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Glucuronoxylomannan of *Cryptococcus neoformans* serotype B: structural analysis by gas-liquid chromatography–mass spectrometry and ^{13}C-nuclear magnetic resonance spectroscopy

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ABSTRACT

The major extracellular polysaccharide (glucuronoxylomannan, GXM) from six strains of *Cryptococcus neoformans* serotype B was characterized by gas–liquid chromatography (g.l.c.), g.l.c.–mass spectrometry (g.l.c.–m.s.), and nuclear magnetic resonance (n.m.r.) spectroscopy. Ultrasonic irradiation (u.i.) was used to reduce the mol.wt. of native GXM from 9.75×10^5 to 1.15×10^5 without apparent change in its composition (GXM-S). The Xyl*p*:Man*p*:Glc*p*A molar ratio of the GXM and GXM-S from the six strains of *C. neoformans* serotype B is approximately 3.5:3.0:0.6. GXM-S was *O*-deacetylated (GXM-D) by treatment with NH_4OH. The ^{13}C-n.m.r. analysis of GXM-D gave spectra that served as characteristic fingerprints of the structure and also facilitated the assignment of the anomeric carbon resonances to specific structural moieties present in GXM-D. The GXM-D from each serotype B strain was found to be similar by ^{13}C-n.m.r. spectroscopy. The structure contains a linear (1→3)-α-D-Man*p* backbone substituted with 2-*O*-β-Glc*p*A and 2-*O*-β-Xyl*p*. β-Xyl*p* is also O-4 linked to the Man*p* substituted with Glc*p*A. In addition, a model for the disposition of the Xyl*p* and Glc*p*A side chain substituents along the mannopyranan backbone is proposed, based upon results from the combination of g.l.c.–m.s. and ^{13}C-n.m.r. spectroscopy.

INTRODUCTION

The major capsular polysaccharide of *Cryptococcus neoformans* is a high mol. wt. glucuronoxylomannan (GXM)[1,2] that appears in quantity in the medium during growth of the yeast. GXM is antiphagocytic[3] and tolerogenic[4], and it is believed to be responsible for serotype (A, B, C, or D) specificity[5]. The major structural element of GXM from each of the serotypes is a linear 6-*O*-acetyl(1→3)-α-D-mannopyranan that is substituted with β-D-xylopyranosyl (Xyl*p*) and β-D-glucopyranosyluronic acid (Glc*p*A) residues[1,2]. However, the proposed models of the GXM structure are based on data obtained from one or two strains of each serotype.

Blandamer and Danishefsky[6] first reported the composition of the soluble capsular polysaccharide of serotype B. However, the data showed that the analyte was a

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composite of cell-envelope polysaccharides. Bhattacharjee *et al.*[7] later purified the major exopolysaccharide of *C. neoformans* serotype B by ion-exchange chromatography. The methylation data showed that all the mannopyranosyl (Man*p*) residues were mono- or di-substituted with Xyl*p* and Glc*p*A. Two conflicting sets of methylation data were unintentionally presented. One set showed Glc*p*A residues in a (1→4) linkage, while the second set of data was consistent with Glc*p*A in a (1→2) linkage. This report is the sole source of structural data for serotype B. Herein, we present data from six isolates of *C. neoformans* serotype B to substantiate its GXM model structure and to determine its constancy.

EXPERIMENTAL

Native and modified D-*glucuronoxylomannan.* — *(a) (*O-*Acetyl)-*D-*glucuronoxylomannan (GXM).* NIH strains 444, 3939, 184, 409 and Centers for Disease Control strains B4545 and B4544 were grown in 2 L of a chemically defined broth containing 2% glucose for 5 days at 30° as previously described[8]. Exopolysaccharide was recovered from the culture supernatants by precipitation with ethanol. The acidic polysaccharide was further purified by selective precipitation with hexadecyltrimethylammonium bromide (CTAB)[8].

(b) Sonicated GXM (GXM-S)[9]. GXM was dissolved in deionized water (5 $mg.mL^{-1}$) with the aid of ultrasonic irradiation (u.i.) (10–15 min) at a power setting of 7 and a 40% pulse (Cell Disrupter, Heat Systems-Ultrasonic Inc., Model R225R). Nitrogen was bubbled through the solution, and the temperature was maintained below 20° with a circulating bath. The u.i. treatment was continued for 2 h after complete dissolution of the GXM was observed. Aliquots (0.5 mL) were removed at timed intervals for the determination of mol. wt. by gel filtration chromatography. The GXM-S in the remaining solution was recovered by lyophilization.

(c) O-*Deacetylated GXM (GXM-D).* Polysaccharide solutions (GXM-S, 2.5 $mg.mL^{-1}$ H_2O) were adjusted to pH 11.0 with concentrated NH_4OH and incubated for 24 h at room temperature. The *O*-deacetylation was followed by ^{13}C-n.m.r. spectroscopy and by the Hestrin[10] method for determination of *O*-acetyl groups. The solution was dialyzed overnight and the *O*-deacetylated GXM-D was recovered by lyophilization.

