Structural differences between the alkali-extracted water-soluble cell wall polysaccharides from mycelial and yeast phases of the pathogenic dimorphic fungus *Paracoccidioides brasiliensis*

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*Paracoccidioides brasiliensis* is a pathogenic dimorphic fungus causing paracoccidioidomycosis, the most widespread systemic mycosis in Latin America. We have studied the structure of the alkali-extracted water-soluble cell wall polysaccharides (F1SS) from both mycelial and yeast phases of this fungus by using chemical analysis and NMR spectroscopic techniques. The F1SS polysaccharide from the mycelial phase consists of a trisaccharidic repeating unit of \((\alpha\text{-Gal} \rightarrow (1 \rightarrow 6) \leftarrow \alpha\text{-Manp} \rightarrow (1 \rightarrow 2) \leftarrow \alpha\text{-Manp} \rightarrow (1 \rightarrow )\). The F1SS polysaccharide of the yeast phase maintains 10% of the structure of the mycelium phase, but the main structure contain a disaccharide repeating unit of \((\alpha\text{-Gal} \rightarrow (1 \rightarrow 6) \leftarrow \alpha\text{-Manp} \rightarrow (1 \rightarrow ))\), alternating with a trisaccharide repeating block of \((\alpha\text{-Gal} \rightarrow (1 \rightarrow 6) \leftarrow \alpha\text{-Manp} \rightarrow (1 \rightarrow ))\).

**Key words:** cell wall polysaccharides/fungi/NMR spectroscopy/*Paracoccidioides brasiliensis*

### Results

**Polysaccharide F1SS from the mycelial phase**

The analyses of the crude alkali-extractable water-soluble cell wall polysaccharidic material from the mycelial phase of *P. brasiliensis* gave glucose (10%), galactose (30%), and mannose (60%). After purification through Sepharose CL-6B, the main component (polysaccharide F1SS) consisted of mannose (70%) and galactose (30%), as shown by gas-liquid chromatography (GLC) of the alditol acetates. The absolute configuration was shown to be D for both sugars (Gerwig et al., 1979). Methylation analysis allowed to deduce the linkage types summarized in Table I. Both polysaccharides are polydisperse, with an average molecular mass around 70 kDa as determined by gel permeation chromatography on Sepharose CL-6B.

### Table I. Linkage types (%) deduced from methylation analyses of the polysaccharides F1SS of the mycelial and yeast phases of *P. brasiliensis*

<table>
<thead>
<tr>
<th>Linkage type</th>
<th>Mycelial (%)</th>
<th>Yeast (%)</th>
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</thead>
<tbody>
<tr>
<td>Manp-(1→)</td>
<td>27.3</td>
<td>31.7</td>
</tr>
<tr>
<td>Galp-(1→)</td>
<td>27.3</td>
<td>30.6</td>
</tr>
<tr>
<td>→2-Manp-(1→)</td>
<td>9.3</td>
<td>10.1</td>
</tr>
<tr>
<td>→6-Manp-(1→)</td>
<td>28.7</td>
<td>29.7</td>
</tr>
<tr>
<td>→2,6-Manp-(1→)</td>
<td>35.0</td>
<td>35.0</td>
</tr>
</tbody>
</table>
The $^1$H-$^n$uclear magnetic resonance (NMR) spectrum contained three anomeric signals at 5.08, 5.04, and 5.02 ppm (Figure 1a) in the proportion 1:1:1, as deduced from integration. The corresponding residues were labeled A–C, from low to high field. A and C appeared as slightly broad singlets ($J_{1,2} \leq 2$ Hz), and B exhibited a clear doublet, $J_{1,2} = 4.4$ Hz, that could be indicative of a galactofuranose with α-configuration (see also the $^{13}$C δ information later and compare with β-configuration, $J_{1,2} \approx 2$ Hz) (Cyr and Perlin, 1979).

The $^{13}$C-NMR spectrum (Figure 1b) showed 18 singlets, 3 of them anomeric, at 103.0, 101.2, and 99.0 ppm. A series of 2D homo-NMR (double quantum filtered homonuclear correlated spectroscopy [DQCOSY], total correlation spectroscopy [TOCSY]) and hetero- (heteronuclear multiple-quantum coherence [HMQC], heteronuclear single quantum coherence [HSQC]-TOCSY) NMR experiments allowed the assignment of all the proton and carbon signals of the three residues (see Table II). Comparison of the chemical shifts values with those reported by Bock and Pedersen (1983) permitted us to deduce the glycosylation positions.

Thus, A is 2,6-di-O-substituted mannopyranose; B, terminal galactofuranose; and C, 6-O-substituted mannopyranose, which is in accordance with the methylation results.

