g/l) and KH_2PO_4 (0.20 g/l) were dissolved in double-distilled water to 1.0 l of solution. One hundred microlitres of different concentrations of polysaccharides (0.125, 0.250, 0.500, 1.000 mg/ml), prepared in culture medium, were added to each well. After 48 h of exposure, the polysaccharide-containing medium was removed, washed with 0.100 ml of PBS and replaced by fresh medium. The cells in each well were then incubated in culture medium with 0.020 ml of a 5 mg/ml solution of MTT for 4 h. After the media were removed, 0.150 ml of DMSO was added to each well. Absorbance at 570 nm (maximum) was determined by a Power Wave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT). The inhibition rate (IR) was calculated according to the formula below:

$$\text{Growth inhibition rate}(\%) = (1 - \text{Absorbance of experimental group} / \text{Absorbance of blank control group}) \times 100\%$$

2.7. Determination of sulfate content in polysaccharides

Sulfate content in polysaccharides was determined by the barium chloride–gelatin method (Lloyd, Dodgson, Price, & Rose, 1961). A standard curve was made as follows: 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.16, 0.18 and 0.20 ml K_2SO_4 standard solution (0.6 mg/ml) were accurately put into tubes; hydrochloric acid was compensated to 0.2 ml solution. Then 3.8 ml of trichloroacetic acid and 1.0 ml of barium chloride-gelatin solution were added, and absorbances of A1 were measured at 360 nm after incubation for 15 min at room temperature, and 0.2 ml hydrochloric acid solution was used as a blank. Absorption of A2 was measured under similar conditions, except that the barium chloride solution was replaced by gelatin solution. A standard curve was made with mass of sulfate (mg) for abscissa and the absorption of (A1–A2) for ordinate.

2.8. Determination of antioxidant activity of polysaccharides

The DPPH free radical-scavenging activities of crude polysaccharides and purified polysaccharides by DEAE-52 anion-exchange chromatography were determined according to the method described by Leong and Shui (2002). Briefly, a 0.1 mM of ethanolic DPPH· solution was prepared. The initial absorbance of the DPPH· in ethanol was measured at 517 nm and did not change throughout

the period of assay. An aliquot (0.1 ml) of each sample (with appropriate dilution if necessary) was added to 3.0 ml of ethanolic DPPH· solution. Discolorations were measured at 517 nm after incubation for 30 min at 30 °C in the dark. Measurements were performed at least in triplicate. The percentage of DPPH· which was scavenged was calculated using the following formula:

$$\text{Scavenging}\% = [1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}] \times 100\%$$

Here, ethanol (3.0 ml) plus sample solution (0.1 ml) was used as a blank and 3 ml of DPPH·–ethanol solution plus ethanol (0.1 ml) was also used as a negative control.

2.9. Statistical analysis

The data were presented as means ± standard deviations of three determinations. Statistical analyses were performed using student's *t*-test and one way analysis of variance. Multiple comparisons of means were done by the LSD (least significance difference) test. All computations were done by employing statistical software (SAS, version 8.0).

3. Results and discussion

3.1. Extraction and ultrafiltration of S. pallidum polysaccharides

Supercritical CO_2 extraction was used to degrease the lipophilic substances in *S. Pallidum*. The effect of extraction pressure, temperature, and extraction time were investigated. The optimum operating conditions for treatment were as follows: temperature, 55 °C; pressure, 45 MPa; flow rate of CO_2, 20 l/h; extraction time, 4 h. The degreased materials were used for extraction of polysaccharides. Before extraction with hot water, the degreased *S. pallidum* powder was disrupted by the ultrasonic apparatus. Then, the resulting sample solution was incubated at 90 °C for 5 h, and centrifuged to remove the pellet. The proteins in the supernatant were removed by adding TCA.

