An antigenic water-soluble glucogalactomannan extracted from cell walls of Paecilomyces fumosoroseus and Paecilomyces farinosus

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The water-soluble fraction (F1S) obtained after solubilizing in alkali the cell walls of four strains of Paecilomyces fumosoroseus and two of Paecilomyces farinosus amounted to 8.3-14.5% of the dry cell wall material. Two polysaccharides, F1S-A (13-20%) and F1S-B (57-68%) were separated from F1S by gel permeation through Sepharose CL-6B. 1H and 13C NMR spectra of F1S-B were recorded and showed analogous structural features in the six isolates of the two species. The fractions isolated from P. fumosoroseus strain CBS 375.70 were subjected to structural analysis and shown to be a (1→4)-α-glucan (F1S-A) and a branched (1→6)-mannan with terminal residues of β-galactopyranosyl (F1S-B). Polyclonal antibodies against the latter polysaccharide were obtained (titre 1/8000). These antibodies reacted specifically with the F1S-B polysaccharides obtained from the four strains of P. fumosoroseus and the two strains of P. farinosus, but they did not react with similar fractions from other species of the same or related genera. The antibodies specifically stained P. fumosoroseus hyphae in indirect immunofluorescence tests.

Keywords: Paecilomyces spp., cell wall glucogalactomannans, ELISA, polyclonal antibodies, immunofluorescence

INTRODUCTION

The genus Paecilomyces comprises a number of species of imperfect fungi, some of which are food contaminants (Douglas King et al., 1969; Engel & Teuber, 1991) and pathogens of humans (Byrd et al., 1985) and animals, especially nematodes (Aoki & Yanase, 1970a, b; Marbannendoza et al., 1992; Naidu & Singh, 1992; Pendland & Boucias, 1993). The genus has been monographed and redefined by Samson (1974) who proposed its subdivision into two sections: Paecilomyces and Isariaidea. The genus Paecilomyces is taxonomically very close to Penicillium, differing only in some morphological features. Since perfect states of Paecilomyces have been classified in Byssashlomys, Talaromyces and Thermoasctls, the genus is heterogeneous, and it seems that morphological criteria alone are not sufficient for species separation.

Fungal carbohydrates are a reliable character for taxonomic classification (Bartnicki-Garcia, 1968; Sugiyama et al., 1985; Weijman et al., 1982; Weijman & Golubev, 1987; Weijman & Van der Walt, 1989; Wessels et al., 1972). The polysaccharides formerly used as chemotaxonomic markers are major components of the cell wall such as the glucan-chitin complex or the α- or β-glucans, which are characteristic of higher fungi (Bartnicki-Garcia, 1968). Recent publications on structural analysis of other cell-wall polymers, such as the water-soluble polysaccharides after alkali extraction, showed differences or similarities among genera or groups of species of the same genus. Therefore, these polysaccharides have chemotaxonomical relevance at the genus or subgenus level. A (1→5)-β-galactofuranan was isolated from most species of Eupenicillium (Leal et al., 1993) and some species of Penicillium and Aspergillus (Leal et al., 1992b). A complex glucomannogalactan was extracted from the cell walls of Talaromyces flavus (Patra et al., 1994) and from other species of Penicillium (Rupérez & Leal, 1987) and Talaromyces (Prieto et al., 1995). In Aphanoascs a (1→2)-(1→6)-α-mannan having a comb-like structure and a (1→4)-α-glucan have been proposed as chemotaxonomic markers (Leal et al., 1992a).

Extracellular polysaccharides (EPS) of many fungi have
been described as immunogenic (De Ruiter et al., 1991; Notermans et al., 1988), and antibodies against them have been used for diagnosis of diseases (De Repentigny et al., 1987, 1989; De Ruiter et al., 1993) and in the detection of fungal contamination in foodstuffs (Notermans & Heuvelman, 1985; Dewey et al., 1990). But not all fungi produce EPS, and their production can be affected by environmental conditions (Leal et al., 1979). However, the water-soluble polysaccharides obtained from cell-wall alkali extracts are stable components which are present in all fungi and may be used as target compounds for immunological detection of fungi in food and clinical samples.