Concerning the anomeric configuration of the mannose residues, a carbon-coupled HMQC experiment allowed the measurement of their $^1J_{C-1,H-1}$ values, giving 173 Hz for units A and C, which are indicative of α-configurations for the mannose residues (Bock and Pedersen, 1974).

To find the connections among residues, we performed a heteronuclear multiple bond correlation (HMBC) experiment, which gives cross-peaks between a proton and the carbons placed at two or three bonds from it. In addition to expected intraring connections, peaks corresponding to H-1A/C-6A’, H-1B/C-6C, and H-1C/C-2A could be observed. We denote a second unit of A as A’.

The NMR spectral data, together with those of the methylation analyses, suggest the following main structure for the cell wall polysaccharide of the mycelial form of *P. brasiliensis*.

The small quantities (<10%) of terminal and 2-O-substituted mannopyranoses observed in the methylation analyses were not detected in the NMR spectra. Therefore, to further investigate those minor components, we selectively hydrolyzed the galactofuranose residues, taking advantage of the lability of the glycosyl linkages of the furanoid rings, as compared with those of the pyranoid rings. Treatment of the polysaccharide with 0.05 M sulfuric acid gave a new polysaccharide composed exclusively of mannose. Methylation analysis gave terminal manno-pyranose (34%); 2-O-substituted, 3-O-substituted, and 6-O-substituted mannopyranoses (23.5%, 1.1%, and 9%, respectively); and 2,6-di-O-substituted mannopyranose.

![Fig. 1](https://example.com/image1.png)

**Fig. 1.** (a) $^1$H-NMR (500 MHz) and (b) $^{13}$C-NMR (125 MHz) spectra in D$_2$O at 40°C for the cell wall F1SS polysaccharide from the mycelial form of *P. brasiliensis*. The anomeric protons have been labeled A–C.

![Fig. 2](https://example.com/image2.png)

**Fig. 2.** (a) $^1$H-NMR (300 MHz) and (b) $^{13}$C-NMR (75 MHz) spectra in D$_2$O at 40°C for the partially hydrolyzed mycelial form of *P. brasiliensis*.

### Table II. $^1$H- and $^{13}$C-NMR chemical shifts (δ) for the alkali-extractable water-soluble cell wall polysaccharide F1SS isolated from the mycelial form of *P. brasiliensis*

<table>
<thead>
<tr>
<th>Units</th>
<th>Proton or Carbon</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
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<tr>
<td>A</td>
<td>H</td>
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<td>C</td>
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<td>B</td>
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<td>C</td>
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<td>C</td>
<td>C</td>
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Numbers in boldface type represent glycosylation sites.
The analysis of the alkali-extractable water-soluble yeast cell wall polysaccharidic material from the yeast form gave mannose (67.9%), galactose (22.1%), and glucose (9.9%). After purification through Sepharose, the main component (polysaccharide F1SS) gave mannose (77%) and galactose (23%). The absolute configuration was shown to be D for both sugars. Methylation analysis led to the results gathered in Table I. Numbers in boldface type represent glycosylation sites. These values may have to be interchanged within each respective row.

### Table III. 1H- and 13C-NMR chemical shifts (δ) for the alkali-extractable water-soluble cell wall polysaccharide F1SS isolated from the yeast form of *P. brasiliensis*.

<table>
<thead>
<tr>
<th>Units</th>
<th>Proton or Carbon</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
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<tr>
<td>D</td>
<td>H</td>
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<td>C</td>
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<td>C</td>
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</table>

Numbers in boldface type represent glycosylation sites.

In addition, a small proportion of short chains of (1→2) linked mannopyranoses is deduced from the methylation analysis, analogously to that observed in the mycelial phase.

### Discussion

*P. brasiliensis* grows as filamentous fungus when cultivated at 23°C and is easily converted to the yeast phase by simply changing the temperature to 37°C. This process is...
reversible, as demonstrated by Nickerson (1948), who also showed that this reversibility was exclusively due to the temperature factor and was independent from other changes in the culture medium.

