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Facilitated Isolation, Purification, and Analysis of Glucuronoxylomannan of *Cryptococcus neoformans*

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Cryptococcus neoformans **was cultured in a chemically defined medium. The culture was adjusted to 0.25% formaldehyde or autoclaved after 5 days of growth at 35°C, and a cell-free supernatant was obtained by centrifugation. Solid calcium acetate was added to the supernatant to give a 5% solution, and the pH was adjusted to ~5 with glacial acetic acid. The polysaccharide (PS) was precipitated by the addition of 3 volumes of 95% ethanol. The PS was dissolved in 0.2 M NaCl, and insoluble calcium salts were solubilized by the addition of several drops of glacial acetic acid. The PS solution was treated by ultrasonic irradiation for 15 min. This concurrently decreased the molecular weight of the PS and reduced the viscosity of the solution. The ultrasonically irradiated PS was precipitated by differential complexation with hexadecyltrimethylammonium bromide at 23°C, the complex was dissolved in 1 M NaCl, and the glucuronoxylomannan was precipitated by adding 3 volumes of ethanol. The glucuronoxylomannan was dissolved in 1 M NaCl and then ultrasonically irradiated for 2 h to reduce the molecular mass to a limiting value of ~100 kDa (GXMS). The purified GXMS was centrifuged, dialyzed, and finally recovered by lyophilization. GXMS was chromatographed on DEAE-cellulose at reasonable concentrations without the complication of high solution viscosity. The sugar composition and structure of GXMS were determined by gas-liquid chromatography, permethylation gas-liquid chromatography-mass spectrometry, and ^{13}C nuclear magnetic resonance spectroscopy. The improved solution characteristics of GXMS were ideal for the determination of its chemical and serological properties.**

The cell envelope of *Cryptococcus neoformans* is composed of a rigid wall, constituted mainly of complex glucans (18); a capsular polysaccharide (glucuronoxylomannan [GXM]) composed of mannose, xylose, glucuronic acid, and *O*-acetyl (2, 8); and at least two minor polysaccharide antigens (8,11,29). GXM is antiphagocytic (6, 20) and tolerogenic (1, 4, 19, 23) and contributes to the virulence of this yeast. The importance of GXM in governing serotype specificity is inferred from the observation that acapsular mutants are untypable (17, 19). *C. neoformans* was reported infrequently in disease until it emerged as a primary cause of opportunistic infections associated with AIDS (7, 13). The original models that depict precise molar compositions and glycosidic linkages for GXM were based on data from one or two isolates representative of the four recognized serotypes (2, 8). A comprehensive study of GXM structure has shown that the original models were an oversimplification (28a). Structural elements thought to be characteristic of one serotype have been identified in others (28a, 29a). Also, heterogeneity in structure within a particular serotype is common. This is particularly true for serotype A strains, the serotype found almost exclusively in patients with AIDS. At present, there is no information available for comparing the structure of GXM elaborated by isolates of *C. neoformans* from AIDS patients with the existing data for GXM obtained from those isolates found in the normal population or the environment.

A data base has been established that distinguishes GXM on the bases of monosaccharide composition, methylation analysis, and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The previously reported methods (2, 8) for the isolation, purification, and structural analysis of the high-molecular-weight and viscous forms of GXM from multiple isolates is time-consuming and can produce misleading results. We now report easier procedures for the complete characterization of GXM from *C. neoformans* obtained from autoclaved culture supernatant.

MATERIALS AND METHODS

Cultures and GXM production. *C. neoformans* serotype A strains 6, 98, and 110 were obtained from T. G. Mitchell (Duke University) (26). The A serotype of the three strains was confirmed in the laboratory of J. E. Bennett (National Institutes of Health). Each isolate was grown in 1 liter of a chemically defined broth containing 2% glucose for 5 days at 35°C as described previously (11).

