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Chemical characterization, antioxidant and antitumor activity of sulfated polysaccharide from *Sargassum horneri*

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ABSTRACT

Three water-soluble polysaccharide fractions (SHP30, SHP60, and SHP80) extracted from the *Sargassum horneri* were obtained by water extraction and radial flow chromatography. The high-performance gel-permeation chromatography analysis showed that the average molecular weight (Mw) of three polysaccharides were approximately 1.58×10^3, 1.92×10^3 and 11.2 KDa, respectively. Their *in vitro* antioxidant activities, antitumor activities were investigated and compared. Among these three polysaccharides, SHP30 with the highest sulfate content and intermediate molecular weight exhibited excellent antioxidant and antitumor activities in the superoxide radical assay, hydroxyl radical assay, reducing power assay, and MTT assay. Then, flow cytometry assay and quantitative real-time reverse transcription-PCR analysis suggested that the accumulation of cells in G_0/G_1 and *S* phase effecting apoptosis-associated gene expressions such as Bcl-2 and Bax might account for the growth inhibition of DLD cells by SHP30. Based on these results, we have inferred that sulfate content and molecular weight were the factors influencing antioxidant and antitumor activities.

1. Introduction

Seaweeds have caused an emerging interest in the biomedical area, mainly due to their contents of bioactive substances which show great potential and advantages as biological medicine production resource (Ye, Wang, Zhou, Liu, & Zeng; Chakraborty & Paulraj, 2010; Wijesinghe, Athukorala, & Jeon). In particular, polysaccharides from marine algae are known to exhibit various biological and physiological activities including antioxidant, anticoagulant, antiviral, antitumor, and antiinflammatory activities (Wang et al., 2012; Becker, Guimarães, Mourão, & Verli, 2007). Indeed, several species of brown algae have been found to contain polysaccharides and glycoproteins with bioactivities (Andradea et al., 2010; Ermakovaa et al., 2013; Ngo & Kim, 2013).

But only a limited numbers of algae species, however, are available for practical use. Among the remaining unexplored species is *Sargassum horneri*, a brown alga growing in the coastal sea, has been used as a food only in limited areas in Japan, but the processed product obtained by boiling has recently appeared in the Japanese market. Little information is available about its chemical composition and nutritional evaluation (Murakami et al., 2011). To enable the practical use of *Sargassum horneri* as a medicinal marine resource, it is important to obtain information about its chemical composition and bioactivities.

Antioxidant and antitumor activities of sulfated polysaccharide isolated from marine algae have been studied by many scientists (Vijayabaskar, Vaseela, & Thirumaran, 2012; Suresh et al., 2013). However, little information was available about the mechanisms and relationships between their biological activities. Therefore, the further evaluation of the polysaccharides bioactivities of algae seems to be imperative for the utilization of these marine resources.

In this study, three different fractions of polysaccharides were obtained by water extraction and radial flow chromatography (RFC) from one brown alga *Sargassum horneri*. The chemical and monosaccharide compositions of polysaccharides were determined. The *in vitro* antioxidant and antitumor activities of all the three polysaccharides were evaluated by superoxide radical assay, hydroxyl radical assay, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays using MKN45 gastric cancer cells and DLD intestinal cancer cells. Furthermore, the apoptotic effect of SHP30 on DLD cells was analyzed by flow cytometry assay and quantitative real-time reverse transcription-PCR analysis since many polysaccharides with antitumor activity act by inducing apoptosis.

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2. Materials and methods

2.1. Materials and chemicals

The *Sargassum horneri* was collected on the coast of Nanji Archipelago (Zhejiang province, China). The authentication of species was done by Prof. Xingqian Ye (Zhejiang University, Hangzhou, China). The collected samples were washed, air-dried and cultured in plastic bags at room temperature.

2,2-Azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS), sodium borohydride ($NaBH_4$), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), ferrous sulphate ($FeSO_4$), potassium ferricyanide ($K_3Fe(CN)_6$), hydrogen peroxide (HO), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), nicotinamide adenine dinucleotide-reduced (NADH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiazolyl blue (MTT) and dimethyl sulfoxide (DMSO), were purchased from Sigma Chemical Co. Ion-exchange resins were obtained from Purolite International Ltd. The RFC column used in the experiment was purchased from Sepragen Corporation, USA. Real-time PCR Master Mix (SYBR Green) was purchased from Toyobo Co., Ltd. The FACSCalibur flow cytometry were purchased from Becton-Dickinson Co. All other chemical reagents used in this experiment were of analytical grade and purchased locally.

2.2. Analytical methods

The content of total sugar was determined by the method of phenol-sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith). The content of sulfate was determined by barium chloride–gelatin method (Kawai et al., 1969). The IR spectra of the polysaccharides were determined using a Fourier transform infrared spectrometer (FTIR) (PerkineElmer 16PC spectrometer, Boston, USA).