From four strains of Paecilomyces variotii (section Paecilomyces) a water-soluble galactomannan was isolated and partially characterized (Domenech et al., 1994). Following a search of cell-wall polysaccharides which may be used as chemotaxonomic markers we report here on: (i) the structure of the alkali- and water-soluble polysaccharides from the cell walls of four strains of Paecilomyces fumosoroseus and two of Paecilomyces farinatus (section Isarioidae); (ii) the immunogenicity of F1S-B polysaccharides and the specificity of the anti-F1S-B serum from P. fumosoroseus CBS 375.70; and (iii) the staining of fungal hyphae of P. fumosoroseus CBS 375.70 by indirect immunofluorescence.

METHODS

Strains and culture media. The fungi used were P. fumosoroseus strains CBS 244.31, CBS 337.52, CBS 339.54 and CBS 375.70, and P. farinatus CBS 334.61 and CBS 250.29. These were maintained on slants of Bacto potato dextrose agar supplemented with 1 g Bacto yeast extract (Difco) l-'. The culture medium and growth conditions were as previously described (Gómez-Miranda et al., 1990).

Wall material preparation and fractionation. Wall material was obtained as reported elsewhere (Gómez-Miranda et al., 1990). Cell wall material (8 g) was repeatedly extracted with 1 M NaOH (300 ml) at 20 °C. After centrifugation, the supernatants were combined and absolute ethanol was added. The precipitate was collected by centrifugation, dialysed against running tap water and then freeze-dried. Five hundred milligrams of this precipitate was also freeze-dried (F1).

Aliquots of fraction F1S (190 mg) were dissolved in 3 ml distilled water and centrifuged at 13000 g for 15 min to eliminate insoluble material. The supernatant was added to a column (90 x 3 cm) of Sepharose CL-6B and eluted with distilled water with a flow of 214 ml h-1. Fractions of 2.5 ml were collected and monitored for carbohydrate by the phenol-sulfuric acid method (Dubois et al., 1956). Appropriate fractions were combined, concentrated to a small volume and freeze-dried.

Chemical analysis. Neutral sugars of all the fractions were released by Saeman hydrolysis (Adams, 1965) in sealed evacuated tubes and neutralized with barium carbonate. Neutral sugars were converted into their corresponding alditol acetates (Laine et al., 1972), and identified and quantified by GLC as described previously (Gómez-Miranda et al., 1981). Protein was determined by the Lowry method.

Methylation analysis. Aliquots of the polysaccharides (1-5 mg) were methylated according to the method of Cucunur & Kerek (1984). The methylated material was extracted with chloroform/methanol (1:1, v/v), dialysed sequentially against water and 50% ethanol, and dried. Methylated fractions, which showed negligible IR absorption for hydroxyl groups, were hydrolysed with 3 M trifluoroacetic acid (121 °C, 1 h) and the products were reduced with NaBD4, then acetylated and subjected to GLC-MS in an Autosystem from Perkin-Elmer using a SPB-1 column (30 m x 0.22 mm internal diameter x 0.25 µm film thickness), a temperature programme (160-200 °C; 1 min initial hold and ramp rate 2 °C min-'-) and a mass detector Q-Mass (Perkin-Elmer). Quantifications were performed according to peak area.

NMR analysis. Spectra were recorded for solution of samples (20 mg) previously deuterated with D2O and dissolved in 0.6 ml 99.9% (v/v) D2O. The experiments were performed at 40 °C with a Varian XL-300 spectrometer (1H, 300 MHz; 13C, 75 MHz). Chemical shifts refer to residual HOD (461 p.p.m. for protons).

Antibody production. Antibodies were obtained by immunization of rabbits with F1S-B polysaccharide of P. fumosoroseus CBS 375.70, P. variotii CBS 990.73A, Expenicillium crustaceum CBS 635.70 and Trychophyton concentricum IFO 31068. Female New Zealand rabbits (3.0-3.5 kg) were injected intradermally at 7 sites in the back with 1 mg polysaccharide dissolved in 0.5 ml PBS (0.145 M sodium chloride, 0.15 M sodium phosphate, pH 7.2) emulsified in 0.5 ml Freund's complete adjuvant. Injections were repeated after 2, 4, 8 and 12 weeks with the same antigen emulsified in Freund's incomplete adjuvant. Animals were bled from the ear vein or by cardiac puncture 1 or 2 weeks after immunization.

