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Purification, molecular properties, structural characterization, and immunomodulatory activities of water soluble polysaccharides from *Sargassum angustifolium*

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ABSTRACT

Sulfated polysaccharides isolated from *Sargassum angustifolium* and purified to determine their structural characteristics and biological activities. Crude polysaccharides and fractions (F_1 and F_2) were chiefly composed of neutral sugars (49.4–68.5%) and sulfates (12.5–23.0%) along with small amounts of uronic acids (1.3–13.6%) and proteins (4.1–4.7%). Polysaccharides were mainly constructed of different levels of fucose (23.9–69.9%) and galactose (22.5–29.8%) sugars. Subfractions with molecular weights ranging from 157.2 to 790.8 × 10^3 g/mol were identified for isolated polysaccharides. Polysaccharides induced RAW264.7 macrophage cells to release noticeable amounts of nitric oxide and cytokines including IL-1β, TNF-α, IL-6, IL-10 and IL-12 through NF-κB and MAPKs signaling pathways. Sulfate esters of fraction F_2 were necessary to its bioactivity and they were located on carbons 2, 4 and 6 of the major sugars. Fraction F_2 was formed of (1 → 4)- and (1 → 3)-linked fucose residues branched at C-2 and C-4 as well as (1 → 6)-linked galactose residues branched at C-3.

1. Introduction

Fucose-rich polysaccharides are anionic polymers abundantly found in marine organisms including invertebrates and algae. While the former source contains a simple and homogeneous fucans with linear chain, the latter one synthesizes a polymer with rather more complex and heterogeneous structure [1]. These polysaccharides which are located in the cell wall of brown seaweeds have been given a general name as fucoidans [2]. Besides to fucose, fucoidans are consisted of sulfate esters and one or more small proportions of galactose, mannose, xylose, rhamnose, glucuronic acid and even acetyl groups [3,4]. The structure of fucoidans might differ from one species to another and yet can be categorized into two major types. Type I which is consisted of (1–3)-α-L-fucopyranose and type II which contains alternating (1–3)- and (1–4)-α-L-fucopyranose [5]. In addition to the main sequences, there are other glycosidic residues involved in the structure of fucoidans which leads to the formation of structural subclasses such as fucogalactan, fucoglucuronomannan and fucoglucuronan [6,7].

The composition of these macromolecules may vary according to the species, extraction protocol and growth condition of seaweeds [2]. These variations in the chemical structures of fucoidans have found to be directly responsible for their wide and irregular biological functions. A broad spectrum of therapeutic effects has been reported for fucoidans including antitumor, immunomodulatory, antioxidant, anticoagulant, antithrombotic and anti-inflammatory activities [3,4]. The exhibition of biological properties by fucoidans is thought to be driven by one or more of their structural features such as molecular weight, amount and position of sulfate, sugar composition, uronic acid and glycosidic linkages [2]. However, these structure-activity relationships (SAR) of fucoidans have been rarely sought and therefore not fully understood resulting in the failure of a proper development of fucoidan products. The relatively undeveloped research and market status of fucoidans have been primarily attributed to the limited number of investigations conducted on SAR of purified fucoidans and the complex nature of their structures [8].

Therefore, the current study was dedicated to the determination of structural and molecular characteristics of purified polysaccharides from *Sargassum angustifolium* and the valuation of their anticancer and immunomodulatory properties on both cellular and molecular levels.

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2. Materials and methods

2.1. Samples and reagents

S. angustifolium was collected from the coast of Bushehr, Iran. The seaweed was previously identified by Agricultural and Natural Resources Research Center of Bushehr under the voucher number 2662 [9]. The fresh seaweed was initially washed with tap water and air dried at 60 °C. The dried biomass was milled using a blender, sieved (< 0.5 mm) and kept in plastic bags at −20 °C. All other chemicals and reagents were of analytical grade. RPMI-1640 medium and fetal bovine serum (FBS) used in cell culture were purchased from Lonza (Walkersville, MD, USA).

2.2. Isolation of crude polysaccharides

Initially, 20 g of seaweed powder was treated with ethanol (80% EtOH, 200 mL) under constant stirring overnight at ambient temperature to remove lipids, pigments and low molecular weight compounds. The mixture was centrifuged at 10 °C and 8000 rpm for 10 min and supernatant were discarded. The residue was rinsed with acetone and dried at room temperature in a fume hood. Distilled water (400 mL) was added into depigmented powder (20 g) and the extraction carried out at 65 °C with stirring for 2 h. The supernatant were collected after centrifugation at 10 °C and 10000 rpm for 10 min. The extraction was carried out twice and the supernatant were combined and concentrated by evaporation under reduced pressure at 60 °C. To remove the alginates, 1% $ClCa_2$ was added into the supernatant which were later kept at 4 °C overnight. The precipitated alginates were discarded after centrifugation. The polysaccharide precipitation was carried out using EtOH (99%) to obtain a final EtOH concentration of 70%. The mixture was kept at 4 °C overnight and the precipitate was obtained after centrifugation at 10 °C and 10000 rpm for 10 min. The polysaccharide precipitates were washed and dehydrated with EtOH (99%), acetone, and then dried at room temperature. The yield of isolated polysaccharide was calculated in relation to the depigmented powder obtained after 80% EtOH treatment.