The solution extracted with hot water was fractionated by ultrafiltration. Here, the membranes with different pore size were selected to find the main fractions of the polysaccharides, and the results are shown in Table 1. The fractions SP-1 ($>1.0 \times 10^{-4}$ mm) and SP-2 (100 kDa–1.0×10^{-4} mm plus 50–100 kDa) produced greater yields, 23.05% and 37.68% by mass percentage, respectively. The yields of other fractions (10–50 kDa, 3–10 kDa, and <3 kDa) were quite low. Thus, the three fractions were collected together to afford SP-3 (<50 kDa).

The membrane flux increased with increasing temperature, owing to the reduction of viscosity and enhancement of diffusion and mass transfer coefficient. The membrane flux increased linearly with operation pressure, which could be explained by the model: $J = PKm/u$, where Km represented the mass transfer coefficient and u represented the viscosity (Mulder, 1996). The separation effect showed a close relationship with the molecular weight distribution of polysaccharide and nominal molecular weight cut-offs of membranes. Membranes with different nominal molecular weight cut-offs combined were effective for fractionation of polysaccharides with wide molecular weight distribution, but were less effective for those with narrow distribution.

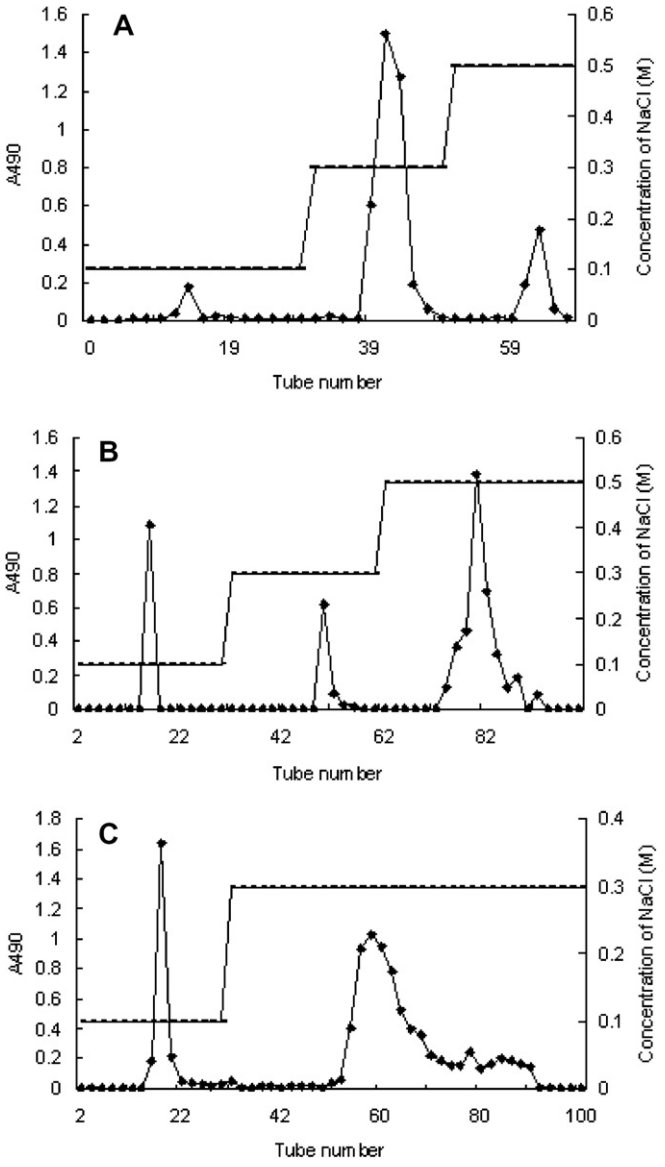

Fig. 1. Elution curve of *S. pallidum* polysaccharides (SP-1, SP-2 and SP-3) from membrane separation by DEAE Cellulose-52 anion-exchange chromatography. (A): SP-1; (B): SP-2; (C): SP-3.

