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Structure and anti-metapneumovirus activity of sulfated galactans from the red seaweed *Cryptonemia seminervis*

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ABSTRACT

The anti-HMPV (human metapneumovirus) activity was determined for sulfated DL-hybrid galactans obtained from the red seaweed *Cryptonemia seminervis* and their depolymerized products obtained by reductive partial hydrolysis. Structural studies carried out in three homogeneous depolymerized fractions DS-1, DS-2e and DS-3 (Mw of 51.6–63.8 kDa) showed that these galactans present different chemical characteristics, as monosaccharide composition, content of sulfate groups (14.1–29.9%) and agaran:carrageenan molar ratio diads, 2.7:1 for DS-1 and DS-2e and 1:1 for DS-3. The sulfate groups are located principally on C-2 of β-D-galactopyranose and 4,6-*O*-(1′-carboxyethylidene)-β-D-galactopyranose residues and on C-6 of α-galactose residues. Sulfated DL-galactans and their depolymerized products exhibited antiviral activity at a very early stage of the viral infection cycle. All fractions, except DS-2e inhibited HMPV replication by binding to the viral particle. Besides depolymerized galactans DS-2e and DS-3 inhibited the recognition of cell receptor by HMPV and penetration to the host cell, respectively.

1. Introduction

Human metapneumovirus (HMPV) member of the family *Paramyxoviridae* is a causative agent of acute respiratory illness (Williams, Edwards, & Weinberg, 2010). First identified in 2001 (van den Hoogen et al., 2001), HMPV has been described worldwide and serological studies have revealed that HMPV seropositivity is almost universal by the age of 5 years (Collins & Crowe, 2007). Re-infection with HMPV is common and currently there is no vaccine available (Arnott et al., 2013). Other than for influenza virus, antiviral therapy for respiratory viruses has not shown satisfactory results. In this way, studies of new compounds with potential anti-HMPV activity could have clinical value. Polysaccharides extracted from seaweeds have been reported to exhibit antiviral activity against a wide spectrum of viruses including important human pathogenic agents such as Human Immunodeficiency virus (HIV), Herpes simplex virus (HSV), Vesicular stomatitis virus (VSV), Cytomegalovirus (CMV) (Cassolato et al., 2008; Duarte et al., 2004; Faria-Tischer et al., 2006; Harden, Falshaw, Carnachan, Kern, & Prichard, 2009; Haslin, Lahaye, Pellegrini, & Chermann, 2001; Matsuhiro et al., 2005; Witvrouw & De Clercq, 1997). Secondary metabolites obtained from green and brown marine algae were able to inhibit HMPV replication (Mendes et al., 2010, 2011). However, in spite of the broad-spectrum of antiviral activities reported for sulfated polysaccharides, as far as we know anti-HMPV activity has not been described for these polymers.

As previously reported red seaweeds from the genus *Cryptonemia* synthesize sulfated galactans named DL-hybrid galactans. These kind of galactans are composed by repeating units of 3-linked β-D-galactopyranosyl (A-unit) and 4-linked α-galactopyranosyl units (B-unit) where the last are present in both D- and L-enantiomeric series (Zibetti, Noseda, Cerezo, & Duarte, 2005; Zibetti et al., 2009). The DL-hybrid family of galactans from

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Cryptonemia seminervis contains the B-units as α-D- and α-L-galactose and 3,6-anhydro-α-D- and 3,6-anhydro-α-L-galactose besides minor amounts of monomethylated galactoses (6-*O*- and 2-*O*-methylgalactose) and 3,6-anhydro-2-*O*-methyl-α-L-galactose. The structural complexity of these galactans is increase by the presence of sulfate groups located at different positions, xylose and/or 4-*O*-methylgalactose as side chain and β-D-galactopyranosyl 4,6-(1′-carboxyethylidene). S2S-4 is one of the homogeneous hybrid galactans (Mw 332.4 kDa) isolated from *C. seminervis*. S2S-4 presents the A-units constituted predominantly by β-D-galactopyranosyl 2-sulfate (11.9 mol%), β-D-galactopyranosyl 2,6-sulfate (18.6 mol%), β-D-galactopyranosyl 2-sulfate 4,6-(1′-carboxyethylidene) (8.8 mol%) whereas the B-units are constituted mainly by 3,6-anhydro-α-L-galactose (7.3 mol%), 3,6-anhydro-α-L-galactose 2-sulfate (14.4 mol%) and α-L- and/or α-D-galactose 2,3,6-sulfated (8.2 mol%). From the enantiomeric analyses, the agaran and carrageenan blocks of S2S-4 are present in a molar ratio of ~1.7:1.

Previous reports have described the antiviral activity against herpes simplex virus and dengue virus of the DL-hybrid galactans obtained from *Cryptonemia crenulata* (Talarico et al., 2004, 2005) however, there are no studies reporting antiviral activity of the sulfated polysaccharides from *C. seminervis.*

The present study describes the chemical structure of the partially depolymerized products obtained from the red seaweed *C. seminervis* and reports for the first time the antiviral activity of sulfated polysaccharides against human metapneumovirus.

