Galactose-Containing Polysaccharides from the Human Pathogens *Sporothrix schenckii* and *Ceratocystis stenoceras*

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Galactose-containing polysaccharides from three strains of *Sporothrix schenckii* and one strain of *Ceratocystis stenoceras* were isolated, and their structures were partially characterized by chemical analysis and ¹³C-nuclear magnetic resonance spectroscopy (¹³C-NMR). *S. schenckii* polysaccharide preparations from all strains were not precipitated by Fehling solution and contained galactomannan (or a mixture of galactan and galactomannan), amylose, and minor amounts of rhamnomannan. *C. stenoceras* polysaccharide contained galactomannan and a smaller proportion of amylose. Conventional chemical techniques and ¹³C-NMR spectroscopy showed that the structures of the two preparations were closely related. The core of the galactomannan consisted principally of nonreducing end units and 2-*O*-, 2,6-di-*O*-, and perhaps 2,3-di-*O*-substituted α -D-mannopyranosyl units. The core was substituted by β -D-galactofuranosyl chains; the units are interlinked (1 $\rightarrow$ 6). ¹³C-NMR evidence shows that the α -D-mannopyranosyl units are substituted in the two positions by the β -D-galactofuranosyl residues. Galactomannans present at the cell surface of *S. schenckii* represent other potential fungal antigens in addition to the already recognized rhamnomannans and their peptide complexes.

Sporothrix schenckii, the agent of human sporotrichosis, is a dimorphic fungus which is frequently associated with plants and soil (17). In these environments, the mycelial phase predominates, with hyphal and conidial cell types. Yeastlike forms develop in infected human and animal tissues. Surface antigens of *S. schenckii* have been studied in both the mycelial and yeast phases (18, 22). The chemical structures, as well as the immunological reactivity of peptido polysaccharides (15, 24) and isolated rhamnomannans (16, 18, 22), were determined. Polysaccharides from several *S. schenckii* strains and from the morphologically related *Ceratocystis stenoceras* were compared (3, 23). During these studies we observed that galactose-containing polysaccharides could also be isolated, sometimes in addition to the rhamnomannans, from supernatants of both *S. schenckii* and *C. stenoceras* cultures. The surface location of the galactose-containing polysaccharides was shown by the reaction with an anti-*Hormodendrum* serum observed by immunofluorescent staining (K. O. Lloyd, unpublished observation) of *S. schenckii* yeast forms. In the present work the structures of galactomannans from three strains of *S. schenckii* and one strain of *C. stenoceras* were studied.

MATERIALS AND METHODS

Microorganisms. The sources of the three strains of *S. schenckii* (1099.12, 1099.18, and 1099.24) and the strain of *C. stenoceras* (1099.40) are given elsewhere (22, 23). Mycelial cultures were grown in the yeast nitrogen base (Difco Laboratories, Detroit, Mich.)-Casamino Acids (Difco)-vitamins medium (22) at 25°C.

Isolation of polysaccharides. Polysaccharides were isolated from vacuum-concentrated supernatants of cultures by precipitation with 3 volumes of ethanol. The precipitates were treated with 2% KOH at 100°C (for 2 h), the alkaline solutions were neutralized and centrifuged, and the polysaccharides were reprecipitated with ethanol. The dry residue was dissolved in water and treated with Fehling solution. The supernatant was separated from the Fehling precipitate and neutralized with acetic acid, dialyzed against distilled water, and deionized with Amberlite IR-120 (H⁺ form) and Dowex 1-X8 (HCO₃ form). The polysaccharide solutions were then concentrated in vacuo and precipitated with ethanol (4 volumes). Polysaccharide was obtained in yields of 7 mg per liter (3-day culture) and 19 mg per liter (4-day culture) from *C. stenoceras*. Sugar components of all preparations were identified by paper chromatography (solvent *n*-butanol-ethanol-water 40:11:19 [vol/vol/vol]; spray *p*-anisidine-hydrochloride) after hydrolysis in 0.5 M H₂SO₄ for 18 h at 100°C, and by gas-liquid chromatography of the derived alditol acetates (20) using an ECNSS-

M-Gas-Chrom Q (100–120 mesh) column (200 × 0.3 cm) at 190°C.