3.2. Purification of S. pallidum polysaccharides

A lyophilized fraction of polysaccharides SP-1 was chromatographed on a DEAE Cellulose-52 anion-exchange column to yield two peaks, SP-1-1 and SP-1-2 (Fig. 1). In a similar manner, SP-2-1, SP-2-2 and SP-2-3, SP-3-1 and SP-3-2 were obtained from SP-2 and SP-3, respectively. The seven polysaccharide fractions were separately collected, dialyzed with water and lyophilized for further study of antitumor and antioxidant activities. The recoveries of SP-1-1 and SP-1-2 were 32.5% and 8.67%, respectively. The recoveries of SP-2-1, SP-2-2 and SP-2-3 were 10.5%, 9.20% and

Table 1
Fractionation of *S. pallidum* polysaccharides by ultrafiltration using different pore size membranes

Nominal molecular weight cut-off	1.0×10^{-4} mm (pore size)	100 kDa	50 kDa	10 kDa	3 kDa	–
Polysaccharide molecular weight scope	$>1.0 \times 10^{-4}$ mm	$<1.0 \times 10^{-4}$ mm > 100 kDa	50–100 kDa	10–50 kDa	3–10 kDa	<3 kDa
Polysaccharide concentration ($\times10^{-3}$ mg/ml)	86.6	62.5	64.8	26.4	18.2	15.0
Retention ratio (%)	23.1	19.4	18.3	7.01	4.76	4.70

36.3%, respectively. The recoveries of SP-3-1 and SP-3-2 were 19.5% and 57.3%, respectively. Anion-exchange chromatography had a certain exchange capacity. If the concentration of sample was too high, exchange capacity of the chromatography column could not reach experimental requirement, resulting in loss of samples. If the concentration of sample was too low, column efficiency was low and handling costs increased. Another important factor of anion-exchange operations was elution rate. Although slower velocity afforded a good separation effect, it took a long time. If elution rate was too fast, two-phase balance could not be fully established between solid and liquid, so the separation effect was poor and eluting peaks were not concentrated. Large sample volume would also make eluting peaks less concentrated (Xing, Zhou, Wang, Song, & Zhang, 2002). So operating conditions would also make eluting peaks less concentrated.

3.3. Growth inhibition on the HepG2 cells, A549 cells and MGC-803 cells

Fractions of polysaccharides SP-3-1 and SP-3-2 presented significantly higher antitumor activity against the HepG2 cells, A549 cells and MGC-803 cells *in vitro* than did blank control groups, and the inhibition ability was dose-dependent (Table 2, Table 3, and Table 4). At 1.000 mg/ml, the inhibition rates of SP-3-1 on the HepG2 cells, A549 cells, and MGC-803 cells were 62.2%, 64.8% and 79.6%, respectively. SP-2-3 only presented significantly higher antitumor activity against the HepG2 cells (81.4%) *in vitro* than a blank control at 1.000 mg/ml, dose-dependently. SP-1-1 and SP-2-2 also showed obvious antitumor activity against the HepG2 cells (59.7% and 63.0%, respectively) at 1.000 mg/ml. SP-2-1 exhibited obvious antitumor activity against the A549 cells (50.0%) at 1.000 mg/ml. These differences in antitumor activities may be attributed to their different molecular weights, charge characteristics and monosaccharide distributions (Dias et al., 2005).

It has been reported that the polysaccharide bioactivities of brown seaweed are closely related to several structural parameters, such as the degree of sulfation, the molecular weight, the sulfation position, type of sugar, and glycosidic branching (Duarte et al., 2001). Also, the sulfate content of a sulfated polysaccharide is one of the most important factors for its biological effects. Therefore, the sulfate contents of the polysaccharides fractions isolated from *S. pallidum* were analyzed. SP-3 had the highest sulfate content (22.6%), while SP-1 and SP-2 had similar sulfate contents (15.7% and 15.3%, respectively). In this work, fractions of polysaccharides SP-3-1 and SP-3-2 showed markedly higher antitumor activity against the HepG2 cells, A549 cells and MGC-803 cells *in vitro*. It seems that polysaccharides with higher sulfate contents exhibit stronger antitumor activity *in vitro*. Furthermore, SP-3-1 and SP-3-2 from SP-3 with relatively lower molecular weight (<50 kDa) showed significantly higher antitumor activity against the HepG2 cells, A549 cells and MGC-803 cells *in vitro*, which may be related to their molecular weights.