ELISA. To determine the titre of antibodies raised in rabbits against fraction F1S-B the following ELISA was carried out. Ninety-six-well microtitre plates (Costar 3590) were coated with 0.1 mg antigen (40 µg ml-1) diluted in PBS. After overnight incubation at room temperature, the antigen solution was removed, the plates washed twice with PBS and the wells saturated for 3 h with 1% (w/v) gelatin diluted in PBS (0.1 ml for each well). Plates were washed 3 times with PBS and incubated for 2 h with 50 µl serial dilutions of rabbit serum diluted in 0.05% gelatin in PBS. The plates were washed 3 times with PBS, incubated for 60 min with 50 µl goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma) diluted 1/1000 in 0.05% gelatin in PBS. The plates were washed 6 times with PBS and 50 µl o-phenylenediamine (0.66 mg ml-1) in phosphate/citrate buffer pH 5 containing 0.04% hydrogen peroxide were added per well. After 20 min incubation at room temperature the reaction was stopped with 50 µl 1.5 M sulfuric acid. Enzyme activity was determined spectrophotometrically in a Titertek Multiscan MC at a dual wavelength, and values are the result of subtracting A660 from A490. The titre was arbitrarily taken as the reciprocal of the serum dilution giving half-maximal absorption in ELISA.

The following controls were included: (i) no antigen, (ii) no antibody, (iii) preimmune serum and (iv) a non-related antiserum.

Inhibition experiments (competitive ELISA). For inhibitory studies the following polysaccharides were used: (a) (1→5)- and (1→6)-β-galactofuranan from Penicillium expansum (Patra et al., 1993) and Penicillium erubescens (unpublished); (b) (1→2)- and (1→3)-β-galactofuranan from Talaromyces wortmannii (Prieto et al., 1995); (c) complex glucomannogalactans from Talaromyces
Antigenic glucogalactomannans from Paecilomyces

Fractionation of polysaccharides from F1S

The eluate from the Sepharose CL-6B column was collected in two fractions (Fig. 1): F1S-A and F1S-B, which accounted for 13-20% and 57-68%, respectively, of the weight of the starting material. F1S-A had the highest amount of glucose and lower percentages of mannose and galactose. F1S-B was composed of galactose (31.8-37.0%), mannose (39.9-40.4%) and a small amount of glucose (64-68%). Fraction F1S-B from P. fumosoroseus CBS 375.70 eluted as a single peak when rechromatographed under the same conditions.

NMR analysis

The NMR spectra of F1S-B of the six strains of the two species were similar with minor differences in signal intensities so only $^{13}$C NMR (Fig. 2a) and $^1$H NMR (Fig. 2b) spectra of F1S-B of P. fumosoroseus CBS 375.70 are shown as the fingerprint of these polysaccharides. The polysaccharides are quite irregular, as can be deduced from the $^{13}$C NMR spectra which show clusters of signals for the mannan backbone (C-1: 100-1020 p.p.m.; C-6: 660-680 p.p.m.) rather than sharp peaks. The galactopyranose showed a $\beta$-configuration (H-1: 4.43 p.p.m.; $J_{H,H}$: 7.8 Hz), while the glucopyranose appeared in an $\alpha$-configuration. (H-1: 4.92 p.p.m.; $J_{H,H}$ < 4 Hz).

Methylation analysis

The results from fraction F1S-B from P. fumosoroseus are shown in Table 2. The amounts of 2,3-Me$_2$-mannitol, 2,3,4-Me$_3$-mannitol and 2,3,4,6-Me$_4$-galactitol suggested a (1 $\rightarrow$ 6)-linked mannan backbone, with branch points (33.4%) at C-4 or C-5 and galactopyranose (29.1%), glucopyranose (7.4%) and galactofuranose (10%) as terminal residues. Other minor components were detected. In F1S-A, the main products detected were 2,3,6-Me$_3$-glucitol (83.1%) and 2,3,6-Me$_3$-glucitol (82.2%) indicating that the polysaccharide is a glucan (1 $\rightarrow$ 4)-linked with branch points in some units at C-6. Glucopyranose (67%) occurred as terminal residues.

