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Preliminary studies on the chemical characterization and antioxidant properties of acidic polysaccharides from *Sargassum fusiforme*[*]

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Abstract: In order to investigate the antioxidant properties of the polysaccharides from the brown alga *Sargassum fusiforme*, the crude polysaccharides from *S. fusiforme* (SFPS) were extracted in hot water, and the lipid peroxidation inhibition assay exhibited that SFPS possessed a potential antioxidant activity. Hence, two purely polymeric fractions, SFPS-1 and SFPS-2 were isolated by the column of DEAE (2-diethylaminoethanol)-Sepharose Fast Flow, with their molecular weights of 51.4 and 30.3 kDa determined by high performance gel permeation chromatography (HPGPC). They were preliminarily characterized using chemical analysis in combination of infrared (IR) and nuclear magnetic resonance (NMR) spectroscopies and found to contain large amounts of uronic acids and β-glycosidical linkages. The antioxidant activities of these two SFPS fractions were evaluated using superoxide and hydroxyl radical-scavenging assays. The results show that the antioxidant ability of SFPS-2 was higher than that of SFPS-1, probably correlating with the molecular weight and uronic acid content.

Key words: *Sargassum fusiforme*, Polysaccharide, Antioxidant activity, Uronic acid, Molecular weight
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INTRODUCTION

Antioxidants are substances that delay the oxidation process, inhibit the polymerization chain initiated by free radicals and other subsequent oxidizing reactions (Halliwell and Aruoma, 1991) and thereby help prevent cancer, heart disease, diabetes mellitus, neurodegenerative and inflammatory diseases (Butterfield *et al*., 2002). Although those synthetic antioxidants seem to be promising, their toxicity and side effects rule out their extensive prescription. Therefore, new interest has been developed to search natural and safe antioxidative agents from natural sources. Among various naturally occurring substances, polysaccharides prove to be one of the useful candidates in search for effective, non-toxic substances with antioxidant activity (Liu *et al*., 1997; Zhang *et al*., 2003; Peterszegi *et al*., 2003; Zhang and Lin, 2008). Previous studies indicated that the bioactivities of polysaccharides can be affected by many factors including chemical components, molecular weight, structure, conformation, even the extraction and isolation methods (Leung *et al*., 2006; Zhang *et al*., 2007; Tseng *et al*., 2008). Im *et al*.(2005) showed that *Aloe* polysaccharides larger than 400 kDa had only marginal immunomodulatory activity, while polysaccharides smaller than 400 kDa have potent immunomodulatory activity. The inhibitory effects of fucans on both coagulation and cell proliferation were dependent on their sulfation degree and molecular weight (Haroun-Bouhedja *et al*., 2000).

Marine algae provide us a large source of bioactive compounds such as polysaccharides, minerals, vitamins and non-caloric dietary fibers (Yan *et al*., 1998; Dawczynski *et al*., 2007). More recent reports revealed marine algae to be a rich source of antioxidant compounds (Park *et al*., 2004; Kuda *et al*., 2005; Duan *et al*., 2006). *Sargassum fusiforme* is one kind

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of marine algae classified as the brown algae. It is an ingredient of Chinese herbal medicine that has been applied as a therapeutic for thousands of years (Zhu *et al*., 2003). The polysaccharides extracted from this alga [*S. fusiforme* polysaccharides (SFPS)] in medicine have been demonstrated to have multiple functions including vibriosis resistance, enhancing immunity (Huang *et al*., 2006), antitumor (Ji *et al*., 2007), inhibition to calcium oxalate (CaOxa) urinary stones (Wu *et al*., 2006), and antihyperlipidemia (Mao *et al*., 2004). However, to the best of our knowledge, the investigations of antioxidant activity of the polysaccharides from *S. fusiforme* are very limited.

Therefore, the present study focused on the investigation of antioxidant activity of the polysaccharides from *S. fusiforme*. The antioxidant property of the crude polysaccharide was evaluated by the traditional lipid peroxidation assay. Further phytochemical investigation on the crude polysaccharide led to two major pure fractions (SFPS-1 and SFPS-2), and their in vitro antioxidant activities were evaluated using hydroxyl and superoxide radical-scavenging assays. Compared with the elementary structure information, the relationship between the chemical characterization and antioxidant activities was discussed.