Primary isolation of PS. The culture of *C. neoformans* was autoclaved for 25 min at 121°C. Sometimes cultures were treated with 0.25% formaldehyde and not autoclaved. Cells were removed by centrifugation at 16,000 × *g* for 4 h (autoclaved cultures) or 16,000 × *g* for 16 h (nonautoclaved cultures). The supernatant culture medium was decanted, and the cells were discarded. Occasionally, centrifugation of nonautoclaved culture supernatants was repeated to remove any remaining yeast cells. Polysaccharide (PS) was precipitated by the slow addition of 3 volumes of 95% ethanol, and the flask was stored at 4°C overnight. Much of the ethanol was decanted, and the remaining suspension was centrifuged to recover the polysaccharide (PS-E). The ethanol supernatant fluid was reserved. PS-E was collected by centrifugation and then it was transferred to a Buchner funnel with 80% ethanol. PS-E was washed with 95% ethanol and triturated with acetone and then with ether. After air-drying, PS-E was stored desiccated. One volume of acetone was added to the original ethanol supernatant, and the precipitate (PS-E-A) was isolated as described above. The supernatant culture

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medium from a separate flask was adjusted to 5% calcium acetate by the addition of solid reagent, and the pH was adjusted to ~5 by adding glacial acetic acid (~0.74 ml/g of calcium acetate). The formation of a small amount of precipitate was ignored. Three volumes of alcohol were added, and the precipitated polysaccharide (PS-E-CA) was isolated and stored as described above for PS-E.

Purification of GXM. The three polysaccharide fractions PS-E, PS-E-A, and PS-E-CA were purified in an identical manner. The PS was dissolved in 0.2 M NaCl (10 mg/ml). Dissolution often required overnight stirring, and insoluble calcium salts were dissolved by adding several drops of glacial acetic acid. The solution was treated with ultrasonic irradiation (UI) for 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°C with a circulating bath (29a).

Hexadecyltrimethylammonium bromide (CTAB), 3 mg per mg of polysaccharide, was added slowly to the stirred solution at 23°C. A small amount of insoluble material was removed by centrifugation at 27,000 × *g* for 15 min at 23°C. A 0.05% solution of CTAB was added slowly and with stirring to the supernatant. As the NaCl concentration decreased, the GXM-CTAB complex precipitated. The completeness of the precipitation was checked after the addition of 2 volumes of 0.05% CTAB; an aliquot of the suspension was centrifuged, and a few drops of 0.05% CTAB were added to the supernatant. If the supernatant remained clear, the entire volume was centrifuged at 12,000 × *g* for 1 h at 23°C. If more precipitate was observed, additional 0.05% CTAB was added, and the test for complete precipitation of the complex was repeated. The CTAB-GXM complex was washed with 10% ethanol in the centrifuge. The CTAB-GXM complex was dissolved in 1 M NaCl, with one-half the volume of the original solution used to dissolve the PS. The GXM solution was UI treated in 100-ml batches for 2 h (28a). The solution was dialyzed (Spectrapore 3 dialysis tubing; molecular weight cutoff, 3,500) versus running tap water for 3 days and then versus distilled water at 4°C for 2 days, clarified by centrifugation, and lyophilized. The resulting preparation was called GXMS. A portion of GXMS (125 mg in 25 ml of H_2O) was adjusted to pH 11.25 with concentrated NH_4OH and incubated for 24 h at 23°C. The sample was dialyzed and lyophilized to give the de-*O*-acetylated derivative of GXMS (GXMS-D).

Analytical methods. (i) Colorimetric assays. Detection of neutral carbohydrate was done by the phenol sulfuric acid method of Dubois et al. (14). Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen (4). *O*-Acetyl was estimated by the Hestrin procedure with dulcitol hexaacetate as the standard (16).

