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Carbohydrate Polymers 49 (2002) 225–230

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Structures of phosphonogalactomannans isolated from mycelia of *Aspergillus versicolor*

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Received 6 February 2001; revised 22 June 2001; accepted 16 August 2001

Abstract

The phosphonogalactomannans of *Aspergillus versicolor* mycelia of various ages were isolated via hot alkaline extraction followed by gel filtration chromatography. Their complex structures were determined mainly by ^{31}P, ^{1}H, and ^{13}C NMR spectroscopy (400 MHz in respect to proton), and methylation analysis. The polysaccharides contained 2-*O*-, 6-*O*-, and 2,6-di-*O*-substituted α-mannopyranosyl units, principally in the core, and these were substituted at O-2 and O-6 with side chains of (1 → 5)-linked β-galactofuranosyl units, which became shorter with increasing culture age. NMR data suggested that phosphorodiester groups were present principally as bridges between C-1 of units of α-mannopyranose and C-6 of those of 5-*O*-substituted β-galactofuranose. Present in earlier stages of growth were very small amounts of α-mannopyranosyl and/or β-galactofuranosyl nonreducing end-units phosphorylated at HO-6. Also 6-phosphorylated were 2-*O*-substituted β-galactofuranosyl-(1 → groups involved in phosphorodiester bridges. Each phosphorylated structure was from side chains and was gradually removed with culture age. The youngest mycelial preparation contained small amounts of glycogen.

Keywords: *Aspergillus versicolor*; Mycelia; Phosphonogalactomannans; NMR; ^{13}C,^{31}P coupling; Structure

1. Introduction

The mycelia of *Aspergillus niger*, other *Aspergillus* spp. (Barreto-Bergter, Travassos & Gorin, 1980), and *Aspergillus fumigatus* (Barreto-Bergter, Gorin & Travassos, 1981a) contain galactomannans. Extracts were analysed by methylation and ^{13}C NMR spectroscopy at 25 MHz and found to contain an α-D-mannan core and side chains of (1 → 5)-linked β-D-galactofuranosyl units, which became shorter with increasing culture age. A polysaccharide now isolated from another species, *Aspergillus versicolor*, appears to have a similar structure, except for the presence of phosphorodiester groups. Accordingly, galactomannans from 5-, 15-, and 30-day old mycelia were isolated, of which the structures of the 5- and 30-day polysaccharides were analysed in more detail.

2. Experimental

2.1. Microorganism and growth conditions

A. versicolor, strain 550, was obtained from the Mycology Sector of the Escola Paulista de Medicina (UNIFESP), São Paulo, State of São Paulo, Brazil. Hyphae were grown at 25°C with shaking in Erlenmeyer flasks (3 l) in Sabouraud medium containing (g l^{-1}): Difco peptone, 10; Difco yeast extract, 5; Difco agar, 10; glucose, 40. They were filtered off, washed with water, and maintained in a frozen state.

2.2. Isolation and purification of polysaccharides

Each sample of thawed 5-, 15- and 30-day old mycelia (200 g) was extracted with 6% KOH in H_2O (600 ml) for 6 h at 100°C. The suspension was then neutralised (HOAc), filtered, the filtrates concentrated to a small volume, and the polysaccharides precipitated by addition of 3 vols. of EtOH. These were filtered off and dried (yields: ~100 mg). The products were deionised with a mixed aqueous suspension of Amberlite 120 (H^+ form) and Dowex 1 × 8 (OAc^- form), again precipitated with EtOH,

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0144-8617/02/$ - see front matter
PII: S0144-8617(01)00325-3

and then applied to a column of Sephacryl S-300 (100 × 0.9 cm i.d.), previously calibrated with blue dextrans of different average molecular weights. It was eluted with 0.05 M NaOAc at a flow rate of 8 ml h^{-1} and fractions of 2 ml were collected and each one was assayed for carbohydrate by the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The polysaccharides from 5- and 30-day mycelia gave single peaks, distinct from the void volume.

2.3. Analytical procedures

2.3.1. General analyses

Total carbohydrate contents of the polysaccharides were determined by the method of Dubois et al. (1956), phosphate by that of Ames (1966) and hexosamine by that of Belcher, Nutten and Sandbrook (1954). Specific rotations were measured using 1% aqueous solutions at 25°C.

