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Characterization and immunomodulatory activities of sulfated polysaccharides from *Capsosiphon fulvescens*

Supatra Karnjanapratum[a], Mehdi Tabarsa[a], MyoungLae Cho[b], SangGuan You[a,*]

[a] Department of Marine Food Science and Technology, Gangneung-Wonju National University, Gangneung, Gangwon 210-702, Republic of Korea
[b] Marine Natural Products Laboratory, Korea Ocean Research and Development Institute, Ansan, Gyeonggi 426-744, Republic of Korea

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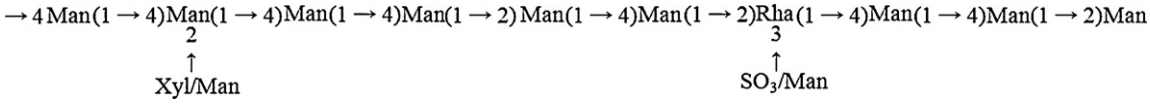
ABSTRACT

Sulfated polysaccharides isolated from *Capsosiphon fulvescens* and fractionated using ion-exchange chromatography were investigated to determine their chemical and molecular characteristics and biological activities. The crude and fractionated polysaccharides (F_1, F_2, and F_3) consisted mostly of carbohydrates (28.9–67.0%), uronic acids (1.6–9.2%) and sulfates (5.2–13.4%) with various amounts of proteins (2.1–53.7%). Their monosaccharide levels were significantly different including rhamnose (20.8–65.2%), xylose (13.0–37.1%) and mannose (11.6–65.1%). The polysaccharides contained one or two subfractions with molecular weights (M_w) ranging from 401.7×10^3 to 6232×10^3 g/mol. These polysaccharides (the crude and fraction F_2) strongly stimulated macrophage cells, RAW264.7 cell line, producing considerable amounts of NO, PGE_2 and cytokines which suggested that they could be strong immunostimulators. The main backbone of the most immunoenhancing polysaccharide (F_2) was suggested by GC–MS and NMR to be the following:



1. Introduction

Sulfated polysaccharides such as fucoidans and carrageenans are commonly found in brown and red seaweeds. These sulfated polysaccharides have been extensively studied because of their various biological activities including antitumor, anti-inflammatory, anti-cancer and immunomodulatory activities [1–4]. The cell walls of green seaweeds also contain sulfated polysaccharides referred to as ulvans. Compared to the sulfated polysaccharides from brown and red seaweeds, little attention has been given to ulvan polysaccharides. However, ulvan polysaccharides have recently garnered attention because they also exhibit various bio-activities. It was reported by Leiro et al. that ulvan polysaccharides extracted from *Ulva rigida* were potent immunomodulators, stimulating RAW264.7 cells and inducing the secretion of prostaglandin E_2 (PGE_2) and nitric oxide (NO) through the expression of COX-2 and iNOS mRNAs [5]. In addition, ulvans from *Enteromorpha intestinalis* showed strong antitumor activity possibly by boosting the host immune system [6]. The thrombin inhibition activity was also observed by ulvans from *Monostroma nitidum*, showing strong anticoagulant activity [7]. Furthermore, Ivanova et al. reported an antiviral activity of ulvans against human and avian influenza viruses through the suppression of the viral reproduction [8]. In a hyperlipidemia study using ICR mice, ulvans from *U. pertusa* exhibited an antihyperlipidemia activity by significantly lowering the levels of plasma total cholesterol, low-density lipoprotein cholesterol and triglyceride but by increasing those of serum high-density lipoprotein cholesterol [9].

Capsosiphon fulvescens is filamentous green seaweed traditionally eaten in the southwestern regions of Korea, showing its great potential to be used as an ingredient in formulated foods as well as in functional foods due to various biological functions of its constituents [10]. Among the constituents in *C. fulvescens*, the sulfated polysaccharides were major active compounds with various bioactivities. In previous studies, the sulfated polysaccharides from *C. fulvescens* considerably improved the proliferation of normal intestinal cells (IEC-6) via stimulating the nuclear translocation of β-catenin and ERK1/2 activation in intestinal epithelial cells, revealing their strong immunomodulating activities [11]. In addition, Park et al. reported that the soluble polysaccharides from *C. fulvescens* suppressed the growth of Sarcoma-180 tumor cells possibly by boosting the host immune system [12].

* Corresponding author. Tel.: +82 33 640 2853; fax: +82 33 640 2340.
E-mail addresses: umyousg@gwnu.ac.kr, umyousg@kangnung.ac.kr (S. You).

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Furthermore, according to Na et al., the purified polysaccharides (glucuronogalactomannan-sulfates) from *C. fulvescens* considerably stimulated RAW264.7 cells and resulted in the secretion of cytokines and chemokines such as TNF-α, IL-6 and NO, through the expression of COX-2 and iNOS mRNAs [13]. The protective effects of the sulfated polysaccharides from *C. fulvescens* on the alcohol-induced gastric injury in rats were also reported by Hwang et al. [14]. The biological activities of the sulfated polysaccharides have been reported to be closely related with their chemical and molecular structures such as molecular weights and sulfate contents [15,16]. Therefore, a better understanding of the primary and secondary molecular structures for the polysaccharides may lead to the successful interpretation of their biological activities. However, despite the abundance and availability of *C. fulvescens*, relatively limited researches have been performed on its molecular structures and bioactivities of sulfated polysaccharides.