(ii) Ion-exchange chromatography. GXMS was dissolved in 0.01 M $NaPO_4$ buffer (pH 7.1) at a concentration of 13 mg/ml, and the solution was applied to a column (13 by 0.7 cm) of DEAE-Sepharose CL-6B equilibrated with the same buffer. The column was washed with 5 column volumes of buffer, and the sample was eluted with 100 ml of eluant over a linear concentration gradient of 0.01 M $NaPO_4$ to 0.01 M $NaPO_4$–1 M NaCl, pH 7.1, at a flow rate of 20 ml/h. The fractions (1 ml) were assayed for neutral carbohydrate (14).

(iii) GLC. For gas-liquid chromatography (GLC), GXMS was hydrolyzed for 1 h at 120°C with 2 N trifluoroacetic acid. Trifluoroacetic acid was removed by extraction with 1 ml of moist ether (five times), the sample was concentrated below 40°C, and then it was dried in vacuo over concentrated H_2SO_4. The constituent monosaccharides from the hydrolysate were identified and quantitated as their per-*O*-acetylated aldononitrile derivatives (PAAN) with a Sigma 1 gas chromatograph (Perkin Elmer) fitted with a RSL-300 capillary column (30 m by 0.25 mm; Alltech) and a flame ionization detector. The initial column temperature of 200°C was held for 2 min. The temperature was increased 7.5°C per min to the final temperature of 220°C, which was held for 8 min. The monosaccharides were quantitated by using ribose as an internal standard (28).

(iv) GLC-MS. For GLC-mass spectrometry (GLC-MS), dried samples (3 to 5 mg) were methylated by the Hakamori (15) procedure as modified by Darvill et al. (12). Methylated polysaccharides were purified with Sep-Pak cartridges (Millipore, Waters Associates) (22). The purified derivatives were hydrolyzed with 88% formic acid (0.5 ml) for 1 h at 100°C. The formic acid was removed in vacuo below 40°C. The residue was hydrolyzed in 2 N trifluoroacetic acid (0.5 ml) for 1 h at 120°C. The samples were dried in vacuo, and the PAAN derivatives were prepared (29). The methylated *O*-acetylated monosaccharides were analyzed with an 8420 capillary gas chromatograph equipped with an ion trap detector (Perkin-Elmer GC/ITD) and associated computer software. The gas chromatograph was equipped with an SPB-5 (Supelco) column (30 m by 0.25 mm). The oven temperature program for the quantitation of the per-*O*-methylated PAAN derivatives was as follows: the initial temperature was 120°C and it was held for 1 min; the temperature was increased to 220°C at a rate of 15°C per min; the final temperature was held for 7 min. Methylated per-*O*-acetylated derivatives were identified by their relative elution compared with 2,3,4,6-tetra-*O*-methyl-Glc*p* and the appearance of chacteristic mass fragments.

(v) ^{13}C NMR spectroscopy. The ^{13}C and ^{1}H NMR spectra were determined with a Varian VXR-400 NMR 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 16,000 data points were collected with a 45-degree pulse repeated at 1.0-s intervals for 18 h. The typical solution contained 60 to 130 mg of GXMS-D in 3.1 ml. Spectra were recorded at 70°C, and chemical shifts were measured relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, taken as 0.00 ppm. The deuterium resonance of the D_2O solvent served as the internal lock.

RESULTS

Purified GXMS. The yield of purified GXMS did not vary appreciably among the methods used in this study (Table 1). However, autoclaved cultures formed firm, compact pellets of dead cells. In contrast, heavily encapsulated cells gave loose mucoid pellets. Sometimes it was difficult to decant the medium free of significant yeast cell contamination without repeating the centrifugation. The autoclave method had an additional advantage: it also sterilized the culture. Therefore, it was not necessary to add formaldehyde and wait 48 h for the cells to die, nor was it necessary to do a cell viability test. Ethanol alone did not precipitate all of the GXM in the medium of the three isolates investigated; a second crop of PS was obtained after the addition of 1 volume of acetone to the ethanol supernatant (Table 1). However, the ethanol precipitation of GXM as its calcium salt at pH 5 resulted in high recovery of PS in one step. A solution of the high-molecular-weight GXM, obtained after the first precipitation, showed extremely high viscosity.