(d) Lithium-treated GXM (XM). The Glc*p*A residues were selectively cleaved by treatment with lithium metal in ethylenediamine[11]. Briefly, dried GXM-S (100 mg) in dry ethylenediamine (20 mL) was stirred with lithium metal for 3 h under argon. Lithium metal was added as necessary to maintain the dark blue color, and methanol (6 mL) was added to terminate the reaction. The residual ethylenediamine was removed *in vacuo* over concentrated H_2SO_4. The slurry was exhaustively dialyzed *vs.* deionized water and then lyophilized. (Yield, 40%).

(e) Reduced GXM (GXM-R). GXM (20 mg) was dissolved in H_2O (5 mL), and the pH was adjusted to pH 4.7. 3-(3-Dimethylaminopropyl)-1-ethylcarbodiimide·HCl (15 mg) was added slowly during 1 h with stirring, while the pH was maintained between pH 4.7 and 5.0 by adding 0.05M HCl[12]. After stirring for an additional 1 h, $NaBH_4$ (0.3 g)

was added in small portions while maintaining the pH between pH 7 and 8. The pH was maintained at pH 7–8 for an additional 1 h after all the $NaBH_4$ was added. The solution was then made slightly acidic (~pH 5) to destroy the remaining $NaBH_4$. The solution was then dialyzed *vs.* deionized water and lyophilized. Complete reduction of all the Glc*p*A residues required three successive treatments with $NaBH_4$. (Yield, 53%).

Analytical methods. — (a) Colorimetric assays. Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen[13]. The *O*-acetyl content was estimated by the Hestrin[10] procedure using D-galactitol hexaacetate as the standard.

(b) Gel-filtration chromatography. The apparent mol. wt. of each isolated GXM-S was determined by gel filtration chromatography on a Sepharose CL-6B (Pharmacia Fine Chemicals) column (89 × 1.5 cm). The column was equilibrated with 0.05M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)–0.14M NaCl buffer (pH 7.6), and then it was calibrated with polysaccharide mol. wt. standards (Polymer Laboratories, Ltd. Stow, Ohio). GXM-S (5 mg) in 1.0 mL of Tris buffer was applied to the column. The column was eluted at a flow rate of 18 $mL.h^{-1}$, and 3.0-mL fractions were collected. The column fractions were analyzed for neutral carbohydrate by the phenol–sulfuric acid method[14].

(c) Ion-exchange chromatography. The purity of each CTAB precipitate was determined by DEAE Sepharose CL-6B column (13 × 0.6 cm) chromatography. Each GXM-S (13 mg in 2.0 mL 0.01M Na_2HPO_4 buffer pH 7.1) was applied to a column equilibrated with the same buffer. The column was washed with five column-volumes of buffer, and the retained GXM-S was then eluted with 100 mL of eluent with a linear concentration gradient of 0.01M Na_2HPO_4 to 0.01M Na_2HPO_4–M NaCl, pH 7.1. The fractions (1 mL) were assayed for the presence of neutral carbohydrate[14].

(d) Gas–liquid chromatography (g.l.c.)[15]. Polysaccharides were hydrolyzed for 1 h at 120° with 2M trifluoroacetic acid (TFA). The TFA was removed by extraction[16] with moist ether (5 × 1 mL), the sample was concentrated below 40°, and then it was dried *in vacuo* over concentrated H_2SO_4. The GXM of strain 409 was analyzed by a timed course hydrolysis in 2M TFA. In addition, this strain was hydrolysed with H_2SO_4 as described by Bhattacharjee *et al.*[7,17] The constituent monosaccharides from each hydrolysate were identified and quantitated as their per-*O*-acetyl aldononitrile derivatives (PAAN)[15] with a Sigma-1 gas–liquid chromatograph (Perkin–Elmer) fitted with a RSL-300 capillary column (30 m × 0.25 mm, Alltech) and a flame-ionization detector. The initial column temperature of 200° was held for 2 min, then the temperature was increased at a rate of 7.5° $.min^{-1}$ to the final temperature of 220°, which was held for 8 min. The monosaccharides were quantitated using D-ribose as an internal standard.

(e) Gas chromatography–mass spectrometry (g.l.c.–m.s.). Dried samples (3–5 mg) were methylated by the Hakamori[18] procedure as modified by Darvill *et al.*[19] Methylated polysaccharides were purified with Sep-Pak cartridges (Millipore, Waters Associates)[20]. The purified derivatives were hydrolysed with 88% formic acid (0.5 mL) for 1 h at 100°. The formic acid was removed *in vacuo* below 40°. The residue was hydrolysed in 2M TFA (0.5 mL) for 1 h at 120°. The samples were dried *in vacuo*, and the PAAN derivatives were prepared as described[15], except the reaction temperature was

increased to 80° and the incubation time was increased to 30 min to obtain complete derivatization of the methylated sugars. The methylated, *O*-acetylated monosaccharides were analyzed with a capillary gas–liquid chromatograph (Perkin–Elmer model 8420) equipped with an ion trap detector (Perkin–Elmer) GC/ITD) and associated computer software. The gas–liquid chromatograph was equipped with an SE-54 (Supelco) column (30 m × 0.25 mm). The oven temperature program for the quantitation of the per-*O*-methyl PAAN derivatives was as follows: the initial temperature was 120°, and it was held for 1 min; the temperature was increased to 220° at a rate of 15° min^{-1}; the final temperature was held for 7 min. Methylated per-*O*-acetylated derivatives were identified by their relative elution compared to 2,3,4,6-tetra-*O*-methyl Glc*p* and published mass fragments[21,22].