Antibodies

Three batches of antisera were obtained per rabbit, after the third, fourth and fifth immunization. Titres of antisera taken after the fourth and fifth injections were similar and high. The serum dilution giving half-maximal absorption in ELISA was 1/8000.

Inhibition studies

Results of inhibition studies using antisera against F1S-B of P. fumosoroseus CBS 375.70 showed that the binding of this serum to the antigen-activated plates was inhibited by the same fraction of three other strains of this species and by two strains of P. farinosus. Fifty percent inhibition was achieved with concentrations of these polysaccharides ranging from 5 to 15 $\mu$g ml$^{-1}$ of a similar F1S-B fraction. The binding of the antibodies was not inhibited by concentrations of 5 mg ml$^{-1}$ of the polysaccharides.

RESULTS

Composition of the different fractions

The alkali-extractable and water-soluble fraction (F1S) obtained from the cell wall of the four strains of P. fumosoroseus and the two strains of P. farinosus amounted to 8.3-14.5% of the dry cell wall material. The alkali-soluble and water-insoluble fraction (F1I) represented a small proportion of the cell wall (0.6-4.6%).

Table 1 shows the neutral sugars released from F1S and F1I hydrolysed by the Saeman procedure. In the two species, the most abundant sugar in F1S was mannose (28.5-53.0%), and there were variable amounts of galactose (8.3-31.6%) and glucose (17.7-38.1%). Fraction F1I contained galactose (47.6-69.9%) and lower proportions of mannose and galactose. Protein was not detected in either of the two fractions.

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</tr>
</thead>
<tbody>
<tr>
<td>F1S</td>
<td>28.5</td>
<td>8.3</td>
<td>17.7</td>
</tr>
<tr>
<td>F1I</td>
<td>47.6</td>
<td>31.6</td>
<td>6.8</td>
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</tbody>
</table>

Two methods were used to determine if the carbohydrate fraction was antigenic. The direct method was indirect immunofluorescence using a whole cell preparation of the whole mycelium. The slides were air-dried and stained with 20 $\mu$l of the test antibody diluted 1:100. Preimmune serum was used as control antibody. As there was some background staining of spores in the absence of the primary antibody, the secondary antibody was pre-adsorbed on anti-rabbit immunoglobulin. The slides were air-dried and stained with 20 $\mu$l of the test antibody diluted 1:100. Preimmune serum was used as control antibody. As there was some background staining of spores in the absence of the primary antibody, the secondary antibody was pre-adsorbed on anti-rabbit immunoglobulin.

Indirect immunofluorescence. The mycelium of P. fumosoroseus CBS 375.70 was grown overnight in a drop of the liquid culture medium on glass coverslips (9 x 9 mm) at room temperature and maintained in a wet chamber. The slides were air-dried and heat-fixed overnight at 60 OC in an oven. The preparation was washed 3 times with 2 ml PBS for 5 min and saturated with 3% (w/v) BSA diluted in PBS for 30 min. The samples were incubated at 1 h at 37 OC with 20 $\mu$l of the test antibody diluted 1/200 in 3% BSA in PBS. After washing twice with PBS and gentle agitation (5 min), the coverslips were overlaid with 20 $\mu$l goat anti-rabbit rhodamine-conjugate (Sigma), diluted 1/100 in 3% BSA in PBS and incubated for 45 min at 37 OC, washed with PBS in the dark and mounted with 0.13 M glycine buffer pH 8.6, 0.2 M NaCl, 70% (v/v) glycerol. Stained mycelium was photographed on Kodak TriX film using a Zeiss Axioplan microscope equipped with epilumination. A 63 x Plan Aploment immersion objective and a Zeiss filter set 15 were used.

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Table 1. Percentages of neutral sugars released from F1S and F1I of four strains of *P. fumosoroseus* and two of *P. farinosus* by Saeman hydrolysis. All values are means of 3 replications (±sd).

