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Isolation and chemical characterization of a novel immunostimulating galactofucan from freshwater *Azolla filiculoides*

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ABSTRACT

Water-soluble polysaccharides were isolated from *Azolla filiculoides* to determine their chemical and structural characteristics as well as anticancer and immunostimulatory activities. Crude and fractions (F_1 and F_2) were mainly composed of neutral sugars (70.0–80.1%), proteins (2.1–14.2%) and uronic acids (1.2–10.8%). The polysaccharides were mostly formed of different levels of fucose (23.8–61.2%), galactose (28.5–38.7%), mannose (7.5–16.7%), xylose (13.3–13.6%), glucose (12.7–13.3%), arabinose (5.5–11.6%) and rhamnose (8.0–9.5%) units. The polysaccharide molecules contained one or more sub-fractions with average molecular weight ranging from 992 to 2162 × 10^3 g/mol. Crude and fractionated polysaccharides induced RAW264.7 macrophages to release pro-inflammatory mediators and cytokines including nitric oxide, IL-1β, TNF-α, IL-6, IL-10 and IL-12 through NF-κB and MAPKs signaling pathways as confirmed by the presence of p-NK-κB, p-JNK, p-ERK and p-38 proteins in the cell cytoplasm. The most immunostimulating polysaccharide, F_2, consisted of alternating →3)-Fuc-(1→ and →4)-Fuc-(1 → residues.

1. Introduction

Anionic polysaccharides are naturally found in the marine organisms with the most well-known resources being brown, red and green macroalgae. The high degree of heterogeneity in the monosaccharide constituents building the polymer chain is the key feature of these marine-originated polysaccharides with the anionic electric charges derived from carboxyl groups and sulfate esters [1,2]. Galactans such as agar and carrageenan are located in the cell walls of red algae consisting of L- and D-galactopyranose residues [3]. Ulvans are polysaccharides from green seaweeds which have α-L-rhamnose as the main monosaccharide unit [4,5]. Fucose-containing polysaccharides (FCPs) are another family of anionic macromolecules which besides to brown algae are included in echinoderms (sea cucumber and sea urchins) and tunicates (ascidians) [1,6]. The synthesis of these polysaccharides is not exclusive to marine habitats and there are many studies reporting the involvement of fucose in the polysaccharide chain of fresh water organisms such as *Tribonema aequale*, *Cryptomonas obovate*, *Chlorella* sp. [7–9].

These polysaccharides that are named as fucoidan consist of a backbone built of (1 → 3)-linked α-L-fucopyranosyl or of alternating (1 → 3)- and (1 → 4)-linked α-L-fucopyranosyl residues [10]. The backbone of fucoidans is substituted with sulfate groups (SO_3^-) on the C-2 and/or C-4 of fucose residues [11]. The molecular weights of fucoidans extracted from brown seaweeds are found to fall in a very divergent range of 13.5 × 10^3 and 1608 × 10^6 g/mol [12]. There is mounting number of publications reporting the promising therapeutic effects of fucoidans in various human diseases such as the inhibition of tube formation and migration of human microvascular endothelial cells [13], the improvement in the state of necrosis and cirrhosis induced by CCl_4 in liver tissue [14], the reduction of serum lipid levels by regulating the expression of cholesterol and triglyceride syntheses enzymes [15] and the inhibition of α-glucosidase activity for the treatment of type 2 diabetes mellitus [16]. The broad spectrum of bioactivities claimed for FCPs are thought to be either individually or cumulatively correlated with their structural characteristics including sugar profile, glycosidic linkages, molecular weight, carboxyl groups and sulfates esters [17].

Azolla filiculoides is a small fern belonging to the Salviniacee family that grows in ponds, wetlands and slow moving streams of temperate and tropical regions. Under optimum conditions, Azolla population growth doubles in nearly 2–4 days [18]. In current study, an anionic polysaccharide was isolated from *Azolla filiculoides* using distilled water and subsequently fractionated by a DEAE Sepharose Fast Flow column. The aim of present study was to investigate the structural and molecular characteristics of purified polysaccharides with anticancer

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Table 1
Yield, chemical composition and molecular properties of the polysaccharides from *A. filiculoides*.

		Crude	F_1	F_2	Red-F_2
Yield (%)		3.04	59.75 ± 0.32	40.25 ± 0.20	–
Neutral sugars (%)		80.14 ± 0.24	72.41 ± 0.10	70.05 ± 0.31	–
Protein (%)		5.41 ± 0.07	14.27 ± 0.14	2.12 ± 0.14	–
Uronic acid (%)		4.54 ± 0.48	1.25 ± 0.20	10.8 ± 0.29	–
Sulfate (%)		ND	ND	ND	–
Monosaccharide composition (%)	Rhamnose	8.05 ± 0.21	9.53 ± 0.29	–	–
	Fucose	23.79 ± 0.24	6.66 ± 0.20	61.25 ± 0.12	53.94 ± 0.41
	Arabinose	5.49 ± 0.47	11.63 ± 0.27	–	–
	Xylose	13.32 ± 0.12	13.60 ± 0.26	–	–
	Mannose	7.50 ± 0.30	16.74 ± 0.24	–	–
	Glucose	12.77 ± 0.12	13.30 ± 0.35	–	–
	Galactose	29.08 ± 0.26	28.54 ± 0.09	38.75 ± 0.12	46.06 ± 0.36
$M_W \times 10^3$ (g/mol)		1516.5 ± 9.12	2162.5 ± 4.24	992.9 ± 13.78	–
R_g (nm)		53.6 ± 0.07	54.4 ± 0.84	52.6 ± 0.21	–
SV_g (cm^3/g)		0.25	0.18	0.37	–