(f) ^{13}*C-N.m.r. spectroscopy*. The ^{13}C- and ^{1}H-n.m.r. spectra were determined with a Varian VXR-400 n.m.r. spectrometer, equipped with a 10-mm, multinuclear probe, operated at 100.58 MHz (^{13}C) or 400.00 MHz (^{1}H). The spectral width was set at 23.980 kHz (^{13}C) or 4.000 kHz (^{1}H), and 16-K data points were collected with a 45° pulse repeated at 1.0-s intervals for 18 h. The typical solution contained 60–130 mg of GXM-D in 3.1 mL (pD adjusted to 7.6 with NH_4OH or by the addition of potassium phosphate). Spectra were recorded at 70°, and chemical shifts were measured relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) taken as 0.00 p.p.m. The deuterium resonance of the D_2O solvent served as the internal lock. The two-dimensional ^{1}H–^{13}C heteronuclear correlated spectroscopy (HETCOR) spectrum was recorded with a ^{1}H spectral width of 1100 Hz and ^{13}C spectral width of 4900 Hz. The average ^{1}H–^{13}C coupling constant (J1XH) was set to 160.0 Hz.

RESULTS

Isolation and molecular weight reduction of GXM (GXM-S). — The average yield of ethanol-precipitated polysaccharide from 2 L of broth was 1.4 ± 0.7 g. GXM comprised approximately 55 ± 18% of the precipitate. Ultrasonic irradiation for 2 h

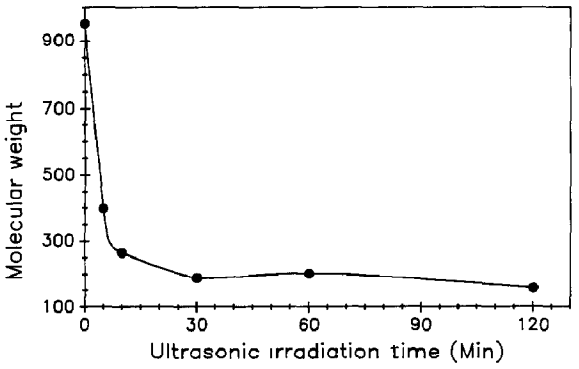


Fig. 1. Reduction of the mol. wt. of *Cryptococcus neoformans* GXM 444 by ultrasonic irradiation. An aliquot was removed at intervals, and molecular weight determined by gel-filtration chromatography.

TABLE I

Carbohydrate composition of GXM[a]

Strain	*Molar ratio*			*O-Acetyl (%)*
	Xylose	*Mannose*	*Uronic Acid*[b]	
444 native GXM	3.78	3.00	0.91	8.0
444 GXM-S	3.67	3.00	0.76	9.0
444 GXM-D	3.56	3.00	0.65	0.0
444 GXM-R	2.74	3.00	0.49[c]	n.d.[d]
3939 GXM-S	3.65	3.00	0.79	9.5
184 GXM-S	3.72	3.00	0.61	6.5
409 GXM-S	4.25	3.00	0.58	7.4
B4545 GXM-S	3.79	3.00	0.72	7.9
B4545 GXM-R	2.88	3.00	0.57	0.0
B4544 GXM-S	3.22	3.00	0.67	7.4

[a] Unless otherwise noted, composition data were obtained on sonicated GXM (GXM-S). [b] Uronic acid content determined colorimetrically. [c] Molar ratio of GlcA determined by g.l.c. as Glc after reduction. [d] Not determined.

reduced the apparent mol. wt. of the native GXM polymer (9.75×10^5) to approximately 1.20×10^5 (Fig. 1). Each GXM-S had a final mol. wt. within $\pm 1.5 \times 10^4$ of this value. In addition, each GXM-S eluted as a single peak on DEAE CL-6B at 0.27 ± 0.05M NaCl.

Carbohydrate composition. — Molar ratios of the substituent sugars were calculated relative to Man*p* taken as 3.00. The average Xyl*p*:Man*p*:Glc*p*A molar ratio of eight GXM samples from strain 444 was 3.78 ± 0.39:3.00:0.94 ± 0.06 (Table I). The Xyl*p*:Man*p* molar ratios of GXM-S and GXM-D were not significantly different from the values obtained with the native GXM. However, the Glc*p*A molar ratio appeared to be lower in GXM-S and GXM-D. The Xyl*p*:Man*p* molar ratio for most of the isolates, except for strain B4544, was similar to that obtained for strain 444 (Table I). The GXM-S from strain B4544 was lower in Xyl*p* content (Xyl:Man 3.22) than any of the other strains. The variation in the Glc*p*A molar ratio for all the GXM-S samples was greater (standard deviation $\pm 15\%$) than the average (standard deviation $\pm 6\%$) of eight GXM samples of strain 444. The percent *O*-acetyl also varied (standard deviation $\pm 16\%$) between strains. The *O*-acetyl values for strains 444 (9%) and 3939 (9.5%) were significantly higher than average (8.0%), while it was below average for strain 184 (6.5%).