Methylation analysis of polysaccharides. Unlike the rhamnomannans, which are readily methylated by the Hakomori procedure (18, 22), the galactose-containing polysaccharides required a more rigorous procedure, which involved two methylation steps by the Haworth procedure followed by three methylation steps by the Kuhn method (8). Methylated polysaccharides were partially hydrolyzed by refluxing with acidified methanol (3% HCl [wt/vol]) for 18 h. After neutralization with Ag₂CO₃ and filtration, the filtrate was evaporated, dried, and hydrolyzed with 10% aqueous H₂SO₄ for 18 h at 100°C. The hydrolysate was neutralized with BaCO₃ and centrifuged, and the supernatant and washes of the precipitate were pooled and concentrated to a syrup. The partially *O*-methylated alditol acetates were prepared (20), and derivatives were identified by gas-liquid chromatography coupled to a mass spectrometer using an ECNSS-M column, as above, at 170°C.

¹³C-NMR spectroscopy. ¹³C-labeled nuclear magnetic (NMR) spectra were obtained at 70°C as described before (3).

Preparation of 6-*O*-β-D-galactofuranosyl-(methyl β-D-galactofuranoside). 3,5,6-Tri-*O*-acetyl-α-D-galactofuranose 1,2-(methyl orthoacetate) (13; 2.0 g) was added to a solution of methyl 2,3,5-tri-*O*-benzyl-β-D-galactofuranoside (3.0 g) and mercuric bromide (100 mg) in nitromethane (4 ml), and the reaction mixture was maintained at 100°C for 3 h. Pyridine (0.4 ml) was then added, and the solution was evaporated to a syrup, which was deacetylated with NaOCH₃ in MeOH, and the product was debenzylated with Pd/H₂ in acetic acid. The resulting mixture was fractionated by cellulose column chromatography with butanol-1 containing 5% H₂O as solvent. Methyl β-D-galactofuranoside was eluted initially followed by 6-*O*-β-D-galactofuranosyl-(methyl β-D-galactofuranoside) (0.57 g), [α]_D²⁵ = -108° (c, 0.7 H₂O) which had a mobility of 0.91 on a paper chromatogram (solvent—butanol-1, ethanol, water 40:11:19 [vol/vol/vol]; spray—ammoniacal silver nitrate) compared with that of ribose. Found: C, 43.52; H, 6.77. Analytical calculation for C₁₃H₂₄O₁₁: C, 43.82; H, 6.79.

The ¹³C-NMR signals (D₂O at 70°C) have shift values expressed as δ_c in ppm, relative to an external standard of Me₄Si, whose shift was determined in a separate experiment. The signals could be assigned by comparison with those of methyl β-D-galactofuranoside (5). The assignments are δ_c 109.9 (C-1), 109.5 (C-1'), 84.8, 84.7 (C-4, C-4'), 82.7, 82.5 (C-2, C-2'), 78.5, 78.4 (C-3, C-3'), 72.5 (C-5'), 64.5 (C-6'), 56.6 (OCH₃). The signals at δ_c 71.2 and 70.7 were assigned to C-6' and C-5, respectively, since the former gave a triplet under coupled conditions and must therefore arise from a CH₂ rather than a CH group.

RESULTS

Galactomannans were purified by treatment of polysaccharide preparations with Fehling solution which precipitated most of the rhamnomannan components as their copper complexes.

The proportions of monosaccharides in preparations, obtained from the mother liquors, are shown in Table 1. The blue reaction with iodine of *S. schenckii* and *C. stenoceras* polysaccharides, as well as the identification of 2,3,6-tri-*O*-methylglucitol acetate by methylation analysis (see below) showed that glucose is present as amylose.