ND: not detected; M_w: mean average molecular weight; R_g: radius of gyration; SV_g: specific volume of gyration; Red-F_2: reduced F_2 polysaccharide.

and immunostimulatory properties and eventually draw a possible relationship between structure and bioactivity.

2. Materials and methods

2.1. Materials

Azolla filiculoides was collected from the paddy fields of Noor, Iran. The collected Azolla was washed with distilled water and dried at 60 °C. The dried sample was ground using a blender, passed through a 0.5 mm sieve and kept in a sealed bag until use. RPMI-1640 medium and fetal bovine serum (FBS) used in cell culture were ordered from Lonza (Walkersville, MD, USA). All other chemical reagents used in this study were of analytical grade.

2.2. Isolation of crude polysaccharides

Dried seaweed powder (20 g) was mixed with 80% ethanol (EtOH, 200 mL) under constant stirring overnight at room temperature to eliminate lipids, pigments and low molecular weight compounds. The mixture was centrifuged at 8000 rpm for 10 min to discard the supernatants. This stage was repeated three times until the supernatant became colorless. The residue was washed with acetone and dried at room temperature in a fume hood. Depigmented powder was extracted with 400 mL of distilled water at 65 °C with stirring for 2 h. The extraction was performed twice and the supernatants were combined for evaporation under reduced pressure at 60 °C. The precipitation of polysaccharides was carried out using 99% EtOH to obtain a final EtOH concentration of 70%. The mixture was kept at 4 °C overnight and the precipitate was collected after centrifugation at 10 °C and 10,000 rpm for 10 min. The polysaccharides were dried using sequential wash with EtOH (99%) and acetone. The yield of polysaccharide was calculated based on the depigmented powder obtained after 80% EtOH treatment.

2.3. Fractionation of polysaccharides

Crude polysaccharides were dissolved in distilled water (250 mg in 10 mL) at 65 °C for 15 min. The solution was filtered using a 3.0-μm filter and then loaded onto a DEAE Sepharose fast flow column (17-0709-01; GE Healthcare Bio-Science AB, Uppsala, Sweden). The column was eluted with distilled water and a stepwise NaCl gradient (0.5 to 2.0 M). All fractions were determined with the phenol-H_2SO_4 assay by measuring the absorbance at 490 nm [19]. The carbohydrate-positive fractions were pooled together, concentrated, dialyzed (3500 Da

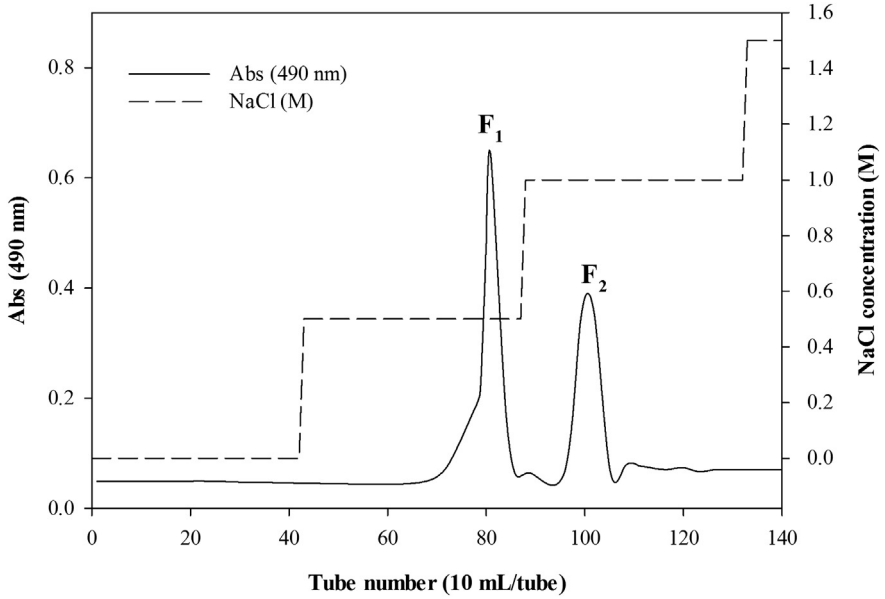

Fig. 1. The DEAE Sepharose FF elution profile of crude polysaccharides from *A. filiculoides*.

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