Galactomannoglucans of lichenized fungi of *Cladonia* spp.: significance as chemotypes

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Abstract

The chemical structures of the glucans, galactoglucomannans and galactomannoglucans of two species of the *Cladonia*, section Cocciferae, *Cladonia miniata* and *Cladonia salmonea*, were determined and compared. α-D-Glucans of the nigeran type were isolated from both species, in common with all *Cladonia* spp., along with galactoglucomannans containing (1→6)-linked main-chains of α-D-Manp units substituted by structurally different and typical side-chains. Isolated were previously unreported galactomannoglucans, with (1→3)-linked main-chains of β-D-Glcp units, substituted at O-2,6 by side-chains. These consisted of β-D-Gal, 6-O-substituted β-D-Galp and 2-O-, 4-O-, 6-O- and 2,3-di-O-substituted α-D-Manp units. According to 13C NMR spectroscopy, a similar galactomannoglucan was isolated from the *Cladonia* spp. *Cladonia signata, Cladonia crispatula, Cladonia penicillata, Cladonia imperialis, Cladonia clathrata, Cladonia connexa, Cladonia substellata* and *Cladonia ibitipocae*. Its presence could also contribute to the classic taxonomy of lichenized fungi. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The best known polysaccharides of lichenized fungi are lichenan, isolichenan and galactomannan, each of the latter having a range of different, but related, chemical structures depending on the species [1]: these are useful in chemotaxonomic studies [2,3]. These storage products have a fundamental role in the biochemistry of fungi and tend to be features conserved in their evolution. Some polysaccharides are taxonomically significant at the highest levels of classification [4]. In lichenized fungi, large genera are usually divided into subgenera and then again into sections, which group very closely related species. Currently, the characteristics that are used in classification are not sufficient to give rise to a correct taxonomy and the sectional division of the genus *Cladonia* is not fully established.
Nigeran, an α-glucan consisting of alternate (1→3)- and (1→4)-linkages, is of interest since it occurred in 10 Cladonia spp. investigated [5,6]. However, it also occurs in four Cladina spp. [5,7] and Flavoparmelia caperata, formerly Parmelia caperata [8].

All Cladonia spp. now examined are found to contain previously undetected galactomannoglucans, which are suggested as a useful chemical marker.

2. Materials and methods

2.1. Lichenized fungi

The Cladonia spp. Cladonia miniata G. Mey., Cladonia salmonia S. Stenroos, Cladonia connexa Vain., Cladonia crispata (Nyl.) Ahti, Cladonia ibitipoca Ahti and Stenroos, Cladonia penicillata Ahti and Marcelli, Cladonia substellata Vain., Cladonia signata (Eschw.) Vain., Cladonia imperialis Ahti and Marcelli and Cladonia clathrata Ahti and L. Xavier were collected in the State of Minas Gerais, Brazil, and are housed at Maria Eneyda P. Kau¡mann Fidalgo Herbarium, São Paulo, Brazil.

2.2. Isolation and purification of the nigerans from Cladonia spp.

Lichenized fungi (40 g) were successively extracted with CHCl3-MeOH, 80% aqueous MeOH (250 ml) and hot 2% aqueous KOH [9]. Resulting polysaccharides were dissolved in hot H2O, the solutions frozen and then thawed at 4°C [7], giving insoluble material. The process was repeated on the supernatants until a precipitate no longer appeared.

The insoluble materials were then extracted with hot aqueous 2% KOH and the neutralized supernatants (HOAc) were dialyzed against tap water. The freeze-thawing process was repeated three times to remove residual heteropolysaccharides. The final precipitated fraction was treated with 4% aqueous HOAc (10 ml) at 100°C for 2 h to remove possible protein aggregates. Dialysis was carried out against tap water for 2 days and the last freeze-thawing step performed to give purified nigerans.

2.3. Preparation of galactoglucomannans and galactomannoglucans

Galactoglucomannans were obtained from above combined supernatants, then treated with Fehling solution (300 ml) and the insoluble Cu complexes were isolated by centrifugation and converted to galactoglucomannans [10]. The supernatants were neutralized with HOAc, dialyzed against tap water and then deionized with mixed ion exchange resins. Solutions were evaporated to ~20 ml and the Fehling treatment was repeated. The resulting supernatant materials were dissolved in H2O and fractionally precipitated in the presence of 1.5% aqueous Cetavlon [11] with the initial pH of 7.0 and then at 8.5, 10.0 and 12.0 in the presence of sodium tetraborate. Precipitates were formed mainly at pH 7.0 and 8.5, the latter was isolated, the borate complexes were decomposed with 2 M HOAc and precipitated with excess EtOH to give material which was then subjected to the same Cetavlon procedure and dissociated with 0.2 M NaCl. The final galactomannoglucans were purified using a gel column of Sepharose CL-6B (46 cm×1.0 cm in diameter). It was eluted with H2O, with a flow rate of 1.0 ml min⁻¹.