2.3.2. Identification and quantification of monosaccharides in polysaccharide samples

Polysaccharide (~1 mg) was hydrolysed with 0.5 M H_2SO_4 for 18 h at 100°C, the solution neutralised ($BaCO_3$), filtered, and the filtrate evaporated to dryness. The residue was successively reduced with aq. $NaBH_4$ and acetylated with Ac_2O–pyridine, and the resulting mixture of alditol acetates was examined by GC-MS on a capillary column of OV-225 (30 m × 0.25 mm i.d.), programmed from 50°C (40°C min^{-1}) to 220°C, then hold.

2.3.3. Methylation analysis

Polysaccharide (~5 mg) was methylated three times by the procedure of Ciucanu and Kerek (1984), being agitated vigorously with a Vortex–Genie (Scientific Instruments Inc., Massachussets) for 1 h in a mixture of DMSO (1 ml), MeI (0.20 ml), and powdered NaOH (80 mg). After leaving overnight, the solution was neutralised (HOAc) and dialysed. The fully *O*-methylated product was hydrolysed with 4 M TFA at 100°C for 4 h, the solution evaporated to dryness and the resulting mixture of *O*-methylaldoses reduced with aqueous NaB_2H_4. The product was then acetylated with Ac_2O–pyridine (1:1, v/v).

The mixture of partially *O*-methylated alditol acetates was examined by GC-MS with a capillary OV-225 column with the same dimensions and temperature programming as described above. It was also similarly examined with a capillary column of DB-210, in order to overcome the problem of overlapping peaks.

2.3.4. ^{31}P-, ^{1}H-, and ^{13}C NMR spectroscopy

All solutions of polysaccharide (50 mg) were in D_2O at pD 7.0 and were examined using a Bruker DRX 400 MHz NMR spectrometer with a 5 mm multinuclear probe as follows:

^{31}P: temperature 30°C; 30° pulse with proton decoupling; pulse interval, 1.00 s; acquisition time, 0.64 s; no. transients, 264.

^{1}H: temperature 60°C with presaturation; 90° pulse; pulse interval, 1.00 s; acquisition time, 1.99 s; no. transients 4.

^{13}C: temperature 30°C; 30° pulse; pulse interval, 1.0 s; acquisition time 1.03 s, no. transients 16,000–25,000.

DEPT: 135° pulse, other conditions as for ^{13}C spectra.

^{1}H (observe),^{13}C HMQC: temperature 60°C with presaturation, ^{13}C values corrected to those at 30°C. The analyses were carried out according to our Bruker manual BIRD sequence, phase sensitive using TPPI with decoupling during acquisition (Bax & Subramanian, 1986).

δ_C and δ_H resonance values are downfield relative to Me_4Si ($\delta = 0$), obtained in a separate experiment and those of δ_P are to orthophosphoric acid ($\delta = 0$).

2.3.5. Degradation of 30-day galactomannan to its main chain

The galactomannan was partially hydrolysed and then treated with an *exo*-α-mannosidase under previously described conditions (Gorin, Spencer & Eveleigh, 1969), except that the enzyme from jack bean was employed instead of that of *Arthrobacter* GJM-1. The H-1 region of the ^{1}H NMR spectrum of the product was examined.

3. Results and discussion

Preliminary experiments were carried out on galactomannans of mycelia of *A. versicolor*, isolated after 5 and 30 days of growth in shake culture. These were obtained via extraction with hot aqueous potassium hydroxide followed by neutralisation with acetic acid, and then precipitation with ethanol. Each preparation was chromatographed on a column of Sephacryl S-300, using water as eluant, and gave symmetrical single peaks in the range 30–80 kD (this step removed inorganic phosphate). The 5-day galactomannan ($[\alpha]_D = -8°$) contained 92% carbohydrate with mannose, galactose, and glucose in a molar ratio of 25:67:7 and a trace (0.6%) of hexosamine. The 30-day polysaccharide ($[\alpha]_D = -14°$) contained 96% carbohydrate with a monosaccharide ratio of 53:46:1 and a trace (0.5%) of hexosamine.

