International Journal of Biological Macromolecules 66 (2014) 332–337

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Chemical characterization and antiherpes activity of sulfated polysaccharides from *Lithothamnion muelleri*

Bruna G. Malagoli[a], Francielle T.G.S. Cardozo[b], Jose Hugo S. Gomes[a], Vany P. Ferraz[c], Cláudia M.O. Simões[d], Fernão C. Braga[a,*]

[a] Department of Pharmaceutical Products, Faculty of Pharmacy, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31.270-901 Belo Horizonte, Brazil
[b] Department of Microbiology, Immunology and Parasitology, Center of Biological Sciences, Universidade Federal de Santa Catarina, Campus Trindade, 88.040-900, Florianópolis, Brazil
[c] Department of Chemistry, ICEX, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31.270-901 Belo Horizonte, Brazil
[d] Department of Pharmaceutical Sciences, Center of Health Sciences, Universidade Federal de Santa Catarina, Campus Trindade, 88.040-900 Florianópolis, Brazil

ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form 18 February 2014
Accepted 23 February 2014
Available online 6 March 2014

Keywords:
Lithothamnion muelleri
Polysaccharides
Sulfated xylogalactans
HSV
Antiherpetic activity

ABSTRACT

We report herein the chemical characterization and antiherpes activity of polysaccharides from the red alga *Lithothamnion muelleri* (Hapalidiaceae). The polysaccharide-rich fractions B1 and B2 were obtained by extraction with Na_2CO_3 and were purified by size exclusion chromatography to afford Fra-B1 and Fra-B2. The polysaccharides were characterized by FT-IR and chemical analysis (total contents of carbohydrates, proteins, sulfate and uronic acid), whereas their average molecular weights were estimated by high performance gel permeation chromatography. The monosaccharide analysis detected galactose, glucose, xylose, mannose, rhamnose and arabinose in the four polysaccharide samples. Antiherpetic *in vitro* assays showed that B1 and B2 inhibited Herpes Simplex Virus types 1 and 2 (HSV-1 and HSV-2) when added simultaneously to viral infection affording selectivity indices ($SI = CC_{50}/EC_{50}$) higher than 20. Investigation of the mechanism of action indicated that B1 and B2 act on the initial steps of HSV replication, mainly inhibiting viral adsorption but also viral penetration into the cells.

1. Introduction

Sulfated galactans are typical constituents of the intercellular matrix and nonfibrillar cell walls of red seaweeds [1]. Calcareous red algae belonging to the family Corallinaceae have a distinct polysaccharide composition [2] and sulfated xylogalactans have been obtained from *Corallina pilulifera*, *Bossiella cretacea* and *Clathromorphum nereostratum*, along with alginic acids, also found in *Amphiroa fragilissima* and *Corallina mediterranea* [2]. The occurrence of sulfated xylogalactans has been also described for *Lithothamnion heterocladum* (Hapalidiaceae), a species belonging to another family of the order Corallinales [1]. In general, species of Corallinales produce low amounts of polysaccharides, a feature attributed to their strong calcified cover [2].

Lithothamnion muelleri Lenormand ex Rosanoff (Hapalidiaceae) is a red seaweed found in Brazilian coastal areas [3]. There is an increasing interest in the biological activities of *Lithothamnion* species for further development as products in nutraceutical, pharmaceutical and cosmeceutical fields. Preparations containing species of *Lithothamnion* are currently commercialized in several countries as source of minerals. The multi-mineral preparation Aquamin®, prepared from *Lithothamnion corallioides*, has been reported to relief the symptoms of osteoarthritis [4,5] and to be of benefit in bone [6] and digestive health [7]. Aquamin® has also been described to reduce LPS-induced TNF-α and IL-1β release *in vitro* [8] and inhibit NF-κB activation [9], along with increasing mineralization in osteoblast cell culture [10].

On its turn, a mineral-rich extract from *Lithothamnion calcareum* has been reported to exhibit anti-inflammatory activity on colon [7] and cortical glial-enriched cell cultures [8], protective effect against CCl_4-induced liver injury [11], preservation of bone structure and function [6], activity against Ca^{2+}-sensitive and -resistant human colon carcinoma cells [12], induction of differentiation in human colon tissue and modulation of stromal function [13]. Interestingly,

* Corresponding author. Tel.: +55 31 3409 6951; fax: +55 31 3409 6935.
E-mail addresses: fernao@netuno.lcc.ufmg.br, fernao.braga@gmail.com (F.C. Braga).

http://dx.doi.org/10.1016/j.ijbiomac.2014.02.053
0141-8130/

the above-cited activities have been credited only to the mineral constituents of both *Lithothamnion* species, and therefore the role of polysaccharides in the biological responses remains to be investigated. In this way, we have recently demonstrated the antiadhesive activity of sulfated polysaccharide-rich fractions from *L. muelleri*, along with their preliminary chemical characterization [14].

Sulfated polysaccharides synthesized by seaweeds are known to possess relevant biological effects such as anticoagulant, antiviral, antioxidant and anti-inflammatory activities [15]. Regarding the antiviral activity, many compounds of this class have been shown to be active against different viruses, including Herpes Simplex Virus (HSV), Human Immunodeficiency Virus (HIV), human cytomegalovirus and dengue virus [16–20]. Within this context, the aim of this work was to characterize the sulfated polysaccharides obtained from a mineral concentrate of *L. muelleri* and to assay their activity against human herpes virus types 1 and 2 (HSV-1 and HSV-2).