Methylation analysis of *S. schenckii* polysaccharide preparations was not markedly different from that of the *C. stenoceras* polysaccharide. For *S. schenckii* 1099.24 polysaccharide, the following acetylated alditols were formed (relative proportions to 3,4-di-*O*-methylmannitol acetate in parentheses): 2,3,4,6-tetra-*O*-methylmannitol (0.47), 2,3,5,6-tetra-*O*-methylgalactitol (0.68), 2,3,4-tri-*O*-methylrhamnitol (0.2), 3,4,6-tri-*O*-methylmannitol (1.6), 2,3,6-tri-*O*-methylglucitol (0.93), 2,3,4-tri-*O*-methylmannitol (0.34), 2,3,5-tri-*O*-methylgalactitol (0.27), 2,4,6-tri-*O*-methylmannitol (0.2), 4,6-di-*O*-methylmannitol (0.2), and 3,4-di-*O*-methylmannitol (1.0). For *C. stenoceras* polysaccharide the acetylated alditols were 2,3,4,6-tetra-*O*-methylmannitol (0.4), 2,3,5,6-tetra-*O*-methylgalactitol (1.0), 3,4,6-tri-*O*-methylmannitol (0.9), 2,3,6-tri-*O*-methylglucitol (0.32), 2,3,5-tri-*O*-methylgalactitol (0.7), 4,6-di-*O*-methylmannitol (0.1), and 3,4-di-*O*-methylmannitol (1.0). The partially hydrolyzed polysaccharide gave similar fragments in a ratio of 0.7:0.74:5.1:1.3:0.48:0.08:1.0, which corresponds to a removal of β-D-galactofuranosyl side chains, some of which are single-unit nonreducing ends, whereas others are from longer chains interspersed with (1 → 6) linkages. A large proportion of 2-*O*-substituted α-D-mannopyranosyl units are present. The number of consecutive (1 → 2) links between α-D-mannopyranosyl units is 3 and 4 since acetolysis of *C. stenoceras* galactomannan followed by deacetylation of the fragments and separation on a paper chromatogram (14) showed spots corresponding to galactose,

TABLE 1. Proportions of monosaccharides in *S. schenckii* and *C. stenoceras* polysaccharide preparations

Strain	Monosaccharide (%)			
	Rhamnose	Mannose	Galactose	Glucose
<i>S. schenckii</i>				
1099.18	10	42	30	18
1099.12	21	43	18	18
1099.24	9	40	31	20
<i>C. stenoceras</i>				
1099.40	<1	40	48	12

mannose (trace), and tetra- and pentasaccharides consisting of mannopyranosyl units.

The ^{13}C -NMR spectrum (Fig. 1) of the *C. stenoceras* galactomannan (4-day culture), which contained 12% of glucose, resembled closely that of *S. schenckii* (Fig. 3). The presence of β -D-galactofuranosyl residues was shown by signals at δ_c 109.5, 107.6, 107.2 (C-1); 84.8, 85.0 (C-4); 82.6, 83.1 (C-2); 78.5 (C-3), and 64.5 (C-6). Also, signals were present at δ_c 102.8 and 100.2, corresponding to 2-*O*- and 2,6-di-*O*-substituted α -D-mannopyranosyl units, respectively (2). It is noteworthy that the proportion of α -D-mannopyranosyl nonreducing end units in the *C. stenoceras* product was insufficient to give a signal at δ_c 103.6 (2) (Fig. 1), whereas it was present in the spectrum of the *S. schenckii* galactomannan (Fig. 3). The signal at δ_c 101.4 corresponds to C-1 of the 4-*O*-substituted α -D-glucopyranosyl units of the amylose component (the C-1 signal of potato starch is at δ_c 101.4). The signal is smaller in the ^{13}C -NMR spectrum of the galactomannan of *C. stenoceras* compared with that of *S. schenckii* polysaccharide. The above structural proposals on *C. stenoceras* polysaccharide are supported by the specific rotation of the galactomannan, $+6^\circ$ (c, 0.8 H_2O), which is reasonable for a polysaccharide with a ratio of galactose to mannose to glucose of 48:40:12. Values of $[\alpha]_D +88^\circ$ have been reported for the α -D-mannopyranan of bakers' yeast (6) and -84° for a (1 $\rightarrow$ 5)-linked β -D-galactofuranan derived from *Penicillium charlesii* (7). Partial hydrolysis of *C. stenoceras* polysaccharide at pH 2 (adjusted with dilute H_2SO_4) at 100°C for 8 h removed most of the galactose units and provided a polysaccharide in 51% yield, which contained