Phosphate was present in 5- and 30-day polysaccharides, but its content diminished from 10.2 to 3.3%. It was present as esters linked to polysaccharide, as shown by the above method of preparation. The ^{31}P NMR spectra (30°C, pD 7.0) each contained two signals with δ 1.70 and 2.95, with respective relative areas of 3.4:1 (5-day) and 3.5:1 (30-day). These arose from phosphorodiester groups, since these shifts are closest to those found at pD 6.85 and 27°C by Costello, Glonek, Slodki and Seymour (1975), whose values were: orthophosphate diesters from δ 1.04 to 2.00, in contrast with orthophosphate monoesters (δ −4.54 – − 3.90) and absent inorganic orthophosphate (δ −2.00).

Many aspects of the overall structures of the 5- and 30-day

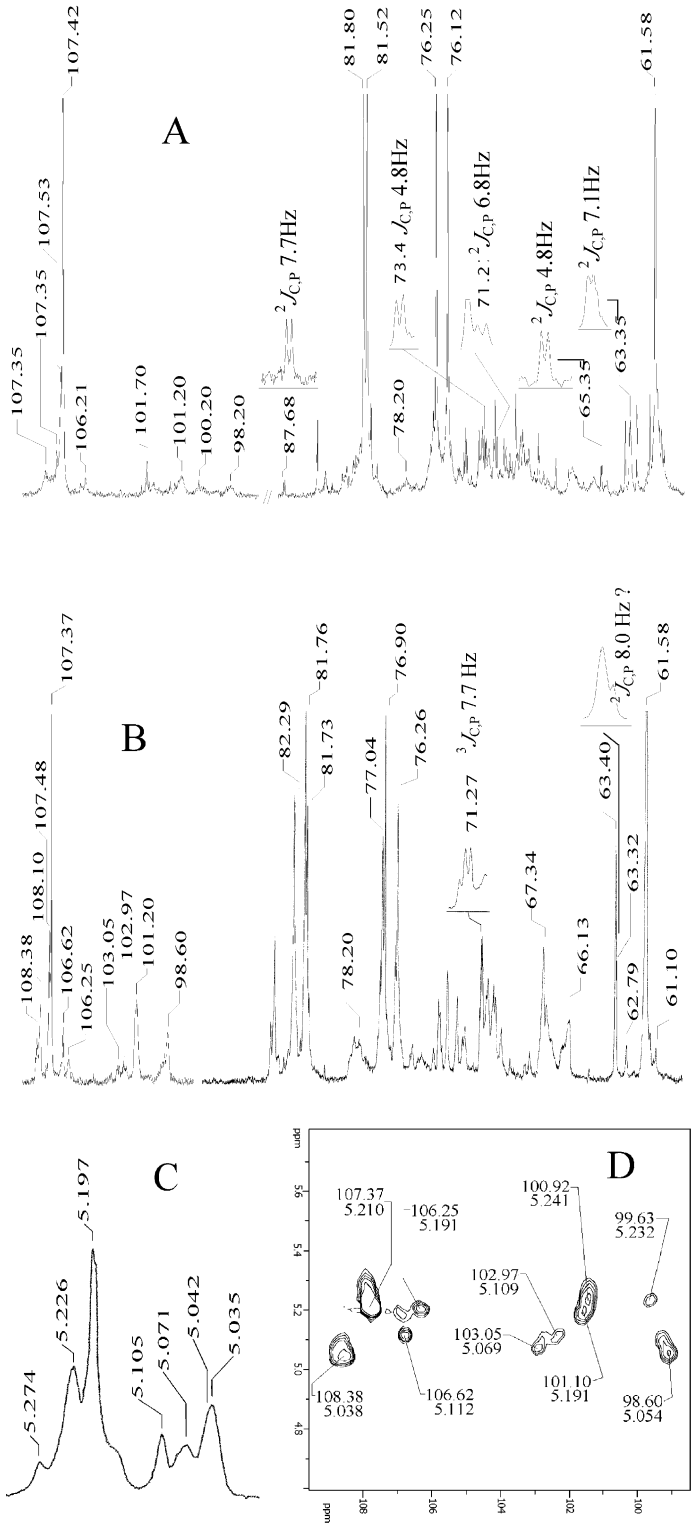


Fig. 1. ^{13}C NMR spectra of the phosphonogalactomannans in D_2O, isolated from 5-day (A) and 30-day mycelia (B). Anomeric regions of ^1H (C) and $^1H,^{13}C$ HMQC spectra (D) of 30-day polysaccharide.

