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A GALACTOXYLOMANNAN ANTIGEN OF *Cryptococcus neoformans* SEROTYPE A

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ABSTRACT

The capsular polysaccharide of *Cryptococcus neoformans* serotype A was fractionated into two chemically and serologically distinct heteroglycans by differential precipitation with cetyltrimethylammonium bromide (CTAB). The major, viscous, acidic glucuronoxylomannan (GXM, 88% w/w) was precipitated with CTAB, while a previously undetected galactoxylomannan (GalXM, 12% w/w) remained in solution. GalXM is characterized by (*i*) molar ratios of galactose:mannose:xylose:glucuronic acid of 1.9:1.8:1:0.2 and 2% of *O*-acetyl; (*ii*) a molecular weight of 275,000 ±25,000, estimated by gel-permeation chromatography; (*iii*) extensive degradation by $NaIO_4$; (*iv*) precipitation in gel by a lectin, concanavalin A, indicating nonreducing mannosyl termini; and (*v*) a distinct, immunoprecipitin arc in counterimmunoelectrophoresis. GalXM was further purified by gel-permeation or ion-exchange chromatography.

INTRODUCTION

The major exopolysaccharide of the zoopathogenic yeast *Cryptococcus neoformans* serotype A is a viscous, acidic glucuronoxylomannan (GXM) of high molecular weight that adheres to the yeast as a capsule, and is sloughed off into body fluids during infection. The composition of GXM was recently reported by our laboratories[1]. The polysaccharide is a (1→3)-α-D-mannan substituted with single (1→2)-D-xylosyl and (1→2)-D-glucosyluronic acid groups. The molar ratios of the components of a serotype A isolate are xylose:mannose:glucuronic acid = 2:5:1.

Interest in cryptococcal polysaccharide derives from its role as an aggressin, a factor potentiating virulence. Like other encapsulated agents of meningitis[2], the cryptococcal capsule enables the yeast to avoid immune surveillance, cross the blood-brain barrier, and proliferate in the central nervous system. Capsular polysaccharides of *C. neoformans* are antiphagocytic[3] and tolerogenic[4], and their circulation in

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cerebrospinal fluid of persons with cryptococcal meningitis forms the basis of specific immunodiagnosis[5]. Immunological studies have used ethanol-precipitated culture filtrates as a source of antigen, on the assumption that the capsular polysaccharide is a single heteroglycan[4,6-8]. Previously, others[9-11] reported galactose in cryptococcal polysaccharide preparations, but, in our studies, none was detected in the major, viscous polysaccharide obtained, which was purified by precipitation with cetyltrimethylammonium bromide (CTAB). We now report that a second, antigenically and chemically distinct polysaccharide, galactoxylomannan, is coproduced by the yeast. The significance of this finding is that reports on immunological properties of unfractionated "cryptococcal polysaccharide" most probably described a composite of responses to at least two different antigens. The occurrence, isolation, and partial characterization of galactoxylomannan (GalXM) are described herein.