<table>
<thead>
<tr>
<th>Strain</th>
<th>F1S</th>
<th>Recovery (%)</th>
<th>F1I</th>
<th>Recovery (%)</th>
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<tr>
<td></td>
<td>Man</td>
<td>Gal</td>
<td>Glc</td>
<td>(%)</td>
</tr>
<tr>
<td><em>P. fumosoroseus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBS 244.31</td>
<td>28·5±2·3</td>
<td>21·5±1·7</td>
<td>38·1±3·2</td>
<td>88·1</td>
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<td><em>P. fumosoroseus</em></td>
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<td>CBS 339.54</td>
<td>53·0±2·8</td>
<td>23·0±3·3</td>
<td>17·7±2·2</td>
<td>73·7</td>
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<td><em>P. fumosoroseus</em></td>
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<tr>
<td>CBS 337.52</td>
<td>41·8±3·9</td>
<td>31·6±2·1</td>
<td>23·4±2·1</td>
<td>96·8</td>
</tr>
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<td><em>P. fumosoroseus</em></td>
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</tr>
<tr>
<td>CBS 375.70</td>
<td>31·7±3·2</td>
<td>18·3±2·0</td>
<td>20·5±1·9</td>
<td>70·5</td>
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<td><em>P. farinosus</em></td>
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<tr>
<td>CBS 250.29</td>
<td>37·6±3·9</td>
<td>21·3±2·3</td>
<td>21·8±2·0</td>
<td>82·4</td>
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<td><em>P. farinosus</em></td>
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<tr>
<td>CBS 334.61</td>
<td>33·1±3·2</td>
<td>8·3±1·5</td>
<td>37·1±2·7</td>
<td>79·6</td>
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</table>

Fig. 1. Column chromatography of F1S from *P. fumosoroseus* CBS 375.70 on Sepharose CL-6B. Fraction A, tubes 42-66; fraction B, tubes 78-114.

Polysaccharide F1S-B of *P. fumosoroseus* CBS 375.70 did not react with sera obtained against the same fraction from *P. variotii*, *E. crustaceum* and *T. concentricum*.

Immunofluorescence microscopy

The mycelium of *P. fumosoroseus* appeared homogeneous and strongly stained after reaction with the polyclonal antibodies obtained against fraction F1S-B, while spores remained unstained. Germ tubes appeared brightly stained where they emerged from the spores (Fig. 3).

DISCUSSION

Cell wall material extracted with 1 M NaOH was separated into two fractions: a water-insoluble glucan (F1I) and a water-soluble polysaccharidic material (F1S).

F1S from all isolates was subjected to gel permeation chromatography on Sepharose CL-6B (Fig. 1) and two fractions were separated. F1S-A was a (1→4)-α-glucan with some branch points at C-6. Polysaccharides having similar characteristics have been found in similar fractions.
### Table 2. GLC-MS data for the methylated alditol acetates from fraction F1S-B of *P. fumosoroseus* CBS 375.70. Methylated samples were reduced with NaBD₄. All values are means of at least three replications.

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Alditol*</th>
<th>Linkage type</th>
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<tr>
<td></td>
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<td>Direct</td>
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<td></td>
<td></td>
<td></td>
<td>analysis</td>
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<tr>
<td>8:37</td>
<td>2,3,4,6-Me₄-Glc</td>
<td>Glcp-(1-</td>
<td>7.4</td>
<td>6.8</td>
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<tr>
<td>8:53</td>
<td>2,3,5,6-Me₄-Gal</td>
<td>Galp-(1-</td>
<td>1.0</td>
<td></td>
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<tr>
<td>8:87</td>
<td>2,3,4,6-Me₄-Gal</td>
<td>Galp-(1-</td>
<td>29.1</td>
<td></td>
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<tr>
<td>10:75</td>
<td>2,3,6-Me₃-Gal</td>
<td>(1-4)-Galp or (1-5)-Galp</td>
<td>8.3</td>
<td>41.3</td>
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<td>10:98</td>
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<td>(1-2)-Galp</td>
<td>2.9</td>
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<td>(1-2)-Manp</td>
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<td>(1-6)-Manp</td>
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<td>14:09</td>
<td>2,3-Me₂-Man</td>
<td>(1-4,6)-or (1-5,6)-Manp</td>
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<td>51.1</td>
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<tr>
<td>14:53</td>
<td>3,4-Me₂-Man</td>
<td>(1-2,6)-Manp</td>
<td>4.4</td>
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* Prepared by hydrolysis with 3 M trifluoroacetic acid for 1 h at 121 °C.