The GXM-S from strain 409 was hydrolysed with H_2SO_4 as described by Bhattacharjee *et al.*[7] The resulting Xyl*p*:Man*p* ratio was 3.19:3.00. In addition, a timed hydrolysis in 2M TFA was carried out on the GXM-S from strain 409. A time-dependent range of Xyl*p*:Man*p* molar ratios was obtained that ranged from 3.9:3.0 to 1.1:3.0. A plot of the release of Xyl*p* and Man*p* *vs.* time showed that the release and degradation of Man*p* was erratic, while Xyl*p* showed a linear decay. The effect of Glc*p*A on the acid-catalyzed hydrolysis of GXM was eliminated by its conversion to Glc*p*, and the

TABLE II

Methylation analysis of GXM[a]

Methylated sugar[b] *(PAAN derivative)*	*444*	*444*[c]	*3939*	*184*	*409*	*4545*	*4545*[d]	*4545*[e]	*4544*
2,3,4-Me_3-Xyl	3.12	2.32	3.55	2.87	3.32	2.76	3.46	3.28	2.61
2,3,4,6-Me_4-Glc		1.01					1.09		
2,6-Me_2-Man								0.94	
4,6-Me_2-Man	2.05	2.03	2.13	2.18	2.04	1.97	2.11	1.90	2.17
6-Me-Man	0.95	0.97	0.87	0.82	0.96	1.03	0.89	0.16	0.82

[a] Unless otherwise indicated, methylation analysis was done on *O*-deacetylated GXM (GXM-D). [b] 2,3,4-Me_3-Xyl = 2,3,4-tri-*O*-methyl-D-xylose, etc. [c] 444 GXM-R. [d] 4545 GXM-R. [e] 4545 XM.

timed hydrolysis was repeated. Extrapolation of the linear decay data for the three substituents (Xyl*p*, Man*p*, Glc*p*) to zero time gave a Xyl*p*:Man*p*:Glc*p* molar ratio of 2.73:3.00:0.78. A similar molar ratio (2.49:3.00:0.83) was obtained when GXM-R strain 409 was hydrolyzed with 2M TFA for 1 h at 120° in 2M TFA.

G.l.c.–m.s. analysis. — The results from g.l.c.–m.s. analyses (see Table II) showed that no major observable differences exist between the serotype B strains. The major variable in the analyses was the amount of 2,3,4-tri-*O*-methylXyl*p*. The average Xyl*p* molar ratio from three methylations of strain 444 GXM-D was 3.03 ± 0.12. The average Xyl*p*:Man*p*:Glc*p*A molar ratio for all strains was 3.03 ± 0.35:3.00:0.91 ± 0.08. As in the g.l.c. analysis, strain B4544 had a slightly lower Xyl*p* content than the other strains (molar ratio 2.61).

The only methylated sugars detected in each GXM-D were 2,3,4-tri-*O*-methyl-Xyl*p*, 4,6-di-*O*-methylMan*p*, and 6-*O*-methylMan*p*. The amount of 2,3,4,6-tetra-*O*-methylGlc*p* released from GXM-R of strains 4545 and 444 was approximately equal to the amount of 6-*O*-methylMan*p*. 2,6-Di-*O*-methylMan*p* appeared in the g.l.c.–m.s. analysis of the XM of strain 4545. The appearance of this derivative was accompanied by the almost total disappearance of 6-*O*-methylMan*p*. Thus, Glc*p*A is linked to the C-2 of Man*p*. Uronic acid analysis of 4545 XM showed that some Glc*p*A (1.7%) remained after the lithium treatment.

^{13}C-n.m.r. spectrum of GXM-D. — Figure 2 illustrates the dramatic improvement in the ^{13}C-n.m.r. spectrum that occurred by the removal of the *O*-acetyl groups from GXM-S. The resonance at 66.12 p.p.m., characteristic of 6-*O*-acetyl substitution in the spectrum of GXM-S is absent in the spectrum of GXM-D. In addition, there was a concomitant increase in the intensity of the resonance at 63.43 p.p.m. due to the generation of an unsubstituted C-6 of Man*p*[16].

Resonance assignments. The ^{13}C-n.m.r. resonances of GXM-D were compared to the chemical shift data reported for a *C. neoformans* serotype A-variant[16] and also that for a glucuronomannan derived from *Tremella mesenterica*[23]. Table III lists the chemical shift assignments for GXM-D. The anomeric resonances of Xyl*p* are located the farthest downfield at 106.70 p.p.m., (1→4)-linked; 106.09 p.p.m., (1→2)-linked; and 105.95 p.p.m., (1→2)-linked. The chemical shifts of carbons C-2–C-5 of Xyl*p* remained