polysaccharides were determined employing their very complex monodimensional ^{13}C NMR spectra. That of 5 days (Fig. 1(A)) contained very large signals of side chains of β-Gal*f* units, which became progressively smaller for those of 15- (not shown) and 30-day galactomannan (Fig. 1(B)), indicating gradual removal of side chains. Each respective C-1 region contained signals with shifts shown in Figs. 1(A) and (B) and Table 1. The two largest β-Gal*f*

Table 1
^{13}C chemical shifts.of structures present in the phosphonogalactomannans, not involved with phosphorodiester linkages

Structure	δ (ppm)
β-Gal*f*-(1 → 5)-β-Gal*f*-(1 → 5)-	107.53 (C-1)
β-Gal*f*-(1 → 5)-β-Gal*f*-(1 → 5)-β-Gal*f*-	107.42 (C-1)
-β-Gal*f*-(1 → 6)-α-Man*p*-	108.00, 108.10, 108.38 (C-1)
-β-Gal*p*-(1 → 2)-α-Man*p*-	106.62 (C-1)
β-Gal*f*-(1 → 6)-α-Man*p*-	68.5 (C-6)
α-Man*p*-(1 → 2)-α-Man*p*-	~ 101.1 (C-1), ~78.2 (C-2)
α-Man*p*-(1 → 6)-[α-Man*p*-(1 → 2)]-α-Man*p*-	98.60 (C-1), ~78.2 (C-2), 66.7 (C-6)
-α-Glc*p*NAc-	61.10 (C-6)

signals of C-1 from the 5-day galactomannan (Fig. 1(A)) were from internal units of (1 → 5)-linked side-chains, being at δ 107.42 (~48%, based on total area of C-1 signals) and those of nonreducing end-units at δ 107.53 (~8%) (Gorin & Mazurek, 1976), corresponding to a side-chain length of ~7 units. The spectrum of the 30-day polysaccharide (Fig. 1(B)) indicated respective area values of 25 and 12%, indicating side chains of ~3 units. This was accompanied by an increase in the relative sizes of the Man*p* C-1 signals at δ ~98.2–98.6 and ~101.2.

The ^{13}C NMR spectrum of the 30-day galactomannan (Fig. 1(B)) was still dominated by β-Gal*f* signals. Apart from the main C-1 signals of β-Gal*f* units at δ 107.37 being greater than that at δ 107.48 (shoulder), smaller ones were present at δ 106.25 (4% of total C-1 signals), 106.62 (6%), 108.00 (4%), 108.10 (4%), and 108.38 (7%). In view of the chemical shifts of methyl β-D-galactofuranosyl-α-D-mannopyranosides obtained at 70°C (Barreto-Bergter, da Cruz & Gorin, 1981b), and now corrected to 30°C values), the signal at δ 106.62 should arise from β-Gal*f* linked to O-2 of the α-Man*p* core, while those at δ 108.00, 108.10, and 108.38 should be from those linked to O-6. Similar signals were probably not previously resolved at 25 MHz, when galactomannan-containing extracts of other *Aspergillus* spp. were examined (Barreto-Bergter et al., 1980, 1981a).

The two C-1 signals of Man*p* units were broad and irregular, consisting of more than one component. That at δ ~101.2 (20% of total C-1 area) should be partly due to α-Man*p*-(1 → 2)-α-Man*p*-, while the other at δ ~98.6 (17%) is consistent with some α-Man*p* residues substituted at O-2,6 with α-Man*p*. A broad signal at δ ~ 78.2 is typical of C-2 of α-Man*p*-(1 → 2)-α-Man*p*- structures (Gorin, 1973) This signal does not represent substitution at HO-2 with β-Gal*f* units since the C-2 resonance of Me β-D-Gal*f*-(1 → 2)-α-D-Man*p* should be at δ 76.0 (Gorin, 1973).