MATERIALS AND METHODS

Plant materials and chemicals

S. fusiforme was collected from Dongtou Town of Zhejiang Province in eastern China. All chemicals used were of reagent grade unless otherwise specified.

Isolation and purification

S. fusiforme (500 g) was defatted with refluxing ethanol (95% (v/v), 2 h, 3 times). The residue was dried in air and then extracted twice with boiled water. The extracted liquids were combined, concentrated and dialyzed against distilled water for 48 h. The non-dialyzable phase was poured with vigorous stirring into three volumes of 95% (v/v) ethanol, and the resulting precipitate was collected by squeezing in fine gauze, washed with absolute ethanol and acetone, and finally dried in a vacuum desiccator at room temperature. The obtained product was deproteinated five times by neutroenzyme-Sevag method (Wu *et al*., 2007) and decolored by D303 anion-exchange resin and HD-1 cation-exchange resin, respectively (Shanghai Huazhen Sci. & Tech. Co., Ltd., China) to afford crude polysaccharide SFPS.

A portion of SFPS (400 mg) was subjected to a column (2.6 cm×40 cm) of DEAE (2-diethylaminoethanol)-Sepharose Fast Flow eluted with 0.3 mol/L NaCl buffer (400 ml) to afford two fractions, SFPS-1 (67 mg) and SFPS-2 (88 mg), as white fluffies. Fractions (4 ml each) were collected and combined according to the results of analysis by the phenol-sulfuric acid procedure (Dubois *et al*., 1956).

Homogeneity and molecular weight determination

The molecular weights of SFPS fractions were determined by high-performance gel permeation chromatography (HPGPC). Samples were separately dissolved in distilled water (1 mg/ml), and applied to HPGPC system incorporating in a Waters 515 instrument fitted with two columns in series (ultrahydrogel 120 and ultrahydrogel 500, Waters, USA). The eluent was 0.1% (w/v) $NaNO_3$ buffer and monitored with Waters 2410 refractive index detector. The HPGPC system was precalibrated with T-series dextran standards. The molecular weight was calculated by the calibration curve obtained by using various standard dextrans.

Components analysis

The content of uronic acids was determined by the carbazole-sulfuric acid method using glucuronic acid as standard (Bitter and Muir, 1962). The composition of neutral monosaccharide was measured by gas chromatography (GC). Briefly, the polysaccharide was hydrolyzed with 2 mol/L trifluoroacetic acid (TFA) at 100 °C for 6 h, and then the hydrolysates were converted into their alditol acetates according to conventional protocols (Crowell and Burnett, 1967). These alditol acetates were analyzed to determine the polysaccharide components in Thermo Quest Trace GC 2000 using a HP-5 fused silica column. D-rhamnose, D-arabinose, D-xylose, D-glucose, D-mannose and D-galactose were used as references and inositol as an internal standard. Column temperature was programmed from 180 °C to 200 °C at 5 °C/min, then increased to 240 °C at 3 °C/min and held for 3 min at 240 °C.

Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopies

FT-IR spectra of SFPS fractions were determined using an FT-IR spectrophotometer (Nicolet NEXUS-470, USA) with KBr pellets in the range 4000~400 cm^{-1} (Wang *et al*., 2004). NMR spectra were recorded on a Bruker AVANCE DRX-500 NMR spectrometer (in D_2O, at 60 °C), using the D_2O signal (δ 4.79, ^1H) and external Me_4Si (δ 0, ^{13}C) as references.

Inhibitory effect of SFPS on lipid peroxidation

The inhibitory effect of SFPS on lipid peroxidation was performed by β-carotene bleaching assay, as reported by He *et al*.(2007). The absorbance of these liposome solutions at 470 nm was monitored by a spectrophotometer (Jasco V530, Japan) before and after incubation. All samples were assayed in triplicate. The antioxidant activity (*AA*) was expressed as percentage of inhibition relative to the negative control using the following equation (Juntachote and Berghofer, 2005):

$$AA\ (\%)=(R_{\text{control}}-R_{\text{sample}})/R_{\text{control}}\times 100,$$

where R_{control} and R_{sample} are the degradation rates of β-carotene in reactant mixtures without antioxidant and with SFPS, respectively.

