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Novel sulfated xylogalactoarabinans from green seaweed *Cladophora falklandica*: Chemical structure and action on the fibrin network

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ABSTRACT

The water-soluble sulfated xylogalactoarabinans from green seaweed *Cladophora falklandica* are constituted by a backbone of 4-linked β-L-arabinopyranose units partially sulfated mainly on C3 and also on C2. Besides, partial glycosylation mostly on C2 with single stubs of β-D-xylopyranose, or single stubs of β-D-galactofuranose or short chains comprising (1 → 5)- and/or (1 → 6)-linkages, was also found. These compounds showed anticoagulant activity, although much lower than that of heparin. The effect of a purified fraction (F1) on the fibrin network was studied in detail. It modifies the kinetics of fibrin formation, suggesting an impaired polymerization process. Scanning electron microscopy showed a laxer conformation, with larger interstitial pores than the control. Accordingly, this network was lysed more easily. These fibrin properties would reduce the time of permanence of the clot in the blood vessel, inducing a lesser thrombogenic state. One of the possible mechanisms of its anticoagulant effect is direct thrombin inhibition.

1. Introduction

Cladophora Kützing is one of the largest green algal genera and has a worldwide distribution. Within the class Cladophorophyceae, the genus *Cladophora* is characterized by its simple thallus architecture: branched, uniseriate filaments of multinucleate cells. *Cladophora* cell walls, which are several micrometers thick, were reported to consist largely of cellulose, which represents about 15% of the biomass (Wissel, Mayr, & Lücke, 2008).

In spite of its wide distribution, which comprises mostly marine, but also fresh water environments, and of its abundance, there is only scarce information about the structure of the sulfated polysaccharides biosynthesized by algae of this genus. Percival and coworkers studied the structural features of the water soluble polysaccharides from *Cladophora rupestris* (Percival & McDowell, 1981). These studies established the presence of arabinose, galactose, xylose, rhamnose, and glucose in the approximate molar proportions of 3.7:2.8:1.0:0.4:0.2 (+8% protein) and 19.6% half ester sulfate. Application of degradative studies provided evidence of a highly branched structure, with xylose and galactose units at the ends of the branches and galactose, arabinose, and rhamnose residues occurring in the inner part of the molecules. Evidence of 6-linked and/or 6-sulfated galactofuranose units was obtained. Partial hydrolysis experiments led to the separation and characterization of the following fragments: L-arabinose 3-sulfate, D-galactose 6-sulfate, 3-linked and 6-linked D-galactobioses, 4- or 5-linked L-arabinobiose 3-sulfate, 4-linked D-xylobiose, a mixture of trisaccharides containing sulfated galactose and arabinose, and a mixture of pentasaccharides in which the molar ratio of arabinose to galactose was 4:1.

Later, the structural characteristics of the polysaccharides obtained from *Cladophora socialis* by extraction with dilute acid were reported (Sri Ramana & Venkata Rao, 1991). A molar ratio

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galactose:arabinose:xylose of 4.5:3.0:1.0 and 16.9% of sulfate were determined. Similar conclusions were obtained regards the arabinose units, but no galactose in the furanose form was detected. In this case, galactose units were found to be 3-linked and sulfated on C4 or C4 and C6.

Between the sulfated polysaccharides, sulfated fucans (SFs) and sulfated galactans (SGs) are currently the marine non-glycosaminoglycan (GAG) sulfated compounds most studied in glycomics. These polysaccharides exhibit therapeutic effects in several pathophysiological systems such as blood coagulation, thrombosis, neovascularization, cancer, inflammation, and microbial infections. As analogs of the largely employed GAGs and due to some limitations of the GAG-based therapies (Anand, Yusuf, Pogue, Ginsberg, & Hirsh, 2003; Kelton & Hirsh, 1980; Kelton & Warkentin, 2008), SFs and SGs comprise new carbohydrate-based therapeutics available for clinical studies. However, the degree and mechanism of action varies greatly, not only with the degree of sulfation, but also with their chemical structure, and other sulfated polysaccharides with different carbohydrate backbones and sulfate distributions have been investigated and were found to have similar properties (Ciancia, Quinitana, & Cerezo, 2010, Fernández et al., 2013).

In a previous paper, the effect in the fibrin network formation of sulfated galactans from green seaweeds of the order Bryopsidales was studied by measuring optical density (OD) at 405 nm *vs* time. It was shown that these galactans caused an impaired assembly of fibrin monomers into the fibrin polymer (Arata et al., 2015).

Kinetics of fibrin formation gives a different insight into the study of the coagulation process, contributing to the understanding of the mechanism of anticoagulant activity. On the other hand, the study of fibrinolysis is also crucial, because currently approved thrombolytic drugs have side effects, such as bleeding, low specificity on account of being indirect plasminogen activators, and high cost. The 'holy grail' for antithrombotic therapy, a drug that prevents coagulation without promoting bleeding, has yet to be found.