On examination of its C-6 signals, a DEPT NMR spectrum of the 30-day polysaccharide contained seven inverted signals of C-6 at δ 61.1, 61.5, and 62.8, one at δ 63.40 greater than 63.32 (shoulder), 66.7, and 68.3. The signal at δ 66.7, when compared with the unsubstituted resonance of α-Man*p* at δ 61.5 suggests an α-shift of 5.2 ppm, close to 4.75 ppm found for the mannan of Baker's yeast and which arises from α-Man*p*-(1 → 6)-α-Man*p*- groups. The signal at δ 68.5 should have arisen from β-Gal*f*-(1 → 6)-α-Man*p*-groups, since Me β-Gal*f*-(1 → 6)-α-Man*p* gives signals at δ 63.9 (C-6′), 68.0 or 68.1 (C-6) (Barreto-Bergter et al., 1981b) [our values corrected to those at 30°C; one of these could be from C-6 as Me α-Man*p* has a C-4 resonance at δ 68.0 (Gorin & Mazurek, 1975)]. Also, Me β-D-Gal*f*-(1 → 2)-α-D-Man*p* has signals at δ 63.8 (C-6′), 61.9 (C-6), and a C-4 signal at δ 68.4.

A very small C-6 signal at δ 61.1 was detected in the NMR spectrum of the 30-day polysaccharide (Fig. 1(B)). This corresponds to units of α-hexosamine (0.5%), which should have a C-6 resonance at higher field than that of α-mannopyranose (Bock & Pedersen, 1983).

The ^{13}C chemical shifts arising of the above-described structures are summarised in Table 1.

As a basis of 2D correlation experiments, the ^{1}H NMR spectrum of the 30-day galactomannan was obtained at 400 MHz and 30°C. Its H-1 region (Fig. 1(C)) was complex with seven signals at δ 5.035, 5.042 (shoulder), 5.071, 5.105, 5.197 (largest), 5.226, and 5.274, corresponding to units of β-Gal*f* and α-Man*p*. Their broadness did not allow detection of $^{3}J_{\text{H-1,P-31}}$ coupling of 6–7 Hz (Costello et al., 1975) arising from phosphorodiester. The overall signal resolution was better in its ^{1}H, ^{13}C HMQC spectrum (Fig. 1(D)), which contained a surprising 15 signals, only the 10 major ones being shown. Of the α-Man*p* ^{13}C signals, the broad one at δ ~ 101.2 (2-*O*-subst.; Fig. 1(B)) arose at δ 101.1 and correlated with the H-1 signal at δ 5.19., while the composite one at δ ~ 98.6 (partly 2,6-di-*O*-substituted) correlated with a main H-1 signal at δ 5.05. Our relative H-1 values for the 2-*O*- and 2,6-di-*O*-substituted structures were similar to signals at δ 5.62 and 5.80, obtained under different conditions (Gorin, Mazurek & Spencer, 1968).

The structures of the phosphorodiester bridges were indicated by the presence of symmetrical or potentially symmetrical doublets. Some of these could be assigned by comparison with spectra of α-D-Man*p*-1-phosphate and αβ-D-Man*p*-6-phosphate.

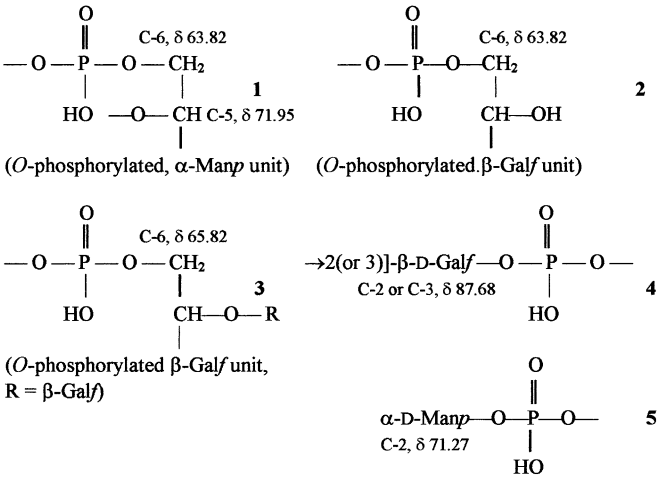
The spectrum of the 5-day polysaccharide (Fig. 1(A)) contained a C-6 signal at δ 63.82 ($^{2}J = 7.8$ Hz), corresponding to that of αβ-Man*p*-6-phosphate at δ 63.7. However, the C-5 signal of the polysaccharide (δ 71.95, $^{3}J = 6.8$ Hz), comparable to that of α-Man*p*-6-phosphate (δ 72.16, $^{3}J = 7.7$ Hz), was much smaller. Thus, the major contribution to the C-6 signal at δ 63.82, must have been from β-Gal*f* units substituted at HO-5 with other β-Gal*f* residues and also phosphorylated at C-6, showing an α-phosphorylation shift of 1.7 ppm from δ 61.58. These data indicate structures **1** and **2**, respectively. The progressive degradation of the side chains was indicated by small signals present in Fig. 1(A), but not in Fig. 1(B). These are at δ 65.82, arising from HO-6 phosphorylation of nonreducing β-Gal*f* end-units (**3**) and at δ 87.68 ($^{3}J = 7.7$ Hz),