2.3. Fractionation of polysaccharides

The crude polysaccharides were fractionated on DEAE Sepharose fast flow column (17-0709-01; GE Healthcare Bio-Science AB, Uppsala, Sweden). To prepare the sample, 250 mg of crude polysaccharides were dissolved in distilled water (10 mL) at 65 °C for 15 min. The solution was filtered using a 3.0-μm filter and then injected into the column which was later eluted with distilled water and a stepwise NaCl gradient (0.5–2 M). All fractions were determined with the phenol-H_2SO_4 assay by measuring the absorbance at 490 nm [10]. The carbohydrate-positive fractions were pooled together, concentrated, dialyzed and lyophilized. The two fractions that were obtained are designated as F_1 and F_2.

2.4. Chemical characterization of polysaccharides

The amount of neutral sugars of the polysaccharides was determined by the phenol–sulfuric acid method using D-fucose as a standard [10]. The amount of protein was determined by the Lowry method [11] using a DC protein assay kit (Bio-Rad, CA, USA). Uronic acid content was determined by a sulfamate/*m*-hydroxydiphenyl assay using glucuronic acid as a standard [12]. The polysaccharides were hydrolyzed with 0.5 M HCl and then $BaCl_2$ gelatin method using K_2SO_4 as a standard was used to measure the amount of sulfate [13].

2.5. FT-IR spectroscopy

Fourier transform infrared (FT-IR) spectra of fucoidans were obtained using a Tensor 27 spectrometer (Bruker Instruments, Billerica, USA). Fucoidans were mixed with KBr to form a 0.5–1 mm thick film and analyzed by ATR-FTIR using absorbance mode. Then, the samples were scanned at wavenumbers ranging from 500 to 4000 cm^{-1} at a resolution of 2 cm^{-1}.

2.6. Monosaccharide composition of polysaccharides

Initially, the polysaccharides were hydrolyzed with 4 M TFA at 100 °C for 6 h. Then, the hydrolysates were reduced in water using $NaBD_4$ and acetylated with acetic anhydride. The final derivatives were analyzed by a gas chromatography-mass spectrometry (GC–MS) (6890N/MSD5973, Agilent Technologies, Santa Clara, CA) equipped with HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, CA).

2.7. Glycosidic linkage analysis

The Ciucanu and Kerek method [14] was used to identify the glycosidic linkages of the polysaccharides. The polysaccharides (3 mg) were solubilized in 0.5 mL DMSO (dimethylsulfoxide) under nitrogen and methylation was performed with 0.3 mL CH_3I and NaOH (20 mg). Then, acid hydrolysis was carried out on the methylated samples with 4 M TFA at 100 °C for 6 h. The reduction of hydrolysates was conducted in distilled water with $NaBD_4$ and acetic anhydride was used for acetylation. The partially methylated alditol acetate derivatives were injected into a gas chromatography mass spectrometry (GC–MS) system (6890 N/MSD 5973, Agilent Technologies, Santa Clara, CA) equipped with HP-5MS capillary column (30 m 0.25 mm 0.25 Lm) (Agilent Technologies, Santa Clara, CA). Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. The temperature program of the oven was as follows: from 160 to 210 °C for 10 min and then to 240 °C for 10 min. The temperature gradient was 5 °C/min and the inlet temperature was kept constant at 250 °C. The mass range was set to measure between 35 and 450 *m*/*z*.

2.8. Preparation of desulfated polysaccharides

Fraction F_2 (100 mg) was dissolved in distilled water (10 mL) and eluted with pyridine from a Dowex 50W resin column (X-8, H^+, 1 × 15 cm). The samples were lyophilized to yield the polysaccharide-pyridinium salts. The solvolytic desulfation of the polysaccharide-pyridinium salts (100 mg) was performed under at 80 °C for 40 min. The desulfated sample was obtained after dialysis of the reaction mixture in a membrane (#3247027, Spectrum Laboratories, Compton, CA, USA) against distilled water [15].

2.9. Determination of molecular properties

Polysaccharides were dissolved in distilled water (2 mg/ mL) and heated for 30 s in a microwave bomb prior to molecular measurement (#4872; Parr Instrument Co., Moline, IL, USA). The samples were immediately filtered through a cellulose acetate membrane (3.0 μm120 pore size; Whatman International). A high performance size exclusion chromatography column (TSK G5000 PW, 7.5 × 600 mm; Toso Biosep, Montgomeryville, PA, USA) linked to a UV detector (Waters, 2487), multi-angle laser light scattering (HELEOS; Wyatt Technology Corp, Santa Barbara, CA, USA) and refractive index detection (Waters, 2414) system (HPSEC-UV-MALLS-RI) were used to analyze the molecular characteristics. The mobile phase, flow rate of 0.4 mL/min, was composed of an aqueous

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