In this study, a sulfated polysaccharide from *C. fulvescens* was extracted in distilled water and subsequently fractionated using an ion-exchange chromatography. The purpose of this study was to investigate the chemical and molecular characteristics of the fractionated polysaccharides, to evaluate their immunomodulatory activity, and finally to obtain the relationship between their structures and bioactivities.

2. Materials and methods

2.1. Materials

The green seaweed *C. fulvescens* was collected from the coast of Wando, Chonnam province, Korea. The seaweed was thoroughly washed with tap water and air-dried at 60 °C. The dried raw material was milled using a blender, sieved (<0.5 mm) and stored in plastic bags at −20 °C before extracting the polysaccharide. All chemicals and reagents used in this work were analytical grade. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Lonza (Walkersville, MD, USA).

2.2. Extraction of crude polysaccharide

The milled green seaweed (20 g) was treated with 85% ethanol (EtOH, 200 mL) under constant mechanical stirring overnight at room temperature. The residual part was separated by centrifugation (10 °C, 8000 rpm, 10 min), rinsed with acetone, and then dried at room temperature. The dried biomass (10 g) was extracted with distilled water (200 mL) at 65 °C with stirring for 2 h. The extracts were centrifuged at 10,000 rpm for 10 min at room temperature, and the supernatants were collected. The supernatants were concentrated by evaporation under reduced pressure at 60 °C to approximately 200 mL. EtOH (99%) was added into the supernatants to obtain the final concentration of 70%, and the solution was placed at 4 °C overnight. The polysaccharide was obtained by the filtration of the solution with a membrane (0.45 μm pore size, Whatman International, Maidstone, UK) and washed with EtOH (99%), followed by acetone, and then dried at room temperature. The precipitated polysaccharide was referred to as the crude polysaccharide and the yield was calculated according to the dried biomass obtained after treating the milled sample with 85% EtOH.

2.3. Fractionation of the polysaccharides

The crude polysaccharide (250 mg) was dissolved in distilled water (10 mL), and then the solution was injected into a DEAE Sepharose fast flow column (17-0709-01, GE Healthcare Bio-Science AB, Uppsala, Sweden). The polysaccharides were eluted with distilled water to obtain a non-adsorbed fraction and developed subsequently with a stepwise NaCl gradient (0.5–2 M) to wash the charged polyanionic polysaccharides. Three fractions were obtained based on the phenol–H_2SO_4 assay, referring to as F_1, F_2 and F_3 [17]. These fractions were concentrated, dialyzed and lyophilized.

2.4. Chemical characterization of polysaccharides

Carbohydrate content of the polysaccharide was estimated by the phenol–sulfuric acid assay using glucose as a standard [17]. Sulfate content of the polysaccharide was determined by the $BaCl_2$ gelatin method using K_2SO_4 as a standard after hydrolysis of the polysaccharides with 0.5 M HCl [18]. Protein content of the polysaccharide was determined by the Lowry method using DC Protein assay kit (Bio-Rad, USA) [19]. Uronic acid content of the polysaccharide was determined by sulfamate/*m*-hydroxydiphenyl assay using glucuronic acid as standard [20].

2.5. Monosaccharide composition of the polysaccharides

The monosaccharide composition of the polysaccharides was performed using a HPLC system, which consisted of a pump (Waters 510, Waters, Milford, MA, USA), an injection valve (Model 7010, Rheodyne, Rohnert Park, CA, USA) with a 20 μL sample loop, a column (Carbohydrate analysis column, 4.6 mm × 250 mm, Waters, Milford, MA, USA) and a RI detector (Waters 410). After the hydrolysis of the polysaccharide (60 mg) in 2 M trifluoroacetic acid (TFA) at 120 °C for 5 h, TFA was removed by the evaporation with a dried stream of nitrogen. The hydrolysates were injected into the HPLC system. Acetonitrile (80%) was used as a mobile phase at a flow rate of 2 mL/min. The following neutral monosaccharides were used as references: rhamnose, xylose, mannose, galactose and glucose.

2.6. Deproteination of F_2 polysaccharide

The proteins in F_2 fraction were hydrolyzed using Flavourzyme (#2384, Novozymes, Tianjin, China) after dissolution of the polysaccharides in 0.1 M phosphate buffer (pH 7.0). The sample solution was incubated at 50 °C for 24 h. The resulting hydrolysate was heated in boiling water for 10 min to inactivate the enzyme, centrifuged at 7000 rpm for 10 min and dialyzed in a membrane (#3247027, Spectrum Laboratories, Compton, CA, USA) against distilled water, and eventually lyophilized.

2.7. Glycosidic linkage analysis

The glycosidic linkage analysis of the most immunoenhancing F_2 polysaccharides was carried out using the method of Hakomori with slight modifications [21]. The partially deproteinized polysaccharide (2–3 mg) was dissolved in DMSO (0.5 mL) under nitrogen, and then methylated with CH_3I (0.3 mL) and dried NaOH powder (20 mg). Partially methylated alditol acetates were prepared from fully methylated samples by acid hydrolysis with 4 M TFA at 100 °C for 6 h, followed by a reduction of the hydrolysates in water using $NaBD_4$ and an acetylation with acetic anhydride. The partially methylated alditol acetates were analyzed by a gas chromatography–mass spectrometry (GC–MS) (6890 N/MSD 5973, Agilent Technologies, Santa Clara, CA) using a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, CA) under previously reported conditions [22].

2.8. Nuclear magnetic resonance (NMR) spectroscopy

The partially deproteinized fraction F_2 (30 mg) was dissolved in D_2O (0.5 mL), and subjected to the spectrometer at 50 °C. ^1H and ^{13}C NMR spectra were recorded on a JEOL ECA-600 spectrometer