Table 2
Growth inhibition of *S. pallidum* polysaccharides at different concentrations against the human hepatoma cell line HepG2 *in vitro*

		1.000 mg/ml	0.500 mg/ml	0.250 mg/ml	0.125 mg/ml
SP-1-1	A_{570}[e]	0.429 ± 0.045^{c}	0.579 ± 0.054^{b}	0.587 ± 0.068^{b}	0.818 ± 0.010^{a}
	IR (%)	59.7	42.3	41.4	14.6
SP-1-2	A_{570}[e]	0.636 ± 0.066^{b}	0.706 ± 0.015^{b}	0.897 ± 0.030^{a}	0.916 ± 0.010^{a}
	IR (%)	35.7	27.6	5.37	3.17
SP-2-1	A_{570}[e]	0.548 ± 0.010^{d}	0.631 ± 0.030^{c}	0.827 ± 0.010^{b}	0.917 ± 0.047^{a}
	IR (%)	45.9	36.3	13.5	3.05
SP-2-2	A_{570}[e]	0.401 ± 0.009^{d}	0.513 ± 0.036^{c}	0.725 ± 0.010^{b}	0.862 ± 0.010^{a}
	IR (%)	63.0	50.0	25.4	9.44
SP-2-3	A_{570}[e]	0.243 ± 0.025^{c}	0.538 ± 0.010^{b}	0.664 ± 0.028^{a}	0.678 ± 0.010^{a}
	IR (%)	81.4	47.1	32.4	30.8
SP-3-1	A_{570}[e]	0.408 ± 0.020^{c}	0.432 ± 0.018^{c}	0.683 ± 0.010^{b}	0.769 ± 0.024^{a}
	IR (%)	62.2	59.4	30.2	20.2
SP-3-2	A_{570}[e]	0.397 ± 0.131^{a}	0.403 ± 0.133^{a}	0.427 ± 0.002^{a}	0.549 ± 0.008^{a}
	IR (%)	63.5	62.8	60.0	45.8

[a,b,c,d] Values within a row followed by different letters are significantly different at $P < 0.05$ using students-t test. Following tables run the same way.
[e] Data are expressed as means ± standard deviation.

Table 3
Growth inhibition of *S. pallidum* polysaccharides at different concentrations against the human lung cancer cell line A549 *in vitro*

		1.000 mg/ml	0.500 mg/ml	0.250 mg/ml	0.125 mg/ml
SP-1-1	A_{570}	0.682 ± 0.009^{c}	0.852 ± 0.018^{b}	0.996 ± 0.084^{a}	1.017 ± 0.031^{a}
	IR (%)	44.1	29.4	16.9	15.0
SP-1-2	A_{570}	0.835 ± 0.009^{c}	0.950 ± 0.01^{b}	1.008 ± 0.051^{ab}	1.063 ± 0.089^{a}
	IR (%)	30.8	20.9	15.8	11.0
SP-2-1	A_{570}	0.614 ± 0.018^{b}	0.655 ± 0.026^{b}	0.670 ± 0.016^{b}	1.114 ± 0.049^{a}
	IR (%)	50.0	46.5	45.2	6.60
SP-2-2	A_{570}	0.820 ± 0.010^{b}	0.846 ± 0.042^{b}	1.106 ± 0.061^{a}	1.131 ± 0.017^{a}
	IR (%)	32.2	29.9	7.30	5.13
SP-2-3	A_{570}	1.009 ± 0.095^{a}	1.019 ± 0.101^{a}	1.038 ± 0.074^{a}	1.160 ± 0.164^{a}
	IR (%)	15.7	14.9	13.2	2.61
SP-3-1	A_{570}	0.444 ± 0.027^{c}	0.722 ± 0.116^{b}	0.848 ± 0.046^{a}	0.930 ± 0.011^{a}
	IR (%)	64.8	40.7	29.7	22.6
SP-3-2	A_{570}	0.419 ± 0.156^{c}	0.883 ± 0.014^{b}	1.073 ± 0.046^{ab}	1.170 ± 0.146^{a}
	IR (%)	67.0	26.7	10.2	1.74