It has been shown (Kanetsuna and Carbonell, 1970; Kanetsuna et al., 1969) that both yeast and mycelial forms have chitin as a common structural polysaccharide, but an α-(1→3) glucan was found in the yeast phase, whereas a β-(1→3) glucan was encountered in the mycelial form. This difference has been suggested as a possible contribution to the distinct morphology of both forms (San Blas and San Blas, 1994). However, a study on the structure of glucans and F1SS polysaccharides in *Eupenicillium*, *Penicillium*, and *Talaromyces* species grown at 25°C (Leal and Bernabé, 1998) revealed that the mycelium from these microorganisms contained either α-(1→3) or β-(1→3) glucans, regardless of their common mycelial shape. On the other hand, studies on mutants of *P. brasiliensis* have suggested a direct relationship between virulence and the presence of variable amounts of α-(1→3) glucan in the cell walls of the mutant strains (San Blas, 1982 and references therein). Major quantities of this glucan determine enhancement of the virulence, whereas a lower amount of the glucan results in a decreased virulence. This behavior has been explained as the result of α-glucan working as a protection mechanism of the fungus against host defences (San Blas, 1982).

Structural variations have also been observed by Mendonça et al. (1976) in the alkali-extractable cell wall polysaccharides from both morphological types of the dimorphic fungus *Sporotrix schenckii*. Nevertheless, they dissociated the effect of the temperature on morphological phase transition because 100% yeast was obtained in a synthetic medium either at 25°C or 37°C. Therefore they concluded that the variations in the structure of the polysaccharides observed must be due to differences in the morphology of both phases and not to modification on growth temperatures.

Nickerson (1948 and references therein) attributed the changes in morphology to reversible denaturation or activation of enzyme processes due to changes in temperature. In this context, it has been shown that exogenous cAMP inhibits the yeast to mycelial transitions, thus favoring the pathogenic yeast form (Borges-Walmsley et al., 2002).

It is not currently possible to interpret the meaning and evaluate the importance of the structural modifications of F1SS polysaccharides observed in the transition from the mycelial to the yeast phase of *P. brasiliensis*. The antigenic relevance of the β-galactofuranose domains of polysaccharides from several pathogenic fungi (Latgé et al., 1991; Notermans et al., 1988; Azuma et al., 1974) is known, yet little or nothing is known of the role of α-galactofuranoses because these residues have only been described in cell wall polysaccharides from a few species belonging to *Onygenales* (Bernabé et al., 2002).

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**Material and methods**

**Strains and growth conditions**

*P. brasiliensis* strain IVIC Pb73 (ATCC 32071) was grown in peptone yeast glucose medium and incubated at either 23°C (mycelium form) or 37°C (yeast form) with continuous shaking.

**Wall material preparation and fractionation**

Cell walls from *P. brasiliensis* were prepared according to Kanetsuna et al. (1969). Fractions and polysaccharides F1SS were obtained and purified following Ahrazem et al. (2000b).

**Chemical analysis**

For analysis of neutral sugars the polysaccharides were hydrolyzed with 3 M trifluoroacetic acid (TFA) (1 h at 121°C), converted into their corresponding alditol acetates (Laine et al., 1972), and identified and quantified by GLC using a SP-2380 fused silica column (30 m × 0.25 mm ID × 0.2 μm film thickness) with a temperature program (210°C to 240°C, initial time 3 min, ramp rate 15°C min⁻¹, final time 7 min) and a flame ionization detector.

The monosaccharides released after hydrolysis were derivatized according to Gerwig et al. (1979) and their absolute configuration was determined by gas chromatography mass spectrometry of the tetra-O-TMSi-(+)-2-butylglycosides obtained.

**Methylation analyses**

The polysaccharides (1–5 mg) were methylated according to the method of Ciucanu and Kerek (1984). The methylated material was treated and processed according to Ahrazem et al. (2000b), with the exception that the partially methylated polysaccharide was hydrolysed with TFA 1.5 M (100°C, 30 min).

**Partial hydrolysis of the polysaccharide F1SS from the mycelial phase**

Eighty milligrams of the polysaccharide were hydrolyzed as described by Prieto et al. (2001).

**NMR analysis**

1D and 2D 1H- and 13C-NMR experiments were carried out at 40°C on a Varian Unity 500 spectrometer with a reverse probe and a gradient unit or a Varian INOVA-300 spectrometer (1H, 300 MHz). Proton chemical shifts refer to residual HDO at δ 4.61 ppm. Carbon chemical shifts refer to internal acetone at δ 31.07 ppm. The polysaccharides F1SS (~20 mg) were dissolved in D₂O (1 ml) followed by centrifugation (10,000 x g, 20 min) and lyophilization. The process was repeated twice, and the final samples were dissolved in D₂O (0.6 ml, 99.98% D).

The 2D NMR experiments (DQ COSY, TOCSY, HMQC, HSQC-TOCSY, and HMBC) were performed by using the standard Varian software.

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Abbreviations

DQ COSY, double quantum filtered homonuclear correlated spectroscopy; GLC, gas-liquid chromatography; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy

References


