Galactofuranose-rich heteropolysaccharide from Trebouxia sp., photobiont of the lichen Ramalina gracilis and its effect on macrophage activation

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A B S T R A C T

A structural study of the carbohydrates from Trebouxia sp., the algal symbiont of the lichen Ramalina gracilis demonstrated a galactofuranan-rich heteropolysaccharide, which was predominated by (1→5)-linked galactofuranosyl units with side-chains in position 6 on approximately 11.0% of the units. The side-chains have very complex branched structures. This polysaccharide showed cell eliciting activity on peritoneal macrophages in vitro at all concentrations tested (1–150 μg/mL), and at 150 μg/mL an increase of 60% of macrophage activation in comparison to the control group was observed. A potential role of these carbohydrates in lichen recognition process is also discussed. © 2008 Elsevier B.V. All rights reserved.

1. Introduction

Lichen thalli, the symbiotic phenotype of lichen-forming fungi in association with their photobiont, are known to contain considerable amounts of polysaccharides. Due to the bi and/or tripartite nature of the lichen symbiosis, the source of the polysaccharides, that is, if they are produced by the mycobiont or photobiont alone or by both in symbiosis, has been an open question along many years. Recently, for lichens of the genera Ramalina, Cordeiro et al. [1–3] showed that the glucans (nigeran and laminaran) and the galactomannan, found previously in the lichen thalli, were also produced by the aposymbiotically cultured mycobiont, while amylose and a (1→5)-linked β-galactofuranan, substituted in a small proportion at O-6 by β-Galf units were solely found in extracts of aposymbiotically cultured photobiont Trebouxia sp.

Polysaccharides from various sources (e.g. plants, fungi and lichens) are well known to have biological activities, such as anti-tumor, anti-inflammatory or immunomodulating activity [4–6]. These polysaccharides have a great variety of chemical structures, being linear or branched homopolysaccharides (α- and β-glucans) or heteropolysaccharides (galactomannans, pectic polysaccharides, and heteroxytans) [5].

Galactofuranose-containing molecules are absent in green plants, but are widespread components of cell wall glycoconjugates of bacteria, protozoa, and fungi [6]. Recent studies have also demonstrated their presence in green algae (Trebouxia and Asterochloris sp.) and in a cyanobacteria (Nostoc commune) [3,7,8]. Mammals do not produce galactofuranosyl-containing molecules, and they are highly immunogenic in mammals [9–12]. Due to their presence in human pathogens, such as Mycobacterium tuberculosis, Paracoccidioides brasiliensis, Aspergillus fumigatus and Trypanosoma cruzi, the metabolism of galactofuranose has become a promising target for drug design [8,13,14]. Moreover, these saccharides seem to be responsible for the high immunogenity of cell-wall antigens from pathogenic fungi and for toxic effects of phytopathogens [8,15].

The objects of the present work were (1) to purify and to determine the fine chemical structure of mannogalactofuranans found in the photobiont Trebouxia sp., in order to amplify the knowledge on carbohydrate production in lichen symbiosis and (2) to investigate if this molecule, which belongs to a galactofuranan-rich class of polysaccharides, has macrophage activation properties.

2. Material and methods

2.1. Photobiont isolation and culture conditions

The isolation of the photobiont and the production of biomass were obtained according Cordeiro et al. [3]. Briefly, photobiont cultures were obtained from thallus fragments, using the tissue culture method of Yamamoto et al. [16]. To obtain biomass, the photobiont was cultivated on a modified Bold’s Basal Medium (BBM) (1 L, in Erlenmeyers of 2 L), to which 1.5% glucose and 0.5% peptone were added. The cultures were illuminated with 45 mol/(m2 s) for 12 h, followed by an interval of 12 h in the dark at 21 ± 2 °C. After 30 days,
the algal cells were removed by filtration, washed with distilled water and freeze-dried. Forty grams of biomass were obtained at the end of 12 months of cultivation.

2.2. Extraction and purification of polysaccharides

The photobiont of *R. gracilis* (40.0 g) was defatted and the polysaccharides extracted with hot water and 10% aq. KOH. The alkaline extract was further processed with freeze-thawing and ultrafiltration through membranes with cut-offs of 300, 30 and 10 kDa (Millipore) according to a previously described fractionation process [3] to give 432 mg of a fraction retained in the 30 kDa membrane (fraction SK30R). Fraction SK30R was collected, treated with α-amylase (Sigma) for 48 h, dialysed and deproteinized with 10% trichloroacetic acid. The resulting fraction (420 mg) was further purified through sequential dialysis using dialysis tube with cut-offs of 50, 100, 250 and 500 kDa (Fig. 1). The materials passing through the 100 and 500 kDa dialysis tube were concentrated under reduced pressure and freeze-dried, to give 40 and 33 mg of purified fractions SK100E and SK500E, respectively.

