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Note

Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans*

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The four recognized serotypes of *Cryptococcus neoformans* (A, B, C, and D) produce extensive polysaccharide capsules that are sloughed into the growth medium. Current immunological studies generally rely on antigen preparations obtained simply by treating with ethanol the growth medium in which *C. neoformans* has been cultivated, and isolating the resulting precipitate. The antigen obtained in this way is a composite of several heteropolysaccharides. The major viscous polysaccharide, glucuronoxylomannan (GXM), has been purified from each of the 4 serotypes of *C. neoformans*, and all were found to contain glucuronic acid, xylose, and mannose, and to be partially *O*-acetylated as well[1–7]. Recently, the supernate that resulted from precipitation of GXM with cetyltrimethylammonium bromide (CTAB) was found to contain a novel polysaccharide[8]. The presence of galactose as a major component, in addition to xylose and mannose, was characteristic of this polysaccharide fraction, galactoxylomannan (GalXM).

The mannosyl residues of GXM, which are linked α-(1→3), are unreactive to concanavalin A (con A), whereas the GalXM formed an immunoprecipitin line by double immunodiffusion in gel[8]. These observations formed the basis for the development of a con A affinity-chromatography procedure to fractionate GalXM. Affinity chromatography showed that GalXM is actually composed of two discrete polymers. Therefore, the extracellular polysaccharides of *C. neoformans* are a composite of at least three polymers.

The fractionation of GalXM by con A affinity-chromatography, and the partial characterization of the purified polysaccharides, are described herein.

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EXPERIMENTAL

Analytical methods. — Neutral carbohydrate was detected by the phenol–sulfuric acid method of Dubois *et al.*[9]. Uronic acid was determined by the carbazole[10] and orcinol[11] reactions, with D-glucuronic acid as the standard. Phosphate was estimated by the Ames–Dubin[12] modification of the procedure of Chen *et al.*[13]. Protein content was determined by means of a dye-binding, assay reagent (Bio-Rad Laboratories 500-0006), and by amino acid analysis. The constituent monosaccharides were identified, and quantitated, by gas–liquid chromatography (g.l.c.) after acid hydrolysis of the polysaccharide with 2M trifluoroacetic acid by methods previously described[14].

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded with a JNM/FX60Q n.m.r. spectrometer operated at 15.04 MHz and equipped with a 10-mm, ^{1}H-^{13}C dual probe, an NM-3975 foreground–background unit, and an NM-5471 temperature controller. The spectra were recorded at 70° by using the pulsed, fast Fourier-transform method, and by employing the deuterium resonance of the solvent, a buffer (pH 7.2) of sodium phosphate in deuterium oxide, as an internal lock. The spectra were 4-kHz wide and were collected with 8-k data points in the f.i.d., and by use of a 45° pulse repeated at intervals of 1.1 s. Chemical shifts were determined from spectra that were proton-decoupled by a 1-kHz noise-band centered at 48.00-kHz offset-frequency, and were measured relative to an external capillary containing tetramethylsilane.

Serological procedures. — The reference antiserum was produced in large 3–4-kg, female, New Zealand white rabbits immunized with formaldehyde-killed *C. neoformans* B 551 yeast cells, and the IgG fraction was labeled with horseradish peroxidase[15].

Concanavalin A affinity chromatography. — GalXM (100 mg/25 mL of Tris starting buffer) was applied to a column (2.5 × 11.4 cm) of con-A–Sepharose 4B (Sigma). The column was successively eluted with *1*, 0.5M NaCl–0.01M Tris buffer, pH 7.2, containing $MnCl_2$ (1mM) and $CaCl_2$ (1mM) (starting buffer); *2*, 0.2M $Na_2H_4B_4O_7 \cdot 10\,H_2O$, pH 5.0; and *3*, 0.2M methyl α-D-mannopyranoside (Me α-D-Man*p*). The elution was monitored continuously at 206 nm. Carbohydrate, in the Tris buffer and the borate buffer eluates was detected with the phenol–sulfuric acid assay[9]. Counterimmunoelectrophoresis (c.i.e.)[16] or enzyme immunoassay (e.i.a.)[17] was used to detect the presence of the antigen in the fraction eluted with Me α-D-Man*p*. The column was regenerated by sequential elution with: *1*, 0.5M NaCl–0.1M Tris buffer, pH 7.2; *2*, 0.05M NaCl–0.1M Tris buffer, pH 8.5; and *3*, starting buffer.