2. Materials and methods

2.1. Algal material

Samples of *L. muelleri* were donated by the company Phosther Algamar (Belo Horizonte, Minas Gerais, Brazil) as a whitish granulate (4 kg) known as *marine mineral concentrate*. The granulate was produced by washing the seaweed sequentially with tap water and distilled water to remove salt and all visible epiphytes, following by crushing in a ball mill and drying in a ventilated oven. The species was identified by Dr. Maria Carolina M. de O. Henriques, Instituto Biodiversidade Marinha, Rio de Janeiro, Brazil.

2.2. Extraction and purification of the polysaccharides

The extraction conditions described in our previous work [14] were used with some modifications. Different periods of time (1, 2, 4 and 6 h) were initially evaluated for polysaccharide extraction at 60 °C, and the 2 h period was selected for the work. Portions of the algal material were submitted to extraction with 1% (w/v) Na_2CO_3 aqueous solution at 60 °C, for 2 h, under mechanical stirring, using the proportion of 20% (w/v) granulate in Na_2CO_3 solution leading to extract B1. The residue remaining after this extraction was recovered by vacuum filtration over paper and was re-extracted with 2% (w/v) Na_2CO_3 aqueous solution for 2 h at 60 °C, using the same proportion of algal material/solvent extractor (20%, w/v), which led to extract B2. A rotatory evaporator was used to concentrate the extracts to a tenth of their original volumes, following by the precipitation of the polysaccharides with ethanol (4:1 ethanol/extract, v/v) and centrifugation at 3000 × *g* for 10 min. The precipitates were dialyzed against water through a cellulose membrane (cut off 10,000 Da) for 2 days, followed by drying in a centrifugal vacuum concentrator (Labconco Centrivap, Kansas City, USA), originating the polysaccharide-rich fractions B1 and B2.

2.3. FT-IR spectra analysis

The IR spectra were recorded for B1 and B2 fractions on a Perkin Elmer FT-IR spectrometer (Norwalk, USA), at room temperature, in the infrared region between 4000 and 650 cm^{-1}.

2.4. Fractionation of polysaccharides

The polysaccharide-rich fractions B1 and B2 were fractionated by gel permeation chromatography over Sephadex G-100 column (70 cm × 2.5 cm i.d.) using water as eluent (0.8 mL/min). Portions of each sample (150 mg dissolved in 10 mL water) were applied to the column and fractions of 5 mL were collected. The chromatographic separations were monitored by the phenol–sulfuric acid method adapted for microplate [21]. After combining the fractions according to their elution profile, the solvent was removed in a centrifugal vacuum concentrator (Labconco Centrivap), at a maximum temperature of 50 °C.

2.5. Composition analysis

Total sugar content was determined spectrophotometrically by the phenol–sulfuric acid method [22] adapted to microplate format [21], using galactose as standard. The protein concentration was quantified according to the method of Bradford [23] modified to microplate format [24,25], using bovine serum albumin (BSA) as standard. Sulfate content was determined after hydrolysis of the samples with 2 M HCl, by the barium chloride–gelatin turbidimetric assay [26], using standard solutions of potassium sulfate. The measurement of uronic acid was performed by the *m*-hydroxybiphenyl colorimetric method [27], employing glucuronic acid as standard. In each case, calibration curves were obtained on three different days and proved to be statistically equivalents by ANOVA. Results are expressed as mean ± standard deviation (SD) of three independent experiments.

2.6. Molecular weight determination

The average molecular weights of the polysaccharides were estimated by high-performance size exclusion chromatography (HPSEC). Analyses were performed in a Shimadzu HPLC system equipped with a DID-6A refractive index detector, at room temperature, using an ultrahydrogel linear column (300 mm × 7.8 mm i.d., Waters, Milford, USA) eluted with 0.1 M $NaNO_3$ solution, at a flow rate of 0.5 mL/min. Pullulan standards (P-5, P-10, P-20, P-50, P-100, P-200, P-400, P-800, Shodex Standard P-82; Sigma–Aldrich, St. Louis, USA) were employed to construct the calibration curve. Samples solutions (5.0 mg/mL) were prepared in ultrapure water and aliquots (20 μL) of the solutions were injected onto the HPLC system. The analyses were performed in triplicate.

2.7. Monosaccharide composition analysis

Samples were hydrolyzed and the released monosaccharides were derivatized to their alditol acetates to determine their proportions in the polysaccharides by gas chromatography. Portions of B1 and B2 (5 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 6 h and the excess of TFA removed by evaporation under compressed air flow. The hydrolyzates were resuspended in water and reduced by stirring with sodium borohydride at room temperature for 12 h. After eliminating the excess of $NaBH_4$, the alditols were acetylated with a mixture of pyridine and acetic anhydride (1:1, v/v) under stirring at room temperature, for 12 h [28,29].

The resulting alditol acetates were analyzed on a HP5890 gas chromatography system with a flame ionization detector (GC-FID) (Hewlett-Packard, Palo Alto, USA), fitted with a BP-10 capillary column (25 m × 0.25 mm i.d., SGE). The column temperature was fixed at 200 °C for 1 min and rising of the temperature at 4 °C/min until 250 °C. The injector and detector temperatures were fixed at 270 °C. Hydrogen was used as carrier gas at a flow rate of 2 ml/min. Injections followed a slip ratio of 110:1. The areas and retention times were obtained with Varian Star #1 software. The standard monosaccharides (glucose, galactose, xylose, mannose, rhamnose and arabinose) were subjected to the same procedures for determination of retention times. Results were expressed as the relative molar percentages (mol%) of each monosaccharide on the fractions.