TABLE 1. Isolation of GXMS from autoclaved *C. neoformans* cultures[a]

Strain	Solvent	Amt (g) and type of PS in:		Yield (%)
		1st precipitation	CTAB precipitation	
98	Flask 1			
	Ethanol	1.15 (PS-E)	0.67	58
	Ethanol-acetone	2.37 (PS-E-A)	1.40	59
	Total	3.52	2.07	59
	Flask 2, ethanol-Ca	4.96[b] (PS-E-CA)	1.75	35
	Flask 3, ethanol-Ca	7.0[b] (PS-E-CA)	2.40	41
110	Ethanol	3.0 (PS-E)	1.70	57
6	Ethanol	2.4 (PS-E)	1.54	64
	Ethanol	2.64 (PS-E)	1.75	66
6[c]	Ethanol	1.96 (PS-E)	1.2	61
	Ethanol	3.50 (PS-E)	1.96	56

[a] First precipitation refers to the primary isolation of PS by the addition of ethanol and acetone. One volume of acetone was added to the ethanol supernatant where indicated. All calculations are for 1 liter of medium.
[b] Contained insoluble calcium salts. Solution was adjusted to pH ~5 to dissolve the salts.
[c] These cultures were not autoclaved.

Therefore, the selective precipitation of GXM with CTAB was done on PS whose molecular weight, and therefore its viscosity, had been partially reduced by treatment with UI. This eliminated the complication of coprecipitation of other polysaccharides in subsequent steps (3, 6, 24, 29). The GXM-CTAB complex was dissociated in 1 M NaCl and then treated with UI for 2 h. This further reduced its molecular mass to a limiting value of ~100 kDa (GXMS, Table 1). It was necessary to apply the UI treatments in this sequence because the GXMS-CTAB complex gave a clear gel that was difficult to isolate.

Ion-exchange chromatography. Each GXMS eluted as a single peak on DEAE-Sepharose CL-6B at 0.27 ± 0.05 M NaCl (mean ± standard deviation). A representative elution is shown in Fig. 1.

Carbohydrate composition. Molar ratios of the substituent sugars and *O*-acetyl of GXMS, determined by GLC and colorimetry, were calculated relative to mannose, taken as 3.0 (Table 2). No substituents other than those indicated in Table 2 were identified. The analysis of GXMS obtained after ion-exchange chromatography did not differ significantly from the data shown in Table 2. The molar mannose-xylose-glucuronic acid ratio observed for strains 98 and 110 (3:1:1) was unexpected, since this ratio was previously thought to occur only in serotype D strains (2). The molar ratio for strain 6 (3:2:1) is typical of serotype A isolates (Table 2) (29a).

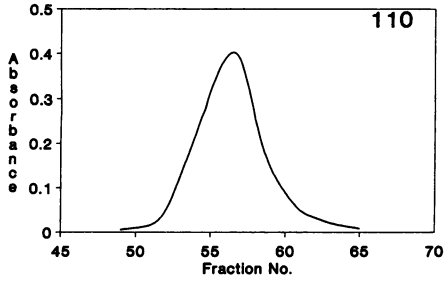

FIG. 1. Ion-exchange chromatography of GXMS from *C. neoformans* 110 on a column of DEAE 52, showing carbohydrate as the A_{490} in the phenol-sulfuric acid assay.