In this paper, structure of the major water-soluble sulfated polysaccharides from *Cladophora falklandica* was determined, and their anticoagulant effect was investigated, not only by general coagulation tests, but also through studies of their influence in fibrin formation and fibrinolysis. Besides, characteristics of the fibrin clot were examined using scanning electron microscopy.

2. Experimental

2.1. Algal sample

Specimens of *Cladophora falklandica* (J.D. Hooker & Harvey) J.D. Hooker & Harvey were collected in Punta Este – Puerto Madryn – Chubut – Argentina (42°46′48″S, 64°57′W), in September 2010. The samples used in this work were in the vegetative state. Thalli of the seaweeds were washed with sea water and analyzed for epiphytic and epizoic contaminants in a Nikon AFX-II macroscope (Nikon, Japan). Voucher specimens were deposited in the Museum Bernandino Rivadavia, Buenos Aires Argentina (collection code: BA: 47426).

2.2. Extraction of the polysaccharides

Algal samples of *C. falklandica* were dried in open air. The extraction procedure was described elsewhere (Ciancia et al., 2007). Briefly, the milled seaweds (100 g) were extracted twice with EtOH 70% (20 g/L) for 3 h at room temperature. The residue from the alcohol extraction was extracted with H2O (20 g/L) at room temperature for 18 h giving a product, which was recovered from the supernatant by dialysis and freeze dried (extract CX1). The residue from the first H_2O extraction was extracted twice more in similar conditions to give extracts CX2 and CX3. The residue from the room temperature H_2O extraction was extracted three times for 3 h with H_2O (20 g/L) at 90 °C, giving extracts CC1–CC3.

2.3. Ion exchange chromatography (IEC)

CX2 was chromatographed on DEAE-Sephadex A-25. The sample (220 mg) was dissolved in water (30 mL), centrifuged, and the supernatant was applied to a column (90 × 1.5 cm id), previously stabilized in H_2O. The first elution solvent was water, and then NaCl solutions of increasing concentration up to 4 M. Fractions of 4 mL were collected. Finally, the phase was boiled in 4 M NaCl solution. The presence of carbohydrates in the samples was detected by the phenol – sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956); after obtaining blank readings, the eluant was replaced by another with higher concentration of NaCl. Eleven fractions were obtained, dialyzed and freeze dried (F1–F11).

2.4. Chemical analyses

The total sugars content was analyzed by the phenol-sulfuric acid method (Dubois et al., 1956). Sulfate was determined turbidimetrically (Dodgson & Price, 1962). Alternatively, ion exchange chromatography with conductimetric detection was used: the sample was hydrolyzed in 2 M CF3CO2H at 121 °C for 2 h, evaporated to dryness under nitrogen and redissolved in high purity water from a Milli-Q system. A DIONEX DX-100 chromatography system (Sunnyvale CA, USA) was used with an AS4A column (4 × 250 mm), an AMMS-II micromembrane suppressor and a conductivity detector, elution was carried out with 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$, at a flow rate of 2 mL min^{-1}. The absence of pyruvic acid and uronic acids was confirmed using the colorimetric determinations of Koepsell and Sharpe (1952) and Filisetti-Cozzi and Carpita (1991), respectively. The protein content was measured by the method of Bradford (1976) (BioRad protein assay), Lowry, Rosenbrough and Farr (1951), and using a kit Pierce™ BCA Protein Assay Kit; however, results were very variable, so microanalysis was performed to determine the amount of nitrogen, a factor of 5 was applied to calculate the amount of protein, according to Angell, Mata, de Nys and Paul (2016). The configuration of galactose and arabinose was determined by the method of Cases, Cerezo and Stortz (1995) through their diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino) alditols. To determine the monosaccharide composition, samples were derivatized to the alditol acetates (Stevenson & Furneaux, 1991). Number average molecular weight of CX2 and F1 was estimated by the method of Park and Johnson (1949). Dialyses were carried out with tubing with molecular weight cutoff of 3500 Da.

2.5. Desulfation of F1 and CC2

The reaction was carried out by the microwave-assisted method described by Navarro, Flores and Stortz (2007). The sample (40 mg) was converted to the pyridinium salt and dissolved in 10 mL of DMSO containing 2% of pyridine. The mixture was heated for 10 s intervals and cooled to 50 °C (×6). It was dyalized 3 days against tap water and then 24 h against distilled water and lyofilized. An aliquot was methylated as described bellow without previous isolation of the product.

2.6. Partial acid hydrolysis of F1

The reaction was carried out according to Bilan, Vinogradova, Shashkov, and Usov (2007). The sample (50 mg) was heated in 1%