Polysaccharide antigens. — Unfractionated GalXM and GalXM-CL-6B were prepared from *C. neoformans* B2550 (serotype A) as previously described[8].

RESULTS AND DISCUSSION

The GalXM (ref. 8) was fractionated on a column of con A–Sepharose 4B by

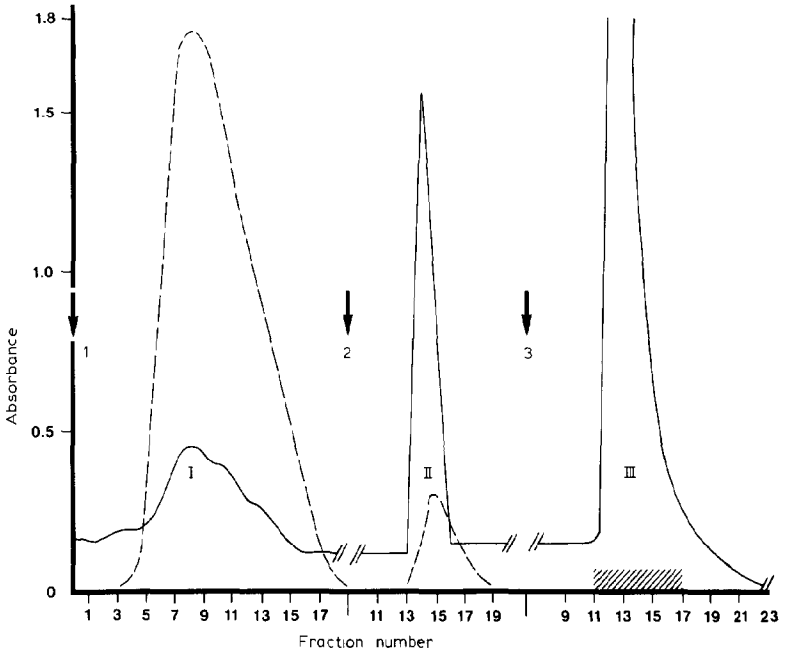

Fig. 1. Concanavalin A affinity-chromatography of GalXM. [Each fraction contained 2.0 mL of eluate collected at 10-min intervals. Key: ——, at 206 nm; ------, total carbohydrate at 490 nm; and shaded, serologically active.]

sequential elution with 3 different buffer-systems. A typical elution-pattern obtained at 206 nm is shown in Fig. 1. Peak I was not retained by the affinity matrix, and 70% of the sample applied was recovered in the starting buffer. Peak II, the borate-buffer eluate, accounted for only 3% of the original column load. Peak III, which was eluted with Me α-D-Man*p* and, therefore, was most probably bound to the immobilized con A, consisted of 12% of the applied sample.

TABLE I

COMPOSITION ANALYSIS OF GalXM FRACTIONS

GalXM	*Molar ratios*						
	Xyl	*Man*	*Glc*	*Gal*	*GlcA*	*Phosphate*	*Peptide (%)*
Unfractionated Sepharose CL-6B–con A	1	2.1 ±0.3[a]	0–0.06	1.9 ±0.3[a]	0.12 ±0.04[a]	0.20	10[b]
Peak I	1	1.8	0	1.9	0.20	0.01	3[c]
Peak II	1	1.5	0.03	1.8	0.17	0.02	2[b]
Peak III	1	7.5	0	1.3	0.08	2.2	n.d.[d]
	1	7.6	0	0.8	0.04	1.8	21[c]

[a]95% confidence limit. [b]Determined by ninhydrin after acid hydrolysis. [c]Determined by amino acid analyses. [d]n.d., not determined.