TABLE 2. Molar ratios of GXM from *C. neoformans*

Strain (precipitate)	Molar ratio			
	Mannose	Xylose	Glucuronic acid	*O*-Acetyl (% by wt)
98 (PS-E)	3.0	1.0	0.9	2.1 (10.3)
98 (PS-E-A)	3.0	0.8	0.8	2.0 (10.5)
98 (PS-E-Ca)	3.0	1.0	1.0	2.2 (10.8)
110 (PS-E)	3.0	1.0	0.9	1.9 (9.7)
6 (PS-E)	3.0	2.0	0.9	1.9 (8.2)
6 (PS-M-E)	3.0	2.0	1.0	2.4 (10.2)
201[b]	3.0	2.0	0.6	1.9 (8.8)

[a] Polysaccharides were obtained from autoclaved medium unless indicated otherwise: E, ethanol; E-A, ethanol and acetone; E-Ca, calcium and ethanol; M-E, medium was not autoclaved before the addition of ethanol. Mannose and xylose were determined by GLC (28). Glucuronic acid (4) and *O*-acetyl (16) were determined colorimetrically.
[b] Typical data for an A serotype strain obtained in a previous study, for comparison (28a).

GLC-MS. The results from the GLC-MS analyses of methylated GXMS (Table 3) were consistent with molar ratios obtained for the GXMS polysaccharides (Table 2). Strains 98 and 110 had significant amounts of 2,4,6-tri-*O*-methylmannose that reflected the lower degree of substitution of the α-(1→3)-mannopyranan by β-(1→2)-xylose. Strain 6 gave a substitution pattern that was typical of other serotype A isolates (2, 8). All three strains had some 6-*O*-methylmannose, which indicated the presence of disubstitution at *O*-2 and *O*-4 of some mannose residues.

^{13}C NMR spectroscopy of de-*O*-acetylated GXMS. Figure 2 illustrates the anomeric carbon region of the ^{13}C NMR spectra for the de-*O*-acetylated GXMS of strains 98, 110, and 6 along with similar data from typical serotype A (NIH 201) and serotype D (NIH 430) isolates. The resonance at ~105.8 ppm was found in the five spectra, and it is due to β-(1→2)-D-xylose. The resonances at ~105.3 ppm and ~104.7 ppm have been identified in several serotype D (see 430 in Fig. 2) isolates and in glucuronomannan (10). These resonances occurred in 98 and 110 but were absent in the more typical serotype A strains (see 201 in Fig. 2 and strain 6); they are due to unsubstituted mannose residues (Fig. 2 and 3). The probability of the occurrence of this sequence is reduced in

TABLE 3. GLC-MS methylation analysis of GXM from *C. neoformans*[a]

Strain	Methylated PAAN derivatives (mol%)				
	Tri-*O*-methylated		Di-*O*-methylated		*O*-Methylated (6-Mannose)
	2,3,4-Xylose[b]	2,4,6-Mannose	2,6-Mannose	4,6-Mannose	
98 (PS-E)	23.1	37.1	2.2	31.3	6.3
98 (PS-E-A)	30.2	28.7	2.3	30.9	7.9
98 (PS-E-Ca)	22.1	39.5	2.5	26.4	9.6
110 (PS-E)	26.8	34.3	0.0	28.5	10.4
6 (PS-E)	41.2	4.9	0.0	48.2	5.7
6 (PS-M-E)	42.3	3.9	0.0	45.4	8.4
201[c]	35.6	8.2	0.0	53.6	2.4

[a] See Table 2, footnote *a*.
[b] Calculated values based on the degree of substitution of mannose.
[c] Typical data for a serotype A strain obtained in a previous study, for comparison.