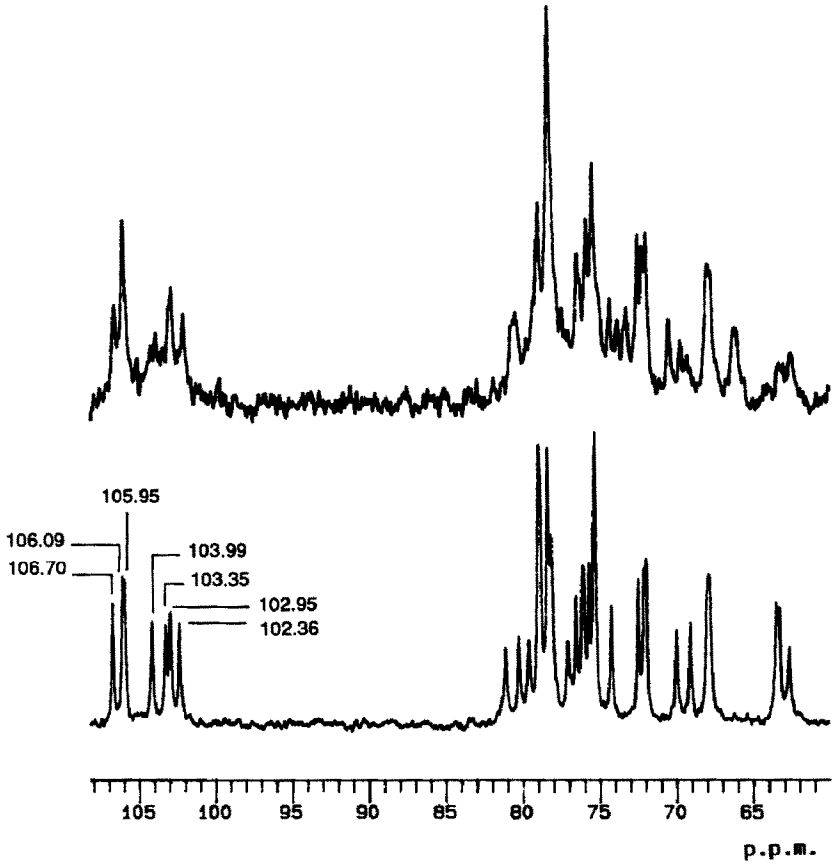


Fig. 2. Proton-decoupled ^{13}C-n.m.r. spectra (70°, 100 MHz) of GXM-S 409 (sonicated, top) and GXM-D (sonicated and *O*-deacetylated, bottom).

essentially unchanged from free Xyl*p*, as expected. The anomeric carbon resonances due to Man*p* residues were observed the farthest upfield (relative to other anomeric resonances) (Fig. 2). The resonances at 102.36 and 102.95 p.p.m. are due to the C-1 of Man*p* residues linked at C-2 by β-Xyl*p*. The resonance at 103.35 p.p.m. is due to the Man*p* disubstituted by 4-*O*-β-Xyl*p* and 2-*O*-β-Glc*p*A (see below). Unfortunately, a lack of model compounds for the fully substituted mannopyranan backbone made it impossible to assign many of the chemical shifts due to the carbons of the Man*p* residues, other than those of the anomeric carbons.

The resonance at 103.99 p.p.m. is assigned to the C-1 of β-Glc*p*A. This resonance, as well as the other assignments for Glc*p*A, were tentatively made by comparing spectra obtained at pD 4.5 and pD 8.0 (ref. 23). The resonance due to the C-1 of Glc*p*A shifted upfield (from 104.21 p.p.m. to 103.99 p.p.m.) as the pD was changed from pD 4.5 to 8.0 (Table IV). The resonance which gave the greatest shift downfield (102.93 p.p.m. to 103.35 p.p.m.) was tentatively assigned to the Man*p* to which Glc*p*A was attached. The remaining carbon resonances of Glc*p*A shifted downfield by varying amounts.

TABLE III

^{13}C-N.m.r. resonance assignments of GXM-D

Residue	*Saccharide*	*Chemical shift of residue carbons*[a]					
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
β-Xyl*p*	Meβ-Xyl*p*[b]	106.35	75.29	78.18	71.66	67.48	
	DGXM-S[b]	105.55	75.32	78.21	71.87	67.70	
	GXM-D						
	-(1→4)	106.70	75.35	78.33	71.96	67.80	
	-(1→2)	106.09	75.35	78.33	71.96	67.80	
	-(1→2)	105.95	75.35	78.33	71.96	67.80	
β-Glc*p*A	Meβ-Glc*p*A[b]	105.8	75.3	78.0	73.8	77.1	179.1
	DGXM-S[b]	104.98	75.18	78.00	74.21	79.31	176.80
	GXM-D	103.99	75.33	77.15	74.33	79.30	179.97
α-Man*p*	DGXM-S[b]	102.91	72.34	81.27	69.60	N.D.[c]	63.20
	GXM-D						
	disubstituted with (1→2) Glc*p*A and (1→4) Xyl*p*						
		103.35	N.D.	N.D.	N.D.	N.D.	N.D.
	substituted with only (1→2) Xyl*p*						
		102.95	72.12	81.20	N.D.	N.D.	63.43
		102.36	N.D.	N.D.	N.D.	N.D.	N.D.

[a] In p.p.m. measured relative to internal sodium 4,4,-dimethyl-4-silapentane-1-sulfonate (DSS) at pD 8.
[b] Reference 16. [c] Not determined.

TABLE IV

Effect of pD changes on anomeric carbon resonances

Residue	*Chemical shift*[a]	
	pD 8.0	*pD 4.5*
-(1→4)-Xyl*p*	106.69	106.67
-(1→2)-Xyl*p*	106.11	106.03
-(1→2)-Xyl*p*	105.93	105.87
-(1→2)-Glc*p*A	103.99	104.21
Man*p* disubstituted with Glc*p*A and Xyl*p*	103.35	102.93
Man*p* substituted with (1→2)-Xyl*p*	102.94	102.93
Man*p* substituted with (1→2)-Xyl*p*	102.35	102.30

[a] In p.p.m. relative to internal sodium 4,4,-dimethyl-4-silapentane-1-sulfonate (DSS).