Degradation rate of the samples was calculated according to the first-order kinetics as follows:

$$R_{\text{sample}}=(OD_0-OD_{120})/OD_0,$$

where OD_0 (470 nm) is the initial absorbance value at 0 min, and OD_{120} (470 nm) is the absorbance value at 120 min.

Scavenging effect on superoxide radicals

The superoxide radical-scavenging activity was measured by the method of Bae and Suh (2007) with minor modifications. A 0.1 ml of sample solution was added to 1.0 ml mixture of 0.4 mmol/L xanthine (Sigma, USA) and 0.24 mmol/L nitro blue tetrazolium chloride (NBT) (Sigma) in 0.1 mol/L phosphate buffer (pH 8.0). Then, a 1.0 ml of xanthine oxidase (0.049 U/ml, Sigma), diluted in 0.1 mol/L phosphate buffer (pH 8.0), was added and the mixture was incubated in a water-bath at 37 °C for 30 min. And then, the absorbance of NBT was measured at 560 nm immediately. All samples were assayed in triplicate and the percent scavenging of radicals of samples was calculated.

Scavenging effect on hydroxyl radicals

The hydroxyl radical-scavenging activity was measured according to the method of Halliwell *et al*.(1987) with some modifications. Samples of different concentrations were mixed with 1 ml of reaction buffer [containing 100 μmol/L $FeCl_3$, 104 μmol/L ethylenediaminetetraacetic acid (EDTA), 1.5 mmol/L H_2O_2, 2.5 mmol/L deoxyribose (Sigma) and 500 μmol/L ascorbic acid (pH=7.4, 20 mmol/L KH_2PO_4-KOH buffer)]. The reaction solutions were incubated for 1.5 h at 37 °C, and then 1 ml of 0.5% (w/v) thiobarbituric acid (TBA) dissolved in 0.025 mol/L NaOH and 1 ml of 2.8% (w/v) trichloroacetic acid were added into the mixtures. The mixtures were incubated for 30 min at 80 °C and cooled rapidly in an ice bath, then measured at 532 nm. All samples were assayed in triplicate and the percent scavenging of radicals of samples was calculated.

RESULTS AND DISCUSSION

Chemical characterization

Both SFPS-1 and SFPS-2 were shown as single symmetrical peak in their respective HPGPC chromatograms, which indicated that they were homogeneous. Chemical components of SFPS fractions and their molecular weights were summarized in Table 1. The uronic acid contents of SFPS-1 and SFPS-2 were

Table 1 Chemical components and molecular weights of the polysaccharide fractions from *Sargassum fusiforme*

Fractions	Molar ratio of neutral monosaccharide composition						Uronic acid content (%)	Molecular weight (kDa)
	Rha	Ara	Xyl	Man	Glu	Gal		
SFPS-1	30.3	44.0	ND	1.3	5.7	1.0	42.4	51.4
SFPS-2	1.4	1.0	4.5	1.6	6.1	2.3	61.1	30.3

Rha: Rhamnose; Ara: Arabinose; Xyl: Xylose; Man: Mannose; Glu: Glucose; Gal: Galactose; ND: Not detected

42.4% and 61.1% and their molecular weights were 51.4 kDa and 30.3 kDa, respectively. According to gas chromatography (GC) analysis, SFPS-1 was composed of rhamnose (Rha), arabinose (Ara), mannose (Man), glucose (Glu) and galactose (Gal) with molar ratio of 30.3:44.0:1.3:5.7:1.0. SFPS-2 was composed of Rha, Ara, xylose (Xyl), Man, Glu and Gal with molar ratio of 1.4:1.0:4.5:1.6:6.1:2.3.