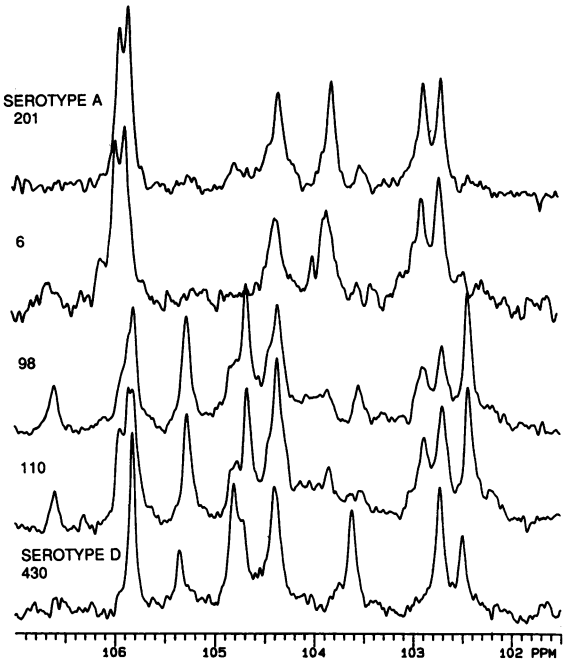

FIG. 2. Anomeric region of proton-decoupled ^{13}C NMR spectra of de-*O*-acetylated GXMS from *C. neoformans*.

GXM having a mannose-xylose ratio of 3:2 or greater. The remaining upfield resonances (~102 to 103 ppm) are due to mannose residues that are substituted *O*-2 with xylose or glucuronic acid (102.4 ppm; present only in strains 98, 110, and 430) enclosed by two unsubstituted mannose residues (Fig. 3A). GXMS from 98 and 110 had a unique combination of resonances in this region that were a composite of those found in 201 and 430 (see the region between 102 and 103 ppm in Fig. 2). The resonance at ~102.9 ppm was present in the four serotype A strains and absent in serotype D. This indicates that a particular 2-*O*-β-xylose is an important component of the serotype A epitope. The resonance at 103.6 ppm found in the typical serotype D strains was of low intensity or completely absent in strains 98, 100, and 6 (Fig. 2). The two resonances at 102.7 and 102.9 ppm observed for strain 6 matched the data for strain 201, the standard serotype A strain. These data indicate that in 98 and 110 there are localized sequences, near the glucuronic acid substituent, that are devoid of xylose which are counterbalanced with localized sequences found in other locations of the PS that have xylose (Fig. 3A). Unequivocal chemical shift assignments are not possible without the availability of model compounds and additional data.

TABLE 4. Precipitation of GXMS from strain 98

Sample wt (mg)	Solvent[a]	Precipitant	Wt recovered (mg)	% Recovered
106	1 M NaCl	Ethanol	81.9	77
101	H_2O	Ethanol + acetone	7.0	7
101	H_2O	Ethanol + Ca + acetate	83.5	83
101	1 M NaCl–0.01 M $NaPO_4$ (pH 7.2)	Ethanol	74.9	74
101	1 M NaCl	Ethanol	72.2	71
101	H_2O	Ethanol	8.6	9
100	0.15 M NaCl–0.01 M $NaPO_4$ (pH 7.2)	Ethanol	80.5	80
100	0.01 M $NaPO_4$ (pH 7.2)	Ethanol	0	0

[a] Each solvent used at 10 ml.

Precipitation of GXMS. GXMS was not recoverable from aqueous solution by the addition of ethanol or ethanol-acetone (Table 4). However, good recovery was obtained in