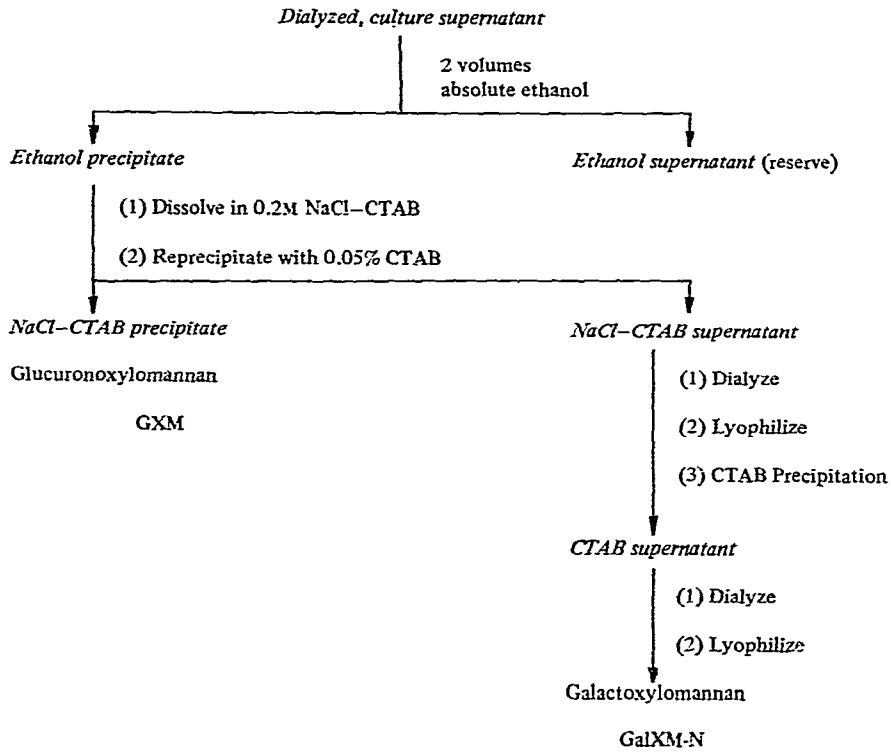
EXPERIMENTAL

Cultures, and isolation of polysaccharide. Cryptococcus neoformans CDC B2550, a large-capsule form of serotype A, was grown in the following medium (g/L): D-glucose (40), $KH_2PO_4 \cdot H_2O$ (1.36), urea (1.29), sodium glutamate (1), and $MgSO_4 \cdot 7\ H_2O$ (0.3), supplemented with thiamine · HCl, 2 mg/L; biotin, 10 μg/L, and trace elements. The pH was adjusted to 7.0. Cultures were incubated at 30° in eight 1-L flasks on a gyratory shaker at 150 r.p.m. After 96 h, cultures were killed with 0.25% formaldehyde, and yeast cells were removed by two cycles of centrifugation, for 40 min at 8,000*g* and then for 30 min at 20,000*g*. Culture supernatant liquors were dialyzed *versus* running tap-water, and then *versus* cold, de-ionized water, and concentrated 10-fold under diminished pressure below 40°. Polysaccharides were precipitated by the addition of cold, absolute ethanol (2 vol.), and dried by washing sequentially with ethanol, acetone, and diethyl ether.

Dry polysaccharides (2.1 g) were dissolved in 0.2M NaCl (480 mL), and CTAB (3 mg/mg of polysaccharide) was added. Then, 0.05% CTAB solution (810 mL) was slowly added until no further precipitation occurred. The precipitate was removed by centrifugation, and reserved. The supernatant liquor was filtered through a 0.45-μm porosity membrane, and the filtrate dialyzed *versus* running tap water and then de-ionized water, and lyophilized (yield, 496 mg).

The precipitate was dissolved in H_2O (70 mL), and 10% CTAB solution was added dropwise until precipitation ceased (2 mL). The suspension was centrifuged at 20,000*g* for 20 min, and the supernatant liquor was dialyzed exhaustively in the usual way and then lyophilized (yield, 277 mg). This fraction was labeled GalXM-N. The precipitate was dried by successively washing with ethanol, acetone, and diethyl ether, and reserved. Four batches of the GalXM-N were prepared, in an average yield of 12%, based on the ethanol precipitate as 100% (see Scheme 1).

The ethanol precipitate from the culture filtrate of the acapsular mutant 67, derived from *C. neoformans* serotype D, was supplied by Dr. E. S. Jacobson, Veterans Administration Medical Center, Richmond, Virginia. The ethanol precipitate was



Scheme 1. Flow diagram for the isolation of GalXM-N.

treated with CTAB as just described. The polysaccharides of this CTAB supernatant liquor were labeled J-67.