The identification of the C-1 resonance of Glc*p*A was also confirmed by HETCOR. The complexity of the ^{13}C (see Fig. 2) and ^{1}H (see Fig. 3) spectra in all regions except the anomeric region prevented detailed analysis of the HETCOR; however, analysis of the anomeric region facilitated the assignment of the proton and carbon resonances due to H-1 and C-1, respectively. The three ^{1}H resonances at ~5.2 p.p.m. are due to the H-1 of the (1→3)-α-D-Man*p* residues since the cross-peaks only correlated with the C-1 mannose resonances. The farthest upfield of these three was assigned to the C-1–H-1 correlation of the Man*p* to which Glc*p*A is attached. The assignment was

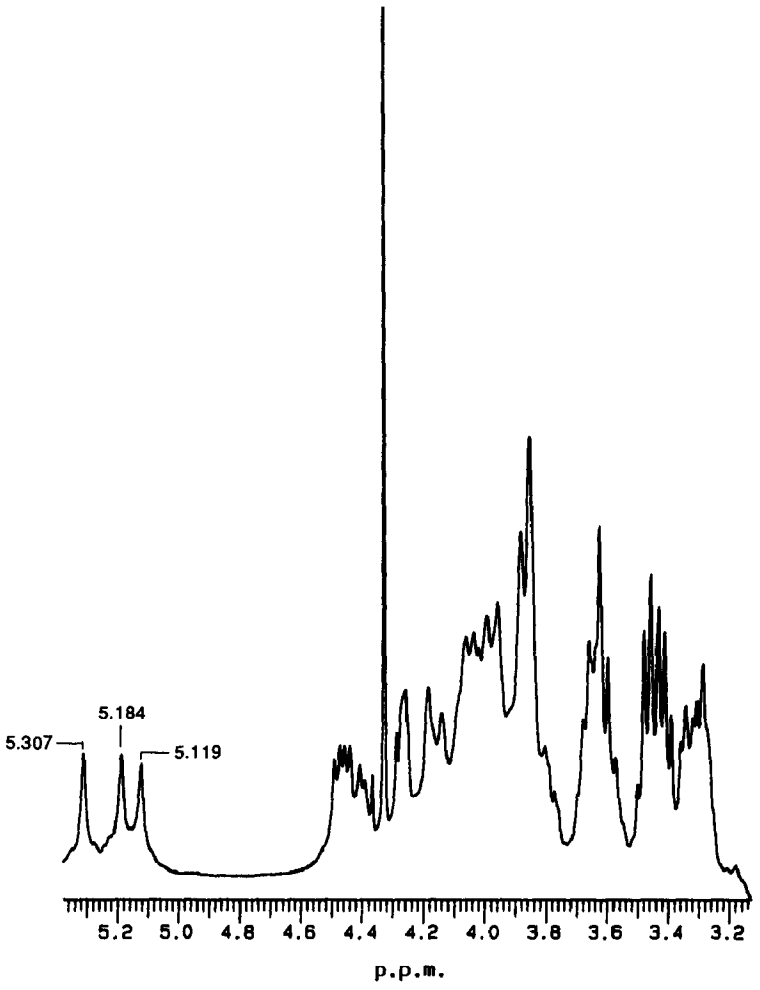


Fig. 3. ^{1}H-n.m.r. spectrum of GXM-D 409 (70°, 400 MHz).

based on the relative location of the C-1–H-1 cross-peak in the glucuronomannan of *T. mesenterica*[23]. The assignment of the upfield cross peak in GXM-D correlated with the assignment of the Man*p* to which Glc*p*A was attached based on the pD-induced change in the chemical shift of the C-1 of Man*p*. Based on the data on the D-glucuronomannan of Tremella[23], the H-1 of Glc*p*A was located in the region of 4.45 p.p.m. This H-1 resonance correlated with the C-1 previously assigned to Glc*p*A based on pD effects. The H-1 of 4-*O*-*β*-Xyl*p* resonated at 4.4 p.p.m. The 2-*O*-*β*-Xyl*p*, which was located the farthest downfield (106.1 p.p.m.) in the ^{13}C-n.m.r. spectrum, resonated at 4.5 p.p.m. in the ^{1}H-n.m.r. spectrum. However, the H-1 of the other 2-*O*-*β*-Xyl*p* (C-1, 105.9 p.p.m.) resonated at 4.25 p.p.m.

Additional evidence for the assignments of the carbon resonances due to the Glc*p*A residues was obtained when GXM-D from strain B4545 was selectively cleaved

with lithium metal to give XM. All of the resonances assigned to the carbons of Glc*p*A were absent in the spectrum of the XM. The two resonances at 103.99 p.p.m. and 103.35 p.p.m., due to the C-1 of β-Glc*p*A and C-1 of α-Man*p* being O-2 glycosylated by β-Glc*p*A and O-4 glycosylated by β-Xyl*p*, were reduced to a single resonance at 104.46 p.p.m. due to the C-1 of α-Man*p* being O-4 glycosylated by β-Xyl*p*.