Table 4
Growth inhibition of *S. pallidum* polysaccharides at different concentrations against the human gastric cancer cell line MGC-803 *in vitro*

		1.000 mg/ ml	0.500 mg/ ml	0.250 mg/ ml	0.125 mg/ ml
SP-1-1	A_{570}	0.817 ± 0.026^{c}	0.863 ± 0.032^{c}	0.967 ± 0.015^{b}	1.053 ± 0.037^{a}
	IR (%)	29.6	25.1	16.2	8.50
SP-1-2	A_{570}	0.752 ± 0.116^{b}	0.786 ± 0.027^{b}	1.033 ± 0.017^{a}	1.056 ± 0.012^{a}
	IR (%)	35.4	32.4	10.3	8.23
SP-2-1	A_{570}	0.749 ± 0.041^{b}	0.773 ± 0.127^{b}	0.782 ± 0.085^{b}	1.118 ± 0.132^{a}
	IR (%)	35.7	33.6	32.8	2.68
SP-2-2	A_{570}	0.828 ± 0.042^{b}	0.871 ± 0.085^{b}	0.899 ± 0.098^{ab}	1.014 ± 0.038^{a}
	IR (%)	28.6	24.8	22.3	12.0
SP-2-3	A_{570}	1.020 ± 0.006^{a}	1.060 ± 0.100^{a}	1.097 ± 0.067^{a}	1.122 ± 0.100^{a}
	IR (%)	11.5	7.88	4.56	2.33
SP-3-1	A_{570}	0.259 ± 0.021^{c}	0.418 ± 0.039^{b}	0.499 ± 0.045^{b}	0.631 ± 0.061^{a}
	IR (%)	79.6	65.3	58.1	46.3
SP-3-2	A_{570}	0.620 ± 0.010^{c}	0.672 ± 0.011^{bc}	0.762 ± 0.010^{b}	0.933 ± 0.113^{a}
	IR (%)	47.3	42.6	34.6	19.2

3.4. Antioxidant activity of S. pallidum polysaccharides

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants (Hu, Lu, Huang, & Ming, 2004). In this work, the antioxidant activities of the three main crude polysaccharides, SP-1, SP-2 and SP-3, were low at the tested concentration of 3.8 mg/ml determined by DPPH free radical-scavenging assay. They were 17.8%, 19.1% and 10.2%, respectively. While the polysaccharide fractions purified by DEAE-52 anion-exchange chromatography showed lower antioxidant activities than did crude polysaccharides, their scavenging effects increased with increasing concentration (Table 5).

Table 5
Antioxidant activities of polysaccharides purified by DEAE-52 anion-exchange chromatography as assessed by DPPH· method

Sample		Concentration (mg/ml)				
		1.0	2.0	3.0	4.0	5.0
Scavenging (%)	SP-1-1	0.84	1.67	2.21	2.81	3.55
	SP-1-2	0.24	0.16	1.47	2.04	3.26
	SP-2-1	2.28	3.21	4.15	5.09	6.60
	SP-2-2	2.73	3.30	3.87	4.41	4.94