2.4. Analysis of polysaccharides

Specific rotations, homogeneity and Mr determinations, methylation analyses, GC-MS determination of monosaccharide contents and partially O-methylated alditol acetates, Smith degradations both total and controlled and NMR spectroscopy were performed as previously described [6,12].

2.5. Partial acetolysis of galactomannoglucan

The polysaccharide from C. miniata was partially acetolized, the product deacetylated [13] and the mixture fractionated by PC on Whatman 3MM paper (solvent: n-BuOH-pyridine-H2O, 2:1:1, v/v). Six fractions were obtained and of these, four were mannose-containing and examination by 1H NMR and HMOC spectroscopy (30°C in D2O) showed typical 1H and 13C signals [14,15] of α-Manp-(1→2)-(α-Manp-(1→2))1-3-α-Manp. Anomeric signals
were at δ 103.15, 5.05 (non-reducing end-units), 101.5, 5.286 and 5.296 (internal units), and 93.3, 5.37 (reducing units), respectively. C-2 signals of 2-O-substituted units were at δ 79.6 (internal units) and 80.3 (reducing units). m/z values of molecular ions were determined by ESI-MS in the positive-ion mode as there forms Na⁺.

3. Results and discussion

Water-insoluble polysaccharides (yields ~0.1%) were obtained via alkaline extraction, followed by freeze-thawing. Gel permeation chromatography of those from *C. miniata* and *C. salmonea* showed that they had a *M*ₗ of 7.0 × 10⁴. ¹³C NMR spectra corresponded to those of nigeran [16].

The galactoglucomannans from *C. miniata* (yield 7.0%, [α]D +51.5°) and *C. salmonea* (yield 5.6%, [α]D +51.5°), *M*ₗ values 1.9 × 10⁶, and mannose, galactose and glucose in ratios of 65:21:14 and 62:28:10, respectively, were superficially similar, but ¹³C NMR spectra (Figs. 1A,B) showed small differences, although they were typical of the species. In general, we have found that the ¹³C NMR spectra are typical of the lichen species, to the extent that they were used for classification and identification [3].

The mother liquors, obtained after successive Fehling precipitations, were fractionated via Cetavlon precipitation and GPC, to give similar homogeneous polysaccharides (yields ~2%, *M*ₗ 1.7 × 10⁶). They had identical ¹³C NMR spectra (Fig. 2), [α]D values −3° and Man, Gal, Glc ratios of 48:40:10 and 43:47:10, respectively. Controlled Smith degradations gave rise to the main-chains of the polysaccharides, whose monosaccharide composition and ¹³C NMR spectra (Fig. 2C) corresponded to that of a (1→3)-linked β-glucan [17].

The ¹³C NMR spectra (Fig. 2) of the heteropolysaccharides each showed a complex side-chain structure with β-Gal/C-1 signals at δ 106.3–108.4 [18]. Methylation analysis showed that the side-chains were highly branched. The main structures were non-reducing end-units of Manp (6%), Galp (10%) and Galf (16%), with 6-O-substituted Galp (5%), 2-O- (19%), 4-O- (8%), 6-O- (8%) and 2,3-di-O-substituted Manp (5%) and 2,3,6-tri-O-substituted Glcp units (10%). Partial acetylation of the polysaccharide from *C. miniata*, followed by deacetylation, gave rise to α-Manp-(1→2)-(α-Manp-(1→2))ₙ₋₃-α-Manp, characterized by their ¹H and ¹³C NMR spectra. These represent the core of the branched side-chains.

The galactomannoglucans of *C. salmonea* and *C. miniata* gave identical ¹³C NMR spectra (Fig. 2), which were in turn similar to those of *C. signata,*...
Fig. 2. $^{13}$C NMR spectrum (D$_2$O; chemical shifts in $\delta$, PPM) of the galactomannoglucans obtained from *C. miniata* (A), *C. salmonca* (B) and the polysaccharide obtained via Smith degradation and partial hydrolysis of *C. miniata* (C).
C. crispulata, C. penicillata, C. imperialis, C. clathrata, C. connexa, C. substellata and C. ibitipocae. Only galactans are present in Cladina stellaris and Cladina confusa [7]. These data show that galactoglucomannans are chemotypes which could have a significant influence in aiding the taxonomy of Cladonia spp. and those of related genera.

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References