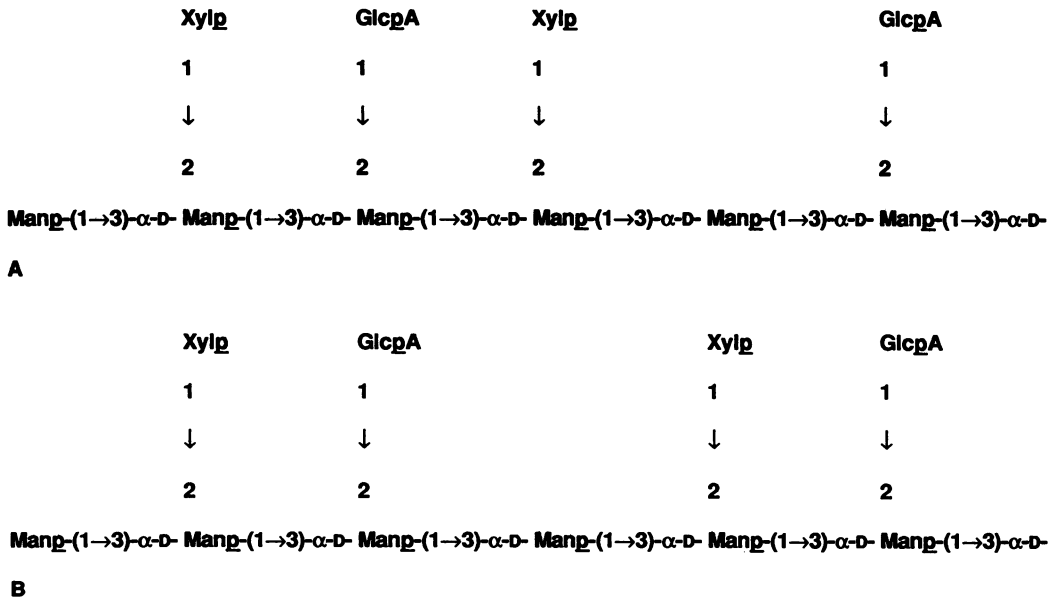

FIG. 3. Model structure of GXMS from *C. neoformans*. (A) Strain 98 or 110; (B) typical serotype D strain.

5% calcium acetate (83%) and in solutions containing from 0.15 to 1.0 M NaCl (Table 4).

DISCUSSION

The isolation and purification of GXM from *C. neoformans* has been one of the major problems for the proficient comparative analysis of its structure. The procedures introduced in this study significantly reduced the amount of time required to obtain substantial amounts of pure GXM. This was accomplished by the use of autoclaved cultures and the precipitation of the calcium salt of GXM directly from the medium by the addition of ethanol. The purification of GXM by selective complexation and precipitation with CTAB was improved by short-term treatment of the PS with UI. This technique reduced the molecular weight of GXM and produced a concomitant decrease in solution viscosity. Consequently, the volumes used in subsequent steps were reduced and coprecipitation of entrapped contaminants was eliminated. UI was used a second time for an interval sufficient to reduce the molecular weight of GXM to a limiting minimum value. A PS preparation, GXMS, that possessed ideal physical properties was obtained that retained all its chemical and serological uniqueness (9, 27). Ion-exchange chromatography of each of the GXMS preparations listed in Table 1 gave single symmetrical peaks indicative of a single polydisperse PS. The results of analyses of GXMS prior to and after chromatography were indistinguishable for all the fractions listed in Table 1. None of the purified polysaccharides contained constituents other then those known to occur in GXM.

The data obtained here were compared with those reported previously by Small et al. (26). The best agreement with the general model of a serotype A structure occurred with strain 6 (2, 8). The data reported here for the molar mannose-xylose-glucuronic acid ratio for strain 98 were only slightly different from those of Small et al. (3:1:1 versus 3:1.24:1) (26). However, we did not detect any galactose, whereas Small et al. reported a substantial amount. A similar comparison of data for strain 110 showed greater discrepancies. Our analytical results for 110 were quite similar to those obtained for strain 98. No evidence for an inositol-like compound was obtained and glucuronic acid was not elevated, as reported by Small et al. (26). As indicated by the molar mannose-xylose-glucuronic acid ratio, strains 98 and 110 are deficient in xylose, and their classification as serotype A was doubted. However, the A serotype of strains 6, 98, and 110 was confirmed in the laboratory of J. E. Bennett. The chemical and serological data for strains 98 and 110 indicate the variable disposition of glucuronic acid and xylose along the mannopyranan backbone (Fig. 3A) compared with an ordered structure found in most serotype D isolates (Fig. 3B). Either of these blocks in conjunction with 6-*O*-acetyl must represent a major epitope of serotype A and serotype D strains, since neither the glucuronomannan nor xylomannan derivatives are serologically active (9, 27). The data for strain 6 showed that it is probably constructed of uniform repeating units of a typical serotype A strain.

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