2.3. Monosaccharide composition of the polysaccharides

Monosaccharide components of the polysaccharides and their ratio were determined by hydrolysis with 2 M TFA for 8 h at 100 °C, followed by conversion to alditol acetates by successive NaBH4 reduction and acetylation with Ac2O-pyridine.

2.4. Determination of homogeneity of polysaccharides and their molecular weights

The homogeneity and molecular weight (Mw) of soluble polysaccharides were determined by high performance steric exclusion chromatography (HPSEC), using a multidetection equipment in which a differential refractometer (Waters) and a multiangle laser light scattering apparatus (MALLS; Dawn DSP-F, Wyatt Technology) were adapted on-line. The eluent was 0.1 M NaNO3, containing 0.5 g/L NaH2PO4. The polysaccharide solution (1 mg/mL) was filtered through a membrane, with pores of 0.22 μm diameter (Millipore).

2.5. Methylation analysis of polysaccharides

Samples were O-methylated using NaOH–Me2SO–Mel [17]. The per-O-methylated polysaccharides were converted into partially O-methylated alditol acetates by successive treatments with 72% H2SO4 for 1 h in ice bath, 8% H2SO4 for 16 h at 100 °C, reduction with NaBD4 and acetylation with Ac2O-pyridine. The products were examined by capillary gas liquid chromatography–mass spectrometry (GC–MS) and identified by their typical electron impact breakdown profiles and retention times [18,19].

2.6. General experimental procedures

Gas liquid chromatography–mass spectrometry was performed using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap model 800 mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection and then programmed at 40 °C min⁻¹ to 220 °C (constant temperature) was used for quantitative analysis of alditol acetates and partially O-methylated alditol acetates. Acetylation of alditols was carried out with Ac2O–pyridine (1:1, v/v) at room temperature for 24 h.

1H and 13C NMR spectra were obtained using a Bruker DRX 400 Avance spectrometer incorporating Fourier transform. Analy-ses were performed at 50 °C in D2O. Chemical shifts are expressed as δ ppm, using the resonances of CH3 groups of acetone internal standard (1H at δ 2.224; 13C, δ 30.2).

2.7. Animals

Male albino Swiss mice (6–8 week old) weighing 25–30 g were used. Animals were acquired from the Central Animal House of the Federal University of Paraná (UFPR), that received a standard laboratory diet (Purina®) and water ad libitum. All recommendations of the Brazilian National Law (no. 6.638.058 nov. 1979) for scientific management of animals were respected and the Institutional Animal Care Committee at UFPR approved all related practices. Experiments were carried out at the Research on Neoplasic and Inflammation Cells Laboratory, UFPR, which has a management program for produced residues.

2.8. Cell culture and microscopy

Macrophages were washed from peritoneal cavities with 10 mL of cold phosphate buffer solution (PBS) pH 7.4. Macrophages were plated in either 24-wells tissue culture plates having glass coverslips. The cells were incubated at 37 °C under 5% CO2 for 15 min and the non-adherent cells were removed by washing with PBS. Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 μg/mL penicillin and 100 U/mL gentamicin was added to culture in the presence or absence of fraction SK100E (1, 5, 10, 50 and 150 μg/mL) for 48 h at 37 °C in a humidified atmosphere containing 5% CO2. Accordingly, >90% of adherent cells were macrophages and the preparation was not further purified.

2.9. Morphological analyses by light microscopy

After the time of culture, the coverslips were washed with PBS pH 7.4 (37 °C), fixed for 5 min in Bouin liquid, stained with Giemsa for 2 h, dehydrated in acetone and xylol, and slides mounted with Entelan. The macrophages adhered to the coverslips were observed.
using a Nikon eclipse E200 microscope to differentiate spreading state of cells. It was examined about 100 macrophages for each coverslip, using an immersion objective resident and activated macrophages were easily differentiated by morphological characteristics [20].