mannose, galactose, and glucose in a ratio of 76:8:16. Its specific rotation was $+72^\circ$ (c, 0.6 H_2O) corresponding to a mixture of α -D-mannopyranosyl, β -D-galactofuranosyl, and α -D-glucopyranosyl units. Its ^{13}C -NMR spectrum (Fig. 2) lacked the low field C-1 signals of β -D-galactofuranosyl units but contained a C-1 signal at δ_c 103.7, suggesting the presence of α -D-mannopyranosyl nonreducing end units, absent in the parent polysaccharide. Such a unit appears to have arisen by cleavage of an *O*- β -D-galactofuranosyl-(1 $\rightarrow$ 2)-*O*- α -D-mannopyranosyl linkage, whose C-1 signal would be shifted to δ_c 102.8 by virtue of a β -*O*-substitution shift (1).

Further structural information on the *C. stenoceras* polysaccharide can be obtained by consideration of the C-1 shifts of the β -D-galactofuranosyl units. Other ^{13}C -NMR studies on model disaccharides, consisting of β -D-galactofuranose attached to methyl α -D-mannopyranoside, showed that the shift of C-1 depended on the position of substitution on the mannosyl residue (Gorin, Barreto-Bergter, and Cruz, submitted for publication). A value of δ_c 109.7 was observed for a (1 $\rightarrow$ 6)-linkage, δ_c 107.7 for a (1 $\rightarrow$ 2)-linkage, and δ_c 106.5 for a (1 $\rightarrow$ 3)-linkage. It appears that the C-1 signals of the polysaccharide at δ_c 107.2 and 107.6 could arise from β -D-galactofuranosyl units linked (1 $\rightarrow$ 2) to α -D-mannopyranosyl units when one considers the formation of 3,4-di-*O* and 3,4,6-tri-*O*-methylmannitol acetates in the methylation experiment and the appearance of a ^{13}C signal at δ_c 103.7 with the partially hydrolyzed polysaccharide. The possibility of a (1 $\rightarrow$ 3)-linkage is not ruled out, however, since 4,6-di-*O*-methylmannitol (2%) was obtained on methylation analysis, al-

FIG. 1. ^{13}C -NMR spectrum of polysaccharide from *C. stenoceras*. Numerical values are chemical shifts (δ_c) in ppm.

though it could arise through incomplete methylation and is present in a very small proportion. The remaining C-1 signal at δ_c 109.5 belongs to a β -D-galactofuranosyl residue attached (1 $\rightarrow$ 6) to another β -D-galactofuranosyl unit. This was confirmed by examination of synthetic 6-O- β -D-galactofuranosyl-(methyl β -D-galactofuranoside), whose C-1' signal is at δ_c 109.5 (the other C-1 signal was assigned to C-1 since its shift, δ_c 109.9 corresponded to that of methyl β -D-galactofuranoside (4; value corrected to 70°C by adding 0.6 ppm). The disaccharide model also served to characterize signals in the ^{13}C -spectra of *C. stenoceras* (Fig. 1) and *S. schenckii* (Fig. 3), at δ_c 70.7 and 71.3, which correspond, respectively,

to C-6 and C-5 of β -D-galactofuranosyl residues substituted at OH-6 with β -D-galactofuranose. Another contribution to the C-1 signal of the polysaccharide at δ_c 109.5 could be made by C-1 of a β -D-galactofuranosyl unit attached (1 $\rightarrow$ 6) to an α -D-mannopyranosyl residue, by analogy with the corresponding disaccharide, whose C-1 signal is at δ_c 109.7.