Table 2
Molar percentages of *O*-methyl alditol acetates obtained on methylation analysis of the polysaccharide extracts of *A. versicolor*

O-Methyl alditol acetate	Molar % of *O*-methyl alditol acetate[a]		
	5-Day-old mycelia	15-Day-old mycelia	30-Day-old mycelia
2,3,4,6-Me_4-Man	3	4	7
2,3,5,6-Me_4-Gal	17	21	30
3,4,6-Me_3-Man	11	13	17
2,3,6-Me_3-Gal	47	37	16
2,3,6-Me_3-Glc	4	–	–
2,3,4-Me_3-Man	11	15	17

[a] Peak areas (mole % of total) of *O*-methylalditol acetates, as averages of values obtained with columns of OV-225 and DB-225.

consistent with 2-*O*- or 3-*O*-substituted β-Gal*f* units (**4**), with their unsubstituted resonances suffering strong downfield α-shifts from δ 81.9 and 77.8, respectively (Gorin & Mazurek, 1975).



The spectrum of the 30-day polysaccharide (Fig. 1(B)) contained fewer assignable, coupled signals, although a large one at δ 71.27 ($J = 7.7$ Hz) was present. This corresponds to the C-2 signal of α-Man*p*-1-phosphate (δ 71.57, $^3J = 6.8$ Hz), as in structure **5**, rather than the above-mentioned C-6 from structure **1**. Furthermore, the *O*-phosphorylated C-6 signal at δ 63.36 was poorly defined, due to the appearance of an overlapping one at δ 63.40.

Other ^{13}C,^{31}P-coupled signals may be present in Figs. 1(A) and (B), but would not be symmetrical due to overlap with another signal. Candidates are present in Fig. 1(A) at δ 107.35, 101.20, the complex 69–74 region, and 63.35 and in Fig. 1(B) at δ 103.05, 102.97, 101.20, ~98.6, 73.80, and 63.36. However, attempts to confirm such coupling via ^1H, ^{31}P decoupled spectra have not yet been successful.

Further attempts to investigate the linkages of β-Gal*f* to α-Man*p* units in the galactomannan were frustrated by the broadness and the complexity of its COSY, TOCSY, ROESY resonances, the latter having many correlations other than those across the glycosidic bonds, and the lack of interpretable correlations in its HMBC spectrum.

The 30-day galactomannan was subjected successively to partial hydrolysis and treatment with the *exo*-α-mannosidase from jack bean. The product gave rise to a proton NMR spectrum with a principal H-1 signal at a relatively high field of δ 4.83, showing that its main chain consisted mainly of (1 → 6)-linked α-Man*p* units (Gorin et al., 1969).

Methylation data of the galactomannans showed an increase in nonreducing end-units compared with 5-*O*-substituted units of Gal*f* (Table 2) over a time period, which agrees with present NMR and previous results on *Aspergillus* spp. (Barreto-Bergter et al., 1980, 1981a; Johnston, 1965) in that gradual enzymic removal of the galactosyl side-chains occurs. Further NMR examination now shows the removal of C-1 phosphorylated moieties, as in structures **1**, **2** or **3**, and **4**. Another time-controlled parameter is the presence of glycogen, which was only detected in the 5-day polysaccharide by the methylation data (4% of 4-*O*-substituted glucopyranosyl units) and its typical blue-brown coloration with iodine. As well as the presence of nonreducing end- and 5-*O*-substituted units of Gal*f*, the methylation results indicated that of nonreducing end- and 2-*O*-, 6-*O*-, and 2,6-di-*O*-substituted Man*p* units, principally of the mannan core.

Acknowledgements

The authors wish to thank Ms Maria de Fatima Ferreira for technical assistence and the Brazilian agencies, CNPq, FINEP-BID, UFRJ, FAPERJ, and PRONEX-CARBOIDRATOS for funding.

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