DISCUSSION

Traditionally, g.l.c. and g.l.c.–m.s. are used to characterize carbohydrates. Although these are highly useful methods, they are destructive and depend on complete hydrolysis of the polysaccharide chain to monomeric sugars. The lability of Xyl*p* in acid solution compared to the (1→3)-mannopyranan backbone and the presence of Glc*p*A makes the total hydrolysis of GXM to its monomeric components impossible without the concomitant decomposition of some Xyl*p* residues; this makes quantitation of the GXM composition difficult. The comparison of data between laboratories is difficult because different hydrolysis methods were used.

The molar ratio of GXM from strain 409 as determined in this study is substantially different from the molar ratio reported by Bhattacharjee *et al.*[7] on strain 444. In addition, even under hydrolysis conditions described by the same group, the Xyl*p*:man*p* molar ratio for the GXM from strain 409 was higher than that previously reported for strain 444. The observed molar ratio of Xyl*p* decreased when GXM purified directly from the ethanol precipitate by ion-exchange chromatography was analyzed (data not shown), rather than that obtained by CTAB precipitation. The ion-exchange purified GXM also contained small quantities of Gal*p* and Glc*p*. Therefore, the molar ratio of Xyl*p* may have been lower because of the presence of contaminating heterologous polysaccharides[24]. Analytical data obtained by the timed hydrolysis of GXM-S and GXM-R demonstrated the difficulty in hydrolyzing native GXM to its component saccharide residues. Other researchers have reported the detection of 2-*O*-(β-D-glucopyranosyluronic acid)-D-Man*p* after hydrolysis[7,25]. The formation of this disaccharide results the erratic release of monomeric Man*p* which interferes with the estimation of true molar ratios for Xyl*p*:Man*p*:Glc*p*A in GXM. If the hydrolysis conditions are extended to release the Man*p* substituents, then some destruction of Xyl*p* is observed.

The methylation data obtained from each strain in this study was similar to the methylation data previously obtained[7]. The ratio of 2,3,4-tri-*O*-methylMan*p*, 4,6-di-*O*-methylMan*p*, and 6-*O*-methylMan*p* obtained by Bhattacharjee *et al.*[7] was 2.5:2.0:1.0. These derivatives were obtained in approximately the same proportions for the six GXM strains described herein. The amount of 2,3,4,6-tetra-*O*-methylGlc*p* in GXM-R was approximately equal to the molar ratio of 6-*O*-methylMan*p*. The almost total absence of 6-*O*-methylMan*p* in XM and the appearance of an equal quantity of 2,6-di-*O*-methylMan*p* showed that all the Glc*p*A substituents were linked O-2 to Man*p*. These data show that Glc*p*A is linked O-2 to a Man*p* which is already substituted O-4 by Xyl*p*. The data clarifies the discrepancy in the two possible structures inadvertently

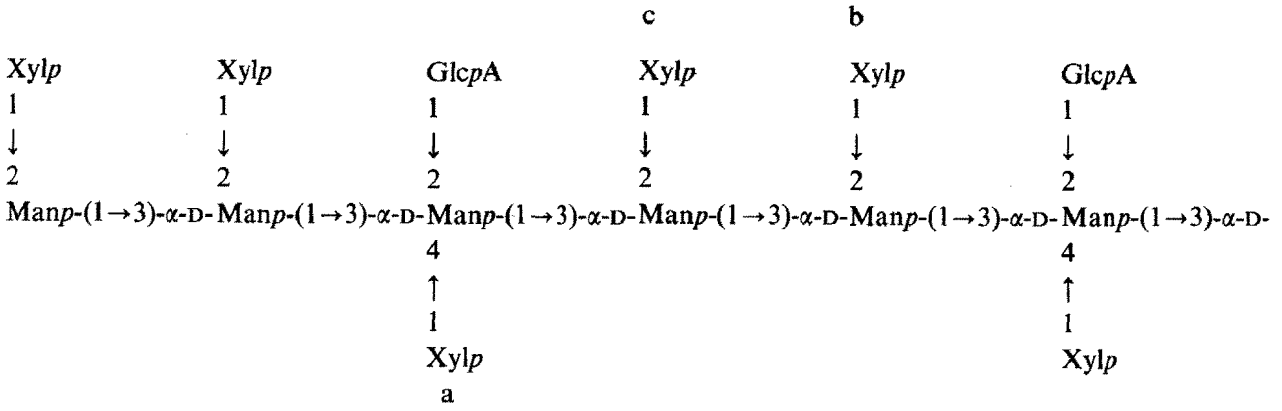
presented by Bhattacharjee *et al.*[7] in which Glc*p*A was shown to be linked O-2 and O-4, respectively.

The characterization of GXM-S from six serotype B strains of *C. neoformans* using identical analytical methods provides a true evaluation of strain-related variability. The composition and structure of the six GXM samples were alike. The Xyl*p*:Man*p*:Glc*p*A molar ratios obtained by g.l.c. and g.l.c.–m.s. analysis of the GXM from each strain were similar. The major difference in composition between strains was the extent of *O*-acetylation. However, the variation in *O*-acetyl content may not reflect a true difference in structure, but may be due to the loss of a portion of the *O*-acetyl substituent during the growth and isolation of GXM.