2.10. Morphological analyses by scanning electron microscopy (SEM)

Differences among the groups were mainly found after 48 h of culture. Thus, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, washed, and post-fixed in 1% osmium tetroxide for 30 min in the dark at room temperature [21]. After washing, the cells were dehydrated using increasing alcohol concentrations. Cells were CO2 critical point dehydrated, metallized and observed using a JEOL JSM e 6360 LV SEM Scanning Electron Microscope at the UFPR Electron Microscopy Center.

2.11. Statistical analysis

Biological assays were performed three times in quadruplicate. Results are expressed as mean ± standard deviations. Data were submitted to analysis of variation (ANOVA) and Tukey’s test to determine the statistical significance (*P<0.05).

3. Results and discussion

The alkaline extract obtained from Trebouxia cells (25.2% yield) was processed with freeze-thawing and the soluble fraction (SK10, 19.5% yield) was submitted to ultrafiltration through membranes with cut-offs of 300, 30 and 10 kDa (Millipore) according to a previously described fractionation process. The material passing through the 10 kDa membrane showed the presence of the β-galactofuranan, similar to that found in the aqueous extract [3].

The materials retained by the 300 and 30 kDa membranes contained a mixture of polysaccharides, as shown by their HPSEC elution profile. The fraction retained in the 30 kDa membrane (fraction SK30R, 430 mg) was treated with α-amylase and deproteinized with 10% trichloroacetic acid. The resulting fraction (420 mg) was composed by rhamnose (12.5%), arabinose (7.5%), xylose (3.0%), mannose (12.5%) and galactose (64.5%) and was further purified through sequential dialysis using dialysis tubes with cut-offs of 50, 100, 250 and 500 kDa (Fig. 1).

The materials passing through the 100 and 500 kDa dialysis tubes were concentrated under reduced pressure and freeze-dried, to give 40 and 33 mg of purified fractions SK100E and SK500E, respectively. The homogeneity of these fractions was confirmed by HPSEC, they were eluted as single peaks (Fig. 2). The dn/dc [3] values found for both fractions were similar (0.186), giving rise to identical Mw 31.500 Da. Moreover, the monosaccharidic, methylation and 13C NMR analysis indicating that they contain the same polysaccharide structure. It consisted mainly of galactose (67.0%) and mannose (22.0%), with small amounts of rhamnose (2.8%) and arabinose (8.2%). Methylation analysis of fraction SK100E (Table 1) indicated that this polymer is a mannogalactofuranan composed by (1→5)-linked galactofuranosyl units in the main-chain with side-chains in position 6 on approximately 11.0% of the units. The side-chains have very complex branched structures, composed mainly by mannoypiranosyl units 4-O-, 2,4-O-, 2,3-O- and 3,6-O-substituted. Rhamnose and arabinose were found only as non-reducing end units, together with non-reducing end units of Galp and Manp. Its 13C NMR spectrum (Fig. 3A) is predominated by signals (δ 107.2 and 108.4) from galactofuranosyl units 5-O- and 5,6-O-substituted, respectively. Moreover, an anomeric β-configuration of these units can be con-

<table>
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<tr>
<th>Partially O-methyl alditol acetate</th>
<th>%a</th>
<th>Linkage typeb</th>
</tr>
</thead>
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<tr>
<td>2,3,5-Me3-Ara</td>
<td>6.7</td>
<td>Ara(1→5)</td>
</tr>
<tr>
<td>2,3-Me2-Ara</td>
<td>1.9</td>
<td>5/S[Ara(1→5)]</td>
</tr>
<tr>
<td>2,4-Me2-Rha</td>
<td>2.2</td>
<td>Rhap(1→5)</td>
</tr>
<tr>
<td>2,3,4,6-Me2-Man</td>
<td>1.6</td>
<td>Manp(1→5)</td>
</tr>
<tr>
<td>2,3,6-Me2-Man</td>
<td>6.0</td>
<td>4-Manp(1→5)</td>
</tr>
<tr>
<td>4,6-Me2-Man</td>
<td>5.0</td>
<td>2,3-Manp(1→5)</td>
</tr>
<tr>
<td>3,6-Me2-Man</td>
<td>2.3</td>
<td>2,4-Manp(1→5)</td>
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<tr>
<td>2,4-Me2-Man</td>
<td>5.3</td>
<td>3,6-Manp(1→5)</td>
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<td>16.5</td>
<td>Galp(1→5)</td>
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<tr>
<td>2-Me-Gal</td>
<td>2.5</td>
<td>3,5,6-Galp(1→5)</td>
</tr>
</tbody>
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a Percentage of peak area of O-methyl alditol acetates relative to total area, determined by GC–MS.
b Based on derived O-methyl alditol acetates.