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**Fig. 3.** (a) Immunofluorescence observed in hyphae of *P. fumosoroseus* CBS 375.70 grown on coverslips that were heat-fixed and stained with serum anti-F1S-B followed by goat anti-rabbit IgG-rhodamine conjugate. Arrows point to germ tubes growing from non-stained spores. (b) Negative control: no immunofluorescence was observed when mycelia were not treated with anti-F1S-B serum. Bar, 11 μm.

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...from *Aphanoascus fulvescens* (Leal et al., 1992a), *Penicillium erythromellis* (Rupérez & Leal, 1987) and in species of *Talaromyces* (Prieto et al., 1995). Therefore this glucan has limited chemotaxonomic relevance.

F1S-B was the main polysaccharide of fraction F1S and according to methylation (Table 2), and ¹H and ¹³C NMR (Fig. 2a, b) analyses, two arrangements of the glycosyl residues are possible: either a (1→6)-linked α-mannopyranose skeleton, branched by α-glucopyranose or β-galactopyranose moieties at position 4 of 70% of the mannose residues, or else (and less likely) a (1→6)-linked β-mannofuranose chain, branched at position 5. In addition, branching points at C-2 of (1→6)-linked mannopyranose residues have been detected. The position of the other minor components detected by methylation analysis has not been elucidated. Additional chemical and NMR studies will be necessary to clarify this question. The ratio
of mannose, galactose and glucose calculated after methyl-
ination analysis coincided with the values determined after
direct analysis (Saeman hydrolysis) of the native poly-
saccharide (Table 2). This polysaccharide differs from
those found in a similar fraction of several strains of P. varioti (Domenech et al., 1994) and other species of
Paecilomyces (unpublished results) or in species of related
genera such as Penicillium (Leal et al., 1992b), Expenicillium
(Leal et al., 1993) and Talaromyces (Prieto et al., 1995).
These results therefore show the relatedness of P. fumosoroseus and P. farinosus and indicate that these
polysaccharides may be used as chemotaxonomic markers
to distinguish these species from other species of Paecilomyces.

Polyclonal antibodies against F1S-B of P. fumosoroseus
CBS 375.70 were obtained with high titres and inhibitory
studies were performed to check the specificity of these
antibodies.

Fraction F1S-B of each of the six isolates strongly
inhibited (50% inhibitory concentration less than 15 µg
ml⁻¹) the binding of antibodies against F1S-B of P. fumosoroseus CBS 375.70 to its specific antigen. These
results were expected as all the polysaccharides have a
similar structure varying slightly in the proportions of the
components. F1S-B polysaccharides with different struc-
tures from related and unrelated species were used in
inhibition experiments against serum anti-F1S-B of P. fumosoroseus CBS 375.70. No cross-reaction was observed
at concentrations of 5 mg ml⁻¹ of the polysaccharides
tested although they may contain in their structure some
residues with the same linkage types as the P. fumosoroseus
antigen.

F1S-B polysaccharide of P. fumosoroseus CBS 375.70 did
not react with sera obtained against the same fraction
from P. varioti, E. crustaceum and T. concentricum, con-
fiming the specificity of the sera obtained against those
polysaccharides.

Hyphae of P. fumosoroseus CBS 375.70 were homogeneous
and strongly stained by indirect immunofluorescence (Fig.
3). With this technique antibodies raised against water-
soluble polysaccharides obtained from alkali extracts of
fungal cell walls could be used to detect fungal hyphae in
contaminated food and biological samples. Antibodies
obtained against extracellular polysaccharides have been
used for fungal detection in animals, foods, agricultural
products and medical samples (De Repentigny et al., 1987;
Notermans et al., 1988; De Repentigny, 1989; Kaufman et
al., 1989; Kamphuis et al., 1992; De Ruiter et al., 1993).

These results confirmed that fraction F1S-B poly-
saccharides are antigenic and that the antibodies raised to
P. fumosoroseus could be used to distinguish these species
from related species lacking this glucogalactomannan.

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