TABLE II

COMPARISON OF % OF TOTAL AMINO ACIDS AND AMINO SUGARS

Amino acid	*Sepharose CL-6B*	*Con A, Peak III*
Alanine	16.6	12.4
Arginine	1.6	1.5
Aspartic acid	10.2	9.5
Glutamic acid	8.0	4.7
Glycine	8.1	6.9
Histidine	0.8	0.7
Isoleucine	2.6	1.5
Leucine	4.0	2.6
Lysine	2.0	1.1
Phenylalanine	2.0	1.5
Serine	*21.0*	*28.7*
Threonine	14.0	16.0
Tyrosine	1.6	1.5
Valine	5.2	2.9
2-Amino-2-deoxyglucose	*1.7*	*8.4*
Total peptide (%)	3.3 ±1	20.6 ±0.1

Peak I contained 75% of neutral carbohydrate, which was present as xylose:mannose:galactose in the molar ratios of 1:1.5:1.8, as determined by g.l.c. These values are substantially different from those observed for the unfractionated GalXM, namely, 1:2.1:1.9 (see Table I). In addition, Peak I also contained 2% of peptide, 3% of glucuronic acid, and 0.3% of phosphate. Glucose, when present in GalXM, was found in Peak I only.

Peak III, the Me α-D-Man*p* eluate, was composed of 53% of neutral carbohydrate, which was present as xylose:mannose:galactose in the molar ratios of 1:7.6:0.8 (see Table I). These values represent a drastic change from those (1:2.1:1.9) observed for the unfractionated GXM (see Table I). In addition to the extensive enrichment in mannose, Peak III also contained 20% of peptide (see Table II), 0.04% of glucuronic acid, and 6% of phosphate. Of particular interest was the large increase in the proportion of 2-amino-2-deoxyglucose, and the high level of serine and threonine (see Table II), as this may indicate that the Peak III polysaccharide is a mannoprotein.

Peak II was very similar to Peak III in neutral carbohydrate content (50%, compared to 53%) and in the xylose:mannose:galactose molar ratios of 1:7.5:1.3, compared to 1:7.5:0.8 (see Table I). Peak II also contained 0.4% of uronic acid and 6% of phosphate.

The proton-decoupled, ^{13}C-n.m.r. spectra of Peak III and *Saccharomyces cerevisiae* mannan are shown in Fig. 2. A comparative examination of the ^{13}C-n.m.r. data pertaining to the resonances assigned to the anomeric centers of yeast mannans[18,19] indicated that a close similarity existed between Peak III and the mannans obtained from *Metschnikowia reukaufii* and *S. cerevisiae* (see Fig. 2).

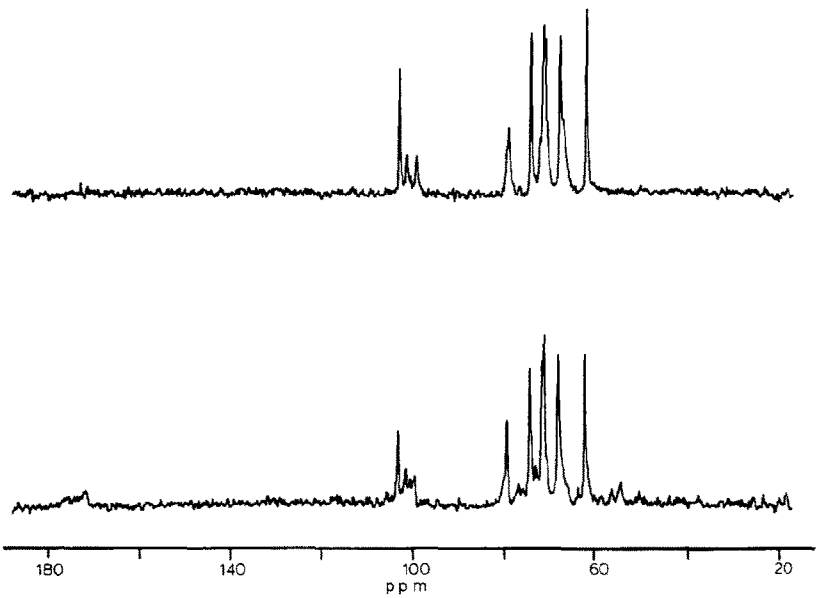

Fig. 2. The ^{13}C-n.m.r. spectra of D-mannans of *Saccharomyces cerevisiae* (top) and *Cryptococcus neoformans*, Peak III (bottom).