Fig. 2. HPSEC elution profile of fractions SK100E (dark line) and SK500E (grey line) obtained from Trebouxia photobiont (refractive index detector).

Fig. 3. 13C-NMR spectrum of (A) mannogalactofuranan (fraction SK100E) of the photobiont Trebouxia sp., photobiont of the lichen R. gracilis.
cluded from their low-field C-1 signals. Others intense signals at δ 82.5 (C-4), 81.8 (C-2), 77.1 (C-3), 75.5 (O-substituted C-5) and 61.5 (C-6) are characteristic of β-(1 → 5)-linked Galf units [3,22–24]. A signal at δ 100.5 correspond to α-mannopyranosyl units. Substitution at OH-6 of the C-6 of the β-Galf and α-Manp units was also shown on DEPT examination (Fig. 3B), which provided an inverted signals at δ 73.8 and 68.3, respectively, corresponding to the substituted CH2OH.

The other fraction, retained in the 300 kDa membrane (fraction SK300R, 1 g) was further purified through sequential dialysis using dialysis tube with cut-offs of 1000 kDa (Fig. 1), giving rise to fractions eluted and retained (SK1000E and SK1000R, respectively). The fraction SK1000R demonstrated higher amounts of rhamnose (26.0%) than SK100E, together with arabinose (9.5%), mannose (12.0%) and galactose (52.5%), and formed very viscous solution. However, this fraction is still heterogeneous, having the presence of two peaks, which could not be separated, although many other purifications steps have been done.

To test if the mannogalactofuranan has immunogenic activity, this fraction was then used in biological assays using peritoneal macrophages in vitro. We analyzed macrophages morphological aspects, characterizing them into resident (Figs. 4A and 5A and B) or activated cells (Fig. 4B). The first are characterized by their small and dark nuclei, few citoplasmatic projections and poor spreading ability, while activated ones have large cytoplasmatic volume, bright and large nuclei and a spreading ability, as observed by various cytoplasmatic projections. After statistical analyzes, it was found that all tested concentrations of mannogalactofuranan induced macrophage activation (Fig. 4C). Approximately 63.9% of the cells incubated with 150 μg/mL of this polysaccharide were morphologically activated, a 60% increase in comparison to the control group. Analyses by SEM confirmed all concentrations tested alters macrophage morphologically (Fig. 5C and D). Macrophages were the target of our experiments since, in addition to the effectors role, they play a central role in the immune modulation. The intercommunication of innate response and adaptive immunity is achieved through macrophages [25]. Considering the macrophages activity, cellular morphology varies considerably with different functional states. As they pass from resident to activated cells, they enhance proliferation, modify their cell morphology acquiring properties such as spreading ability, phagocytosis and reactive species production (such as nitric oxide).

To the best of our knowledge, galactofuranan structures are unusual polysaccharides for algae. Instead, they are common polysaccharides of bacteria, protozoa and fungi, and are highly immunogenic in mammals [6]. In algae, these saccharides were found only in Trebouxia and Asterochloris [3,7], two genera of green algae that are symbionts of lichens. Which role this molecule plays in the lichen symbiosis is still unknown. It is known that lectins produced by the mycobiont and located at the fungal cell wall bind to specific ligands at the algal cell wall [26–28]. These lectins seem to be able to discriminate between compatible and incompatible

![Representative images from resident (white arrow) and activated (black arrow) peritoneal macrophages. (A) Resident macrophage morphology-control group and (B) activate macrophage morphology-treated group (10 μg/mL). Images observed in light microscopy (1000×).](image-url)
algae, thus acting as recognition proteins when the compatible alga
has a specific cell wall receptor, but producing deterioration and cell
dearth when the receptor is lacking [29,30]. Moreover, according to
Fontaniella et al. [31] the monosaccharide unit recognized by nat-
ural lichen lectins is galactose, although they did not distinguish
between Galp or Galf.

Moreover, this study enhances previous studies carried out on
polysaccharide chemistry of photobionts of lichens [3,7] which
observed that the Trebouxia and Asterochloris had different polysac-
charides. These results provide evidences that the determination of
the contents of polysaccharides of the photobionts could be utilized
as an alternative method for chemotyping, and could also be used
as a marker to aid algal symbiont taxonomy.

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