There are some reports in the literature on the antioxidant capacity of algae. Alcoholic and aqueous extracts of seaweeds have been evaluated for antioxidant activity by lipoxygenase inhibition, DPPH· assay and deoxyribose assays (Jiménez-Escrig, Jiménez-Jiménez, Pulido, & Saura-Calixto, 2001; Yan, Nagata, & Fan, 1998). Recently, several marine alginate derivatives, sulfated fucoidans from the brown seaweed *Laminaria japonica*, agar-like sulfated galactans from the red seaweed Nori and sulfated polysaccharides from *Fucus vesiculosus*, have been reported to have antioxidant activity (Rupérez, Ahrazem, & Leal, 2002; Xue, Yu, Hirata, Terao, & Lin, 1998; Xue et al., 2001). However, the relationship between the structure of seaweed polysaccharides and antioxidative mechanisms has not yet been elucidated. Therefore, it is of great interest to have available highly purified and well characterized seaweed polysaccharides with which to elucidate their mode of action.

4. Conclusions

Supercritical CO_2 extraction, ultrasonic-aid extraction and advanced membrane separation technology, were applied to prepare *S. pallidum* polysaccharides, affording SP-1, SP-2, and SP-3 polysaccharides. The high yield of polysaccharide fractions in *S. pallidum* could be cut off by 50 kDa membrane (SP-1 and SP-2), and SP-3 was obtained in lower yield. The resulting three preparations were further purified by DEAE-52 anion-exchange chromatography, and seven polysaccharide fractions, SP-1-1, SP-1-2, SP-2-1, SP-2-2, SP-2-3, SP-3-1 and SP-3-2, were obtained. Furthermore, the antitumor and antioxidant activities, *in vitro*, of the seven fractions were evaluated by MTT assay and DPPH free radical-scavenging assay. SP-3-1 and SP-3-2 showed significantly higher antitumor activity against the HepG2 cells, A549 cells and MGC-803 cells *in vitro*, which may be related to their molecular weights and sulfate contents. Therefore, it is important to define the complete structure of the polysaccharides, including configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharides, position of branching points and the structure–function relationship, which will certainly present a good opportunity to elucidate the biological roles of polysaccharides and develop potential antitumor drugs based on the three-dimensional structures. Further research is in progress.

Acknowledgements

This work was partly supported by a grant-in-aid for scientific research from the National Natural Science Foundation of China (No. 30570415) and a grant-in-aid for Youth Innovation Fund from Nanjing Agricultural University, China (No. KJ04015).