2. Materials and methods

2.1. Collection of specimens

Specimens of the red seaweed *C. seminervis* (C. Agardh) J. Agardh were collected at the southeastern coast of Brazil (Marataízes, Espírito Santo State). A voucher specimen was deposited at the herbarium of the Department of Botany, Federal University of Paraná (Curitiba, Brazil) with the herbarium identification code UPCB-59441. The algal material was cleaned to remove contaminants, washed with tap water, sun-dried, and milled. Whole thalli were used for polysaccharide extractions.

2.2. Extraction of polysaccharides

The milled alga was extracted with phosphate buffer pH 6.5 (1.5%, w/v) at 80 °C under mechanical stirring for 6 h. After centrifugation, the supernatant was concentrated and treated with EtOH (3:1, v/v). The precipitated material was redissolved in distilled water, dialyzed (cut-off 12–14 kDa) sequentially against distilled water, 1.0 M NaCl and distilled water, concentrated and freeze-dried. This extraction process was repeated three times and the four extracts were combined rendering the crude extract CE.

2.3. Fractionation by KCl-treatment

For KCl treatment (Cardoso, Noseda, Fujii, Zibetti, & Duarte, 2007) CE crude extract was dissolved in water (0.25%, w/v) and KCl was added to a concentration of 2.0 M under magnetic stirring for 3 h. The solution was maintained at 4 °C overnight. After centrifugation, the supernatant and precipitate were separately dialyzed (distilled water, 1.0 M NaCl and distilled water) and freeze-dried giving rise to the KCl-soluble fraction S and precipitate fraction P.

2.4. Partial depolymerization

Galactan S (1.1 g) was dissolved in distilled water (1.5%, w/v), the solution was heated to 65 °C and borane-4-methylmorpholine complex (6.6 g) and 1.5 M TFA (25 mL) were added, giving a final concentration of 0.375 M TFA (Usov & Elashvili, 1991; Zibetti et al., 2005, 2009). The solution was maintained at 65 °C for 6 h. Acid was removed by co-distillation with water and the partially depolymerized product was dialyzed (cut-off 6–8 kDa) against distilled water yielding the DS fraction.

2.5. Fractionation of the partially depolymerized product

The DS fraction (790 mg) was submitted to anion-exchange chromatography on a DEAE-Sephacel column (30 cm × 6 cm i.d.) that was sequentially eluted (stepwise) with water and aq. NaCl solutions of increasing concentrations (0.25–4.0 M). The column eluents were analyzed for carbohydrate content by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The eluents obtained were concentrated, dialyzed and freeze-dried, giving rise to the following fractions: DS-W (eluted with water); DS-1 (eluted with 0.25 M NaCl); DS-2 (0.50 M); DS-3 (0.75 M); DS-4 (1.0 M) and DS-5 (1.5 M). The higher yield fraction DS-2, was submitted to a new chromatographic fractionation under the same conditions described above, but with a concentration range of 0.1–1.0 M NaCl with an increment of 0.1 M for each elution step, giving rise to the following fractions: DS-2W (water) and DS-2a–DS-2f (0.1–0.8 M NaCl, respectively).

2.6. Analytical methods

Total carbohydrate content was estimated by the phenol–sulfuric acid method using galactose as standard (Dubois et al., 1956). Sulfate content was determined by the turbidimetric method of Dodgson and Price (1962). Protein content was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as standard. The specific rotation was measured at 20 °C, using a 10-cm cell and the sodium D line (589.3 nm) on a Rudolph Autopol III automatic polarimeter.

The determination of monosaccharide composition was carried out by reductive hydrolysis (Stevenson & Furneaux, 1991) using extra reducing agent [borane·4-methylmorpholine complex (4-MMB)] before and after pre-hydrolysis and hydrolysis steps (Falshaw & Furneaux, 1994; Jol, Neiss, Penninkhof, Rudolph, & De Ruiter, 1999). The hydrolytic process was performed as described by Ferreira et al. (2012). After acetylation (Stevenson & Furneaux, 1991) the alditol acetate derivatives were analyzed by GC–MS in the same conditions described by Ferreira et al. (2012) and were identified by their typical electron-impact fragmentation profiles and GC retention times (Jansson, Kenne, Liedgren, Lindberg, & Lonngren, 1976). A double hydrolysis-reductive amination method was used to determine the absolute configuration of the monosaccharide constituents (Navarro & Stortz, 2003). Chiral 1-amino-2-propanol was used to determine the ratio of D- and L-galactose and its 6-*O*-methyl derivative, whereas the configuration of 2-*O*-methylgalactose, 3,6-anhydrogalactose and their 2-*O*-methyl derivatives were determined using chiral α-methylbenzylamine. The resulting alditol acetates derivatives were analyzed by GC–MS using the conditions described by Ferreira et al. (2012).

2.7. Desulfation

Fractions DS-1, DS-2e and DS-3 in the pyridinium salt form (Stevenson & Furneaux, 1991) were submitted to partial solvolytic desulfation (Nagasawa, Inoue, & Kamata, 1977) yielding DS-1D, DS-2eD and DS-3D, respectively.