Nuclear magnetic resonance (n.m.r.) spectroscopy provides a non-destructive technique for characterizing the structure of oligo- and poly-saccharides. The high mol. wt. and solution viscosity of native GXM gave a poorly resolved ^{13}C-n.m.r. spectrum. It was apparent that the analysis of GXM by ^{13}C-n.m.r. spectroscopy would require alteration of the native polymer. Ultrasonic irradiation reduced the mol. wt. of native GXM (GXM-S). This allowed the analysis of solutions containing increased concentrations of GXM-S (~4%) in D_2O. The ^{13}C-n.m.r. spectrum of the *O*-deacetylated GXM-S demonstrated a dramatic increase in resolution. Obviously, the polysaccharide has undergone a substantial change. Since the molar ratios of the substituent sugars remained the same after *O*-deacetylation, then the increased resolution was probably due to both a change in conformation and in a reduction in the number of different environments of each sugar moiety due to the loss of the *O*-acetyl groups. The increased resolution facilitated the comparison of polysaccharides from related strains. The GXM-D from each strain of *C. neoformans* gave an identical ^{13}C-n.m.r. spectrum. Only one strain (B4544) gave a spectrum that was somewhat different (data not shown). The spectrum of the GXM-D from this strain contained several minor anomeric carbon resonances, with chemical shifts similar to those found in serotype A, in addition to the seven major resonances observed with the other serotype B strains. The significance of these minor resonances was not determined.

N.m.r. spectroscopy provided an alternative technique for the comparison of GXM-D from *C. neoformans*. ^{1}H-n.m.r. spectroscopy gave useful fingerprints for each GXM-D; however, it did not provide structural information owing to the broad resonances, especially in the anomeric region of 4.0–5.4 p.p.m. The broad resonances were probably due to extensive frequency overlap of closely related protons; however, assignments for the anomeric proton of Man*p*, Xyl*p*, and Glc*p*A were made. Previously[17,26], the anomeric protons of Xyl*p* and Glc*p*A were assigned to the resonances in the same downfield region where the Man*p* signals appear (approximately 5.0 p.p.m.). Two of the three downfield resonances of the anomeric protons of Man*p* present in *O*-deacetylated GXM were erroneously assigned to Xyl*p* and Glc*p*A, and the molar ratio of GXM was calculated based on these assignments[17].

The HETCOR analyses done in this study showed that the three downfield resonances were due to Man*p* residues alone and that H-1 of Xyl*p* and Glc*p*A resonate at 4.2–4.5 p.p.m., respectively. An unexpected correlation occurred between the down-



Representative model of GXM-D of *Cryptococcus neoformans* serotype B.

field carbon resonance for 2-*O*-β-Xyl*p* and a proton resonance upfield of the HDO resonance. This represents a significant difference in the environments of the H-1 of the two O-2-linked Xyl*p* residues. However, the structural basis for the environmental differences is not obvious. The presence of an O-2-linked Xyl*p* residue other than those linked to Man*p* were not observed by either n.m.r. spectroscopy or by g.l.c.–m.s.

Resonance assignments were made by comparison with the data from two previous studies[16,23] and by HETCOR. The anomeric resonances were assigned to specific structural moieties present in serotype B GXM; however, most signals owing to the remaining carbon atoms were not assigned.

Removal of Glc*p*A from GXM-4545 did not cause an appreciable change in the resonances of O-2-linked Xyl*p* and 2-*O*-β-D-glycosylated Man*p*. This observation may give some indication of the sequence of substituents on the mannopyranan backbone. The fact that the resonances due to 2-*O*-β-Xyl and the resonances due to α-Man*p* substituted O-2 by β-Xyl remain split after the removal of Glc*p* shows that this splitting is due to two different environments of the Man*p* residue linked O-2 by Xyl*p*. These environments are obviously not related to the presence of Glc*p*A. The construction of a space-filling model (CPK) (not shown) based on a previously described mannopyrannan model[16] shows that an O-4-linked Xyl*p* (Structure 1, Xyl*p* residue "a") residue could interact with the C-1 of a Man*p* two residues from the Man*p* to which the Xyl*p* is attached. It is possible that a Man*p* residue substituted at O-4 by β-Xyl and at O-2 by β-Glc*p*A lies between two Man*p* residues substituted at O-2 by β-Xyl. This would provide two different environments for each 2-*O*-β-Xyl*p* (b and c) and for the Man*p* residues to which they are attached. The effect that 4-*O*-β-Xyl*p* or 4-*O*-β-Glc*p*A has on the secondary and tertiary structure of GXM is not known; therefore, the conformation of the mannopyranan backbone in GXM may be different from the mannopyranan earlier described[16].

The present paper reports the use of extended u.i. to reduce the mol. wt. of the serotype B GXM to a limiting value. The reduction in viscosity due to the lowered mol. wt. improved the spectral resolution in the ^{13}C-n.m.r. spectrum. A further improvement in the resolution of the spectrum was obtained after removal of the *O*-acetyl groups. Resonance assignments were used to characterize the modified polysaccharides. A model for the disposition of the substituents along the mannopyranan backbone is proposed based on the environmental effects on the chemical shifts for the anomeric carbon nuclei.

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