DISCUSSION

The present data indicate a complex structure for the galactomannans from *C. stenoceras* and *S. schenckii*. The polysaccharides consist of a core of α -D-mannopyranosyl units that are 2-O-, 2,6-di-O- and perhaps also 2,3-di-O-substituted.

FIG. 2. ^{13}C -NMR spectrum of acid-degraded polysaccharide from *C. stenoceras*. Numerical values are chemical shifts (δ_c) in ppm.

FIG. 3. ^{13}C -NMR spectrum of polysaccharide from *S. schenckii*. Numerical values are chemical shifts (δ_c) in ppm.

Three and four consecutive (1 → 2) linkages occur between the α -D-mannopyranosyl residues. The β -D-galactofuranosyl units are components of side chains, being (1 → 6)-linked to each other and in turn, at least partly, (1 → 2)-linked to mannose core. Whether the polysaccharide consists of a single galactomannan or is a mixture of galactan and galactomannan is not yet clear; both possibilities are consistent with the methylation data. A range of structural components is indicated, however, by a further fractionation of the galactomannan preparation from *C. stenoceras* via precipitation of the borate derivative by Cetavlon (15), at pH 8.0 and 9.5. At pH 9.5 the precipitated polysaccharide had the same proportions of galactose and mannose as those before Cetavlon treatment. At pH 8.0, however, some fractionation was obtained with the precipitation of a polysaccharide with increased proportion of mannose (Table 2). It is possible that the original preparation contained different chains of polysaccharides and that those having less substitution with β -galactofuranosyl units were preferentially precipitated at pH 8.0.

The galactomannans from three *S. schenckii* strains and from *C. stenoceras* described herein differ from the galactomannan described before (18) by having an increased proportion of galactose and probably by lacking regions with unsubstituted (1 → 6)-linked α -D-mannopyranosyl units in the main chain, which would render the polysaccharide precipitable by Fehling reagent. It seems that the galactomannan described earlier (18) was synthesized by a variant of *S. schenckii* 1099.12 strain which was selected under certain growth conditions and that the galacto-

mannans non-precipitable by Fehling reagent reported in the present work are common in most strains of *S. schenckii*. A possible series of galactomannans with different galactose-to-mannose ratios is suggested.

Serological cross-reactivity occurs between *S. schenckii*, *Ceratocystis* spp., and other microorganisms, and this has been interpreted in terms of common rhamnosyl side chains of component polysaccharides (9, 12, 16, 19, 22). Cutaneous delayed hypersensitivity to antigens from *Sporothrix* spp. and *Ceratocystis* spp., which have mannose, rhamnose, and galactose units in their polysaccharides, has been observed in cutaneous sporotrichotic patients (10, 11). The presence of galactomannan components in *S. schenckii* may explain some immunological cross-reactions among fungal antigens because the antigenic β -D-galactofuranosyl nonreducing end units are present in a variety of other fungi (21). The anti-*Hormodendrum* serum that reacted with *S. schenckii* yeast cells (K. O. Lloyd, unpublished data) also reacted with galactomannans from *Aspergillus fumigatus*, *Cladosporium werneckii*, and *Trichophyton rubrum* (21). For diagnostic serology, antigenic preparations should be checked for the presence of galactose-containing polysaccharides, in addition to the rhamnomannans which are specific of *S. schenckii*. So far, there are no studies on the immune response of human patients of sporotrichosis to individual polymers or molecular complexes isolated from this fungus.

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TABLE 2. Proportions of monosaccharides in *C. stenoceras* and *S. schenckii* polysaccharides^a

Strain	Monosaccharide	% Monosaccharide after Cetavlon-borate precipitation at pH:	
		8.0	9.5
<i>C. stenoceras</i> ^b 1099.40	Rhamnose	Tr ^c	Tr
	Mannose	50	36
	Galactose	50	64
<i>S. schenckii</i> 1099.24	Rhamnose	8	6
	Mannose	56	52
	Galactose	35	42

^a After precipitation with Cetavlon-borate at two different pH's.

^b Starting with a polysaccharide preparation containing rhamnose (11), mannose (33), galactose (67), glucose (tr).

^c Trace.

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