References

Abdel-Fattah, A. F., Hussein, M. M., & Salem, H. M. (1974). Studies of the purification and some properties of sargassan, a sulphated heteropolysaccharide from *Sargassum linifolium*. *Carbohydrate Research, 33*, 9–17.
Blunden, G. (1993). Marine algae as sources of biologically active compounds. *Interdisciplinary Science Reviews, 18*, 73–80.
Chen, Y. L., Lin, S. Z., Chang, J. Y., Cheng, Y. L., Tsai, N. M., Chen, S. P., et al. (2006). *In vitro* and *in vivo* studies of a novel potential anticancer agent of isochaihulactone on human lung cancer A549 cells. *Biochemical Pharmacology, 72*(3), 308–319.
de Sousa, A. P. A., Torres, M. R., Pessoa, C., de Moraes, M. O., Filho, F. D. R., Alves, A. P. N. N., et al. (2007). *In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed *Sargassum vulgare*. *Carbohydrate Polymers, 69*(1), 7–13.
Dias, P. F., Siqueira, J. M., Vendruscolo, L. F., de Jesus Neiva, T., Gagliardi, A. R., Maraschin, M., et al. (2005). Antiangiogenic and antitumoral properties of a polysaccharide isolated from the seaweed *Sargassum stenophyllum*. *Cancer Chemotherapy and Pharmacology, 56*(4), 436–446.
Duarte, M. E. R., Cardoso, M. A., Noseda, M. D., & Cerezo, A. S. (2001). Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. *Carbohydrate Research, 333*(4), 281–293.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.
García Salgado, S., Quijano Nieto, M. A., & Bonilla Simón, M. M. (2006). Optimisation of sample treatment for arsenic speciation in alga samples by focussed sonication and ultrafiltration. *Talanta, 68*(5), 1522–1527.
Hu, F. L., Lu, R. L., Huang, B., & Ming, L. (2004). Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. *Fitoterapia, 75*(1), 14–23.
Jiménez-Escrig, A., Jiménez-Jiménez, I., Pulido, R., & Saura-Calixto, F. (2001). Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture, 81*(5), 530–534.
Khomenko, V. A., & Ovodov, Yu. S. (1975). Polysaccharides of brown seaweeds VIII. The structure of the side chains of the sargassan molecule. *Chemistry of Natural Compounds, 9*(1), 96–97.
Leong, L. P., & Shui, G. (2002). An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chemistry, 76*(1), 69–75.
Lloyd, A. G., Dodgson, K. S., Price, R. G., & Rose, F. A. I. (1961). Polysaccharide sulphates. *Biochimica et Biophysica Acta, 1*, 108–115.
Michio, F., Noriko, I., Ichiro, Y., & Terukazu, N. (1984). Purification and chemical and physical characterization of an antitumor polysaccharide from the brown seaweed *Sargassum fulvellum*. *Carbohydrate Research, 125*, 97–106.
Mulder, M. (1996). *Basic principles of membrane technology* (2nd ed.). Dordrecht: Kluwer Academic Publishers.
Nishino, T., Yokoyama, G., Dobashi, K., Fujihara, M., & Nagumo, T. (1989). Isolation, purification and characterization of fucose-containing sulfated polysaccharides from the brown seaweed *Ecklonia kurome* and their blood-anticoagulant activities. *Carbohydrate Research, 186*, 119–129.
Rupérez, P., Ahrazem, O., & Leal, J. A. . (2002). Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed Fucus vesiculosus. *Journal of Agricultural and Food Chemistry, 50*(4), 840–845.
Sheng, J. C., Yu, F., Xin, Z. H., Zhao, L. Y., Zhu, X. J., & Hu, Q. H. (2007). Preparation, identification and their antimumor activities *in vitro* of polysaccharides from *Chlorella pyrenoidosa*. *Food Chemistry, 105*, 533–539.
Smit, A. J. (2004). Medicinal and pharmaceutical uses of seaweed natural products: A review. *Journal of Applied Phycology, 16*(4), 245–262.
van Reis, R., & Zydney, A. (2001). Membrane separations in biotechnology. *Current Opinion in Biotechnology, 12*(2), 208–211.
Xing, W. L., Zhou, X. G., Wang, H. X., Song, Y., & Zhang, H. (2002). Study on the process of extracting colistin by ion exchange chromatography. *China Veterinary Magazines, 38*(11), 44–46.
Xue, C. H., Fang, Y., Lin, H., Chen, L., Li, Z. J., Deng, D., et al. (2001). Chemical characters and antioxidative properties of sulfated polysaccharides from *Laminaria japonica*. *Journal of Applied Phycology, 13*(1), 67–70.
Xue, C., Yu, G., Hirata, T., Terao, J., & Lin, H. (1998). Antioxidative activities of several marine polysaccharides evaluated in a phosphatidylcholine-liposomal suspension and organic solvents. *Bioscience, Biotechnology, and Biochemistry, 62*(2), 206–209.
Yan, X., Nagata, T., & Fan, X. (1998). Antioxidative activities in some common seaweeds. *Plant Foods for Human Nutrition, 52*(3), 253–262.
Ye, H., Wu, T., & Zhou, C. H. (2006a). Optimization of the extraction of Sargassum sp. polysaccharides. *Food Research and Development, 27*(7), 22–24.
Ye, H., Zhou, C. H., & Bian, F. (2006b). Study on degreasing of *Sargassum* sp. by supercritical carbon dioxide fluid. *Science and Technology of Food Industry, 27*(7), 136–137.
Zhukova, N. V., & Svetashev, V. I. (1999). A high level of dihomogammalinolenic acid in brown alga *Sargassum pallidum* (Turn). *Phytochemistry, 50*(7), 1209–1211.