FT-IR spectra of SFPS-1 and SFPS-2 bore similar characteristic absorption bands at 3422, 2924, 1609, 1420, 1299, 1160~1030, 887 and 821 cm^{-1}. The band at 887 cm^{-1} was ascribed to β-configuration of glycosidic linkage (Barker *et al*., 1954); the bands between 1160 and 1030 cm^{-1} corresponded to the glycosidic linkage stretch vibration of C-O-C and C-O-H. In addition, the signals at 1609 and 1420 cm^{-1} were due to the asymmetric and symmetric stretch vibration of $-COO^-$ of uronic acid (Mao *et al*., 2004; Singthong *et al*., 2004) and 821 cm^{-1} due to the characteristic absorption of mannuronic acid (Mao *et al*., 2004). The band at 1299 cm^{-1} may be the stretch vibration of S=O (Zhu *et al*., 2003; Mao *et al*., 2004).

These two compounds also have some comparabilities in their NMR spectra. In their ^{1}H NMR spectra, all of the relevant signals occurred in three regions. The anomeric regions δ 4.3~4.9 were characteristic of β-anomers, which agreed with the presence of IR bands at 887 cm^{-1}. The ring proton regions δ 3.0~4.2 showed overlapping peaks and were assigned to protons of carbons C2 to C5 (or C6) of the glycosidic ring. And the high field region δ 1.28, observed as two well-separated signals, contributed to the methyl groups of two kinds of glycosyl linkages of rhamnose residues (Chiarini *et al*., 2004; Cozzolino *et al*., 2006). Likewise, in their ^{13}C NMR spectra, a low-field intense signal at δ 175 corresponded to $-CO_2H$ of uronic acids (Simas *et al*., 2006).

Inhibitory effect of SFPS on lipid peroxidation

Biological macromolecules, such as lipids, proteins and DNA, can undergo oxidative damage in the presence of reactive oxygen species (ROS), and especially membrane lipids are sensitive to oxidation through this physiological process (Diplock *et al*., 1998). For this reason, liposomes were used for the investigation of lipid peroxidation as an assessment of oxidative stress.

The inhibitory effect of SFPS on lipid peroxidation was determined by β-carotene bleaching assay with four concentrations (5, 10, 30 and 50 mg/ml), and 5 mg/ml vitamin C (V_C) was used as positive control. As shown in Fig.1, SFPS possessed compatible antioxidant ability with V_C at the same dose of 5 mg/ml. It also should be noted that the inhibition effect on lipid peroxidation (54%, w/v) was optimally observed at the concentration of 10 mg/ml and gradually decreased as the concentration increased. The non-dose-response relationship might indicate the equalizing effect of the crude polysaccharide, whereas a combined effect of some other compounds was also possible. Based on this result, SFPS could be exploited as a potential natural source for antioxidants.

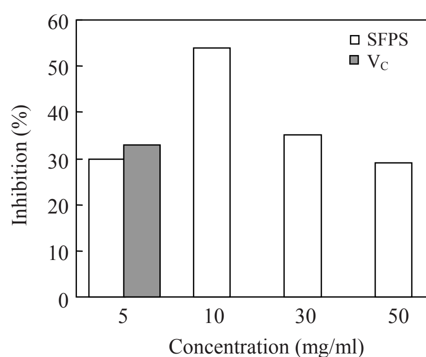

Fig.1 Inhibitory effect of SFPS on lipid peroxidation together with V_C (5 mg/ml) applied as a positive control

Scavenging effects of SFPS fractions on superoxide radicals

Superoxide anions are the most common free radicals in vivo generated in a variety of biological systems and the concentration of superoxide anions increases under conditions of oxidative stress (Lee *et al*., 2002). Hence, an NBT assay was carried out to test the scavenging ability of SFPS fractions on superoxide anions.

As shown in Fig.2, the scavenging effects of SFPS fractions on superoxide radicals showed a dose-response relationship. SFPS-2 exhibited higher scavenging effects than SFPS-1 relatively. Moreover, the rising trend of scavenging ability of SFPS-1 was slowed down after the dose of 0.5 mg/ml, while with respect to SFPS-2, the scavenging effect continuously increased with the concentration in the whole assay.