Analytical methods. — Neutral carbohydrate was detected by the phenol–sulfuric acid method[12]. Uronic acid was determined by the carbazole[13] and orcinol[14] reactions, with D-glucuronic acid as the standard. Phosphate was quantitated by the Ames–Dubin modification[15]. Protein content was estimated by the Coomassie Blue dye-binding assay (Bio-Rad Laboratories, 500-0006), or by amino acid analysis. The constituent monosaccharides were identified, and quantitated, by gas–liquid chromatography (g.l.c.) after hydrolysis with 2M trifluoroacetic acid[16]. The *O*-acetyl content was determined as benzyl ester, with respect to an internal standard of propanoic acid, by g.l.c. in a modification[16] of the Bethge–Lindström procedure[17], or by the Hestrin procedure, with galactitol hexaacetate as the standard[18]. Smith

degradation[19] of the polysaccharide is briefly described: GalXM (10 mg) was dissolved in freshly prepared, 0.04M sodium periodate, and reaction proceeded for 3 days at 4°. Ethylene glycol (15 mg) was added, and, after 1 h at room temperature, sodium borohydride (20 mg) was added. The solution was refrigerated overnight, and then the pH was adjusted to 4.5 with glacial acetic acid. The sample was dialyzed, and then evaporated to dryness *in vacuo* below 40°; the residue was dissolved in H_2O, and half of the solution was hydrolyzed in 2M trifluoroacetic acid, and analyzed by g.l.c.[16]. The other half was treated with 0.1M HCl for 16 h at 37°, dialyzed, and evaporated to dryness. The periodate-oxidation procedure and g.l.c. analysis were repeated on this sample.

Column chromatography. — A portion (10 mg) of GalXM-N polysaccharide (see Fig. 1) was dissolved in 0.05M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris; 1.0 mL) buffer, pH 7.6, and applied to a column (80 × 1.6 cm) of Sepharose CL-6B (Pharmacia) equilibrated with the same buffer. Elution was conducted at a flow rate of 18 mL/h, with continuous monitoring at 206 nm, and analysis of individual fractions with phenol–sulfuric acid[12]. The column was calibrated with standard proteins having a range of molecular weight of 150,000 to 670,000.

GalXM-N (10–25 mg), dissolved in water to a concentration of 10 mg/mL, was applied to a column (16 × 1 cm) of (2-diethylaminoethyl) (DEAE)-Sephadex A25 (Cl^-) (Pharmacia) or DE-52-cellulose (Whatman Chemical Separations, Ltd.), and eluted with 0.2M NaCl at a flow rate of 15 mL/h. The eluate was monitored by the phenol–sulfuric acid reaction[12]. The appropriate, carbohydrate-containing fractions were combined, dialyzed, and lyophilized.

Partial hydrolysis with acid. — GalXM-N (20 mg) was treated with 0.1M HCl for 15 min at 100°. The solution was evaporated *in vacuo* at 40°, and the residual acid was removed by evaporation with absolute ethanol. The residue was dissolved in buffer, and analyzed by chromatography on Sepharose CL-6B.

Serological procedures. — Serological relationships among the various polysaccharide preparations were determined, with specific, rabbit antisera, by double immunodiffusion (i.d.) in gel. Large, female, New Zealand white rabbits were immunized with formaldehyde-killed, *C. neoformans* yeast-cells[20]. Counterimmunoelectrophoresis (c.i.e.) was conducted in barbital buffer (pH 8.6, 0.075 ionic strength), in 1% agarose on a plate (8.3 × 10.2 cm) for 2 h at a constant current of 25 mA.

The specificity of concanavalin A (con A) for various polysaccharide preparations was performed as described for i.d. Con A (5 mg/mL) and polysaccharides (2–2.5 mg/mL) were dissolved in 0.01M Tris–0.5M NaCl (pH 7.2) buffer containing $MnCl_2$ (1mM) and $CaCl_2$ (1mM).

Glycosidase treatment. — α-D-Mannosidase and α- and β-D-galactosidase (Sigma Chemical Company, M-7257, G-9007, and G-9132, respectively) were diluted 1/50 with 0.1M sodium acetate, pH 4.8, and dialyzed against the same buffer at 0°, to remove ammonium sulfate. The diluted enzyme (0.5 mL) was added to a sample that had been predissolved in sodium acetate buffer (2.0 mL). A portion (10 μL) of the mixture was immediately removed, and reserved