These polysaccharide models are composed of a main chain consisting of (1→6)-linked α-D-Man*p*, and are substituted with oligosaccharide side-chains having (1→3)- and (1→2)-linked α-D-Man*p* residues.

The ^{13}C-n.m.r. resonance at 103.6 p.p.m. arises from C-1 of the nonreducing and 3-*O*-substituted internal residues. The absorptions at 101.9 and 99.3 p.p.m. are assigned to 2-*O*- and 2,6-di-*O*-substituted Man*p*, respectively. The resonance ob-

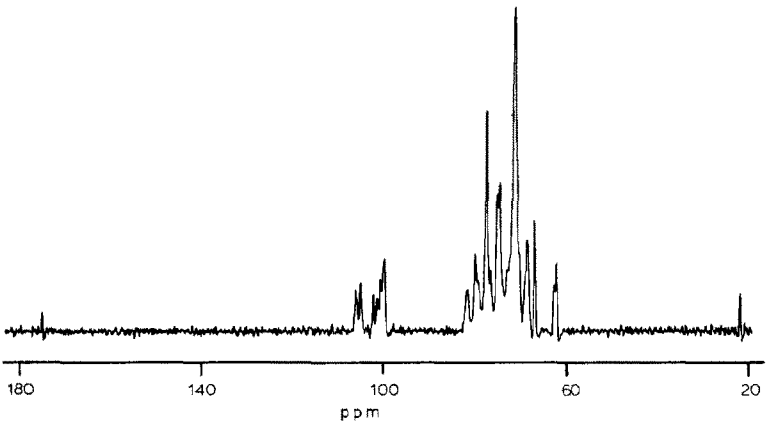

Fig. 3. The ^{13}C-n.m.r. spectrum of *Cryptococcus neoformans*, Peak I.

served at lowest intensity, 101.0 p.p.m., is assigned to unsubstituted, (1→6)-linked α-D-Man*p*. These tentative, linkage assignments are consistent with the observed affinity Peak III exhibited for con A.

The unfractionated, GalXM polysaccharide reacted strongly with antiserum raised against a synthetic glycoprotein containing the hapten[20] α-D-Man*p*-(1→2)-[α-D-Man*p*-(1→6)]-α-D-Man*p*. This observation is consistent with the foregoing linkage assignment, as well.

The major resonances observed between 79.96 and 62.7 p.p.m. are characteristic for mannans. The other, low-intensity resonances observed in the spectrum of Peak III probably result from the xylose and galactose residues known to be present in the sample. It is not known whether they are integral constituents of Peak III, or simply some Peak I polysaccharide which has been co-eluted. The ^{13}C-n.m.r. absorption spectrum obtained for Peak I was extremely complex, and a meaningful interpretation was not possible at this time (see Fig. 3). However, the material in Peak I is partially *O*-acetylated, and that in Peak III is not, as indicated by the presence and the absence, respectively, of the characteristic methyl resonance at 21.9 p.p.m. (see Figs. 2 and 3).

Peak III was found to be ~100 times as active serologically as Peak I, as determined by enzyme immunoassay [20]. This difference in activity may be due to the probability that Peak III is a mannoprotein. An assessment of the applicability of Peaks I and III for use in the specific diagnosis of cryptococcosis has been made[20]. Preliminary studies indicated that polysaccharides equivalent to Peaks I and III may be present in other serotypes, and also in acapsular mutants of *C. neoformans*.

The minor, capsular polysaccharide, the galactoxylomannan of *C. neoformans*, was fractionated, by their differing affinity for con A, into two biochemically distinct entities, a galactoxylo-enriched component (Peak I, nonbound) and a mannoprotein (Peak III, bound). The molar ratios for xylose:mannose:galactose for the mannoprotein and the galactoxylo-enriched polysaccharide, respectively, were 1:7.6:0.8 and 1:1.5:1.8.

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