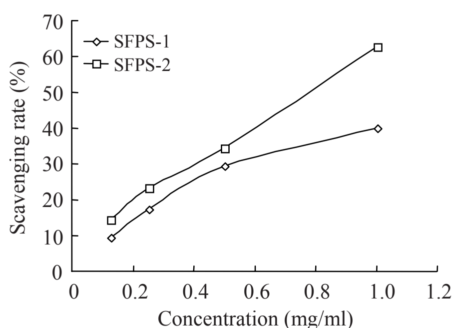

Fig.2 Scavenging effects of SFPS fractions on superoxide radicals

Scavenging effects of SFPS fractions on hydroxyl radicals

Hydroxyl radicals are known to be the most reactive among all the reduced forms of dioxygen and are thought to initiate cell damage in vivo (Rollet-Labelle *et al*., 1998). Further, the effect of SFPS fractions on hydroxyl radicals generated by Fe^{3+} ions was measured by the extent of deoxyribose degradation, an indicator of TBA-MDA (malonic dialdehyde) adducts formation.

The two SFPS fractions were found to have ability to scavenge hydroxyl radicals (Fig.3). In this assay, the scavenging ability of SFPS-2 was also stronger than that of SFPS-1 at the same concentrations of 333 μg/ml and 167 μg/ml. Therefore, the hydroxyl radical-scavenging activities of SFPS fractions in decreasing order were SFPS-2>SFPS-1, which was consistent with superoxide radical-scavenging activities of them.

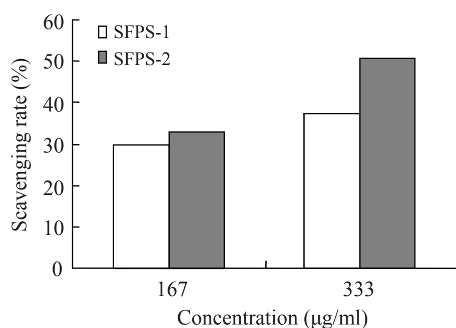

Fig.3 Scavenging effects of SFPS fractions on hydroxyl radicals

Effects of molecular weight and uronic acid content on SFPS

In the chemical characteristic studies, the two polysaccharide fractions (SFPS-1 and SFPS-2), isolated by a column of DEAE-Sepharose Fast Flow, were different from each other in chemical components and molecular weights. According to the NBT assay and the deoxyribose assay, the lower molecular weighted SFPS-2 exhibited higher antioxidant activity. The results suggest that the molecular weights of these polysaccharides played an important role on their bioactivity, which was similar to Zhao *et al*.(2006)'s report that higher antioxidant activities were found when the molecular weight decreased.

Uronic acid also plays an important role on the bioactivities. Many polysaccharides are acid complex carbohydrate, which are composed of uronic acid. In the study of SFPS, we obtained two uronic acid complex polysaccharide fractions, and found that the SFPS-2 exhibited higher antioxidant activities than SFPS-1, while SFPS-2 also contained higher uronic acid content. Referring to the report of Chen *et al*.(2004) that the scavenging effects increased with the increasing uronic acid content in different tea polysaccharide conjugate fractions, the existence of uronic acid might affect the physicochemical properties of the polysaccharides and hence their bioactivities.

CONCLUSION

In this work, several in vitro assays were applied to evaluate the antioxidant potential of the SFPS from *S. fusiforme* and its fractions. The two SFPS fractions with different molecular weights were preliminarily characterized and found to contain large amounts of uronic acids and β-glycosidical linkages. It was found that SFPS had a potential antioxidant activity and that both uronic acid content and molecular weight might play an important role in the antioxidant activity. Among the two SFPS fractions, a relatively low molecular weight and a relatively high uronic acid content appeared to increase the antioxidant activity. The mechanism is not clear now. So, further structural studies are needed to explore the mechanism of antioxidant action of the acidic polysaccharides from *S. fusiforme*. Our current investigation could be a promising beginning for further research on the relationship between structure and bioactivity of *S. fusiforme* polysaccharides.

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