2.3. Extraction of polysaccharides

The air shade dried algae were smashed and screened through mesh size of 100, and were then treated with water extraction methods. Briefly, 30 g of algae powder was mixed with the distilled water at a mass/volume ratio of 1:65. The extraction time was 2 h, and the extraction temperature was 95 °C. After cooling and standing for 1 h, the slurry was filtered and centrifuged at 10,000 rpm for 10 min. Then the supernate was decanted and concentrated to an approximate volume of 180 mL by rotary evaporator. Afterwards, the crude polysaccharides were successively sub-fractionated by graded precipitation at final ethanol concentrations of 30%, 60% and 80%, and obtained by lyophylization.

2.4. Separation of polysaccharides by radial flow chromatography

Three kinds of polysaccharides were obtained after separation by radial flow chromatography, named SHP30, SHP60 and SHP80, respectively. The details were as follows: The above crude polysaccharides extracted from *Sargassum horneri* were configured to 10 mg/mL feed solution with distilled water, then the feed solution was centrifuged at 10,000 rpm, 4 °C for 15 min. The supernatant was pretreated through a membrane with the pore size of 0.45 μm. Then the polysaccharides separated by filtration were purified by ion-exchange chromatography in radial flow column. The crude samples were loaded onto the radial flow column at a flow rate of 5 mL/min. Then the column was washed with distilled water at a flow rate of 40 mL/min. Fractions (8 mL/each tube) were collected at a flow rate of 4 mL/min and monitored using the phenol-sulfuric acid method at 490 nm. After a process purification of the crude polysaccharides, resin regeneration was carried out. The ion exchange resins in the radial flow column were eluted with 2 mol/L NaCl solution at a flow rate of 40 mL/min until the absorbance at 280 nm of the eluent fell to the baseline, then washed with distilled water. At last, the polysaccharide was obtained after condensing under vacuum and drying by lyophylization.

2.5. Molecular weight and monosaccharide composition analysis

The relative molecular weight of SHP was estimated on an Agilent 1100 HPLC system equipped with a refractive index detector (RID) and a TSK-GEL G3000SWxl column (7.5 × 300 mm, Tosoh Corp., Tokyo, Japan). The column was eluted with 0.01 mol/L sodium phosphate buffer (pH 7.0) containing 0.2 mol/L Na_2SO_4 at a flow rate of 0.7 mL/min. Pullulan P-800, P-400, P-200, P-100, P-20, P-10 and P-5 were used as the standards for molecular weight measurement.

The monosaccharide compositions of three sulfated polysaccharides were obtained by reductive hydrolysis. First, 20 mg of sample was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 3 h. The excess acid was removed by vacuum evaporation with methylalcohol (MeOH) after the hydrolysis. The hydrolyzed products were reduced by $NaBH_4$ and acetylated with acetic anhydride, and the standard monosaccharide was derived with same method. Then the alditol acetates were analyzed by gas chromatography (GC) using an Agilent 7890N instrument equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) and a flameionization detector (FID). The applied temperature program was as follows: Oven temperature was initially set at 120 °C, increasing to 240 °C at a rate of 10 °C/min and then held at 240 °C for 6.5 min. The heater temperatures of both injector and detector were kept at 250 °C Nitrogen was used as the carrier gas. Quantitation was calculated from the peak area using response factors.

2.6. Antioxidant activities in vitro

2.6.1. Superoxide radical assay

The superoxide radical scavenging abilities of the sample were assessed by the method of Robak and Gryglewski (1988), with a minor modification. Samples were firstly dissolved in distilled water at different concentrations. Then, 0.1 mL of sample solution, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 557 μM NADH, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 45 μM PMS, and 1 mL of 16 mM Tris–HCl (pH 8.0) containing 108 μM NBT were mixed. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion-scavenging activity. The capability of scavenging the superoxide anion radicals was calculated using the following equation:where A_0 is the absorbance of mixture solution without sample; A_i is the absorbance of the test sample mixed with reaction solution.

2.6.2. Hydroxyl radical scavenging assay

The hydroxyl radical assay of samples was measured according to the method described by Ghiselli, Nardini, Baldi, and Scaccini (1988), with a minor modification. Briefly, sample was dissolved in distilled water at 0.5–6 mg/mL. 0.1 mL of sample solution was mixed with 0.6 mL of reaction buffer [0.2 M phosphate buffer (pH 7.4), 2.67 mM deoxyribose, and 0.13 mM EDTA], 0.2 mL of 0.4 mM ferrous ammonium sulfate, 0.05 mL of 2.0 mM ascorbic acid, and 0.05 mL of 20 mM H_2O_2 were then added to the reaction solution. The reaction solution was incubated at 37 °C for 15 min, and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.0% trichloroacetic acid were added. Afterwards, the mixture reacted at 100 °C for 15 min and cooled down with ice. The presence of hydroxyl radical was detected by monitoring absorbance at 532 nm. The control group contained all the reaction reagents without the samples was prepared and measured as A_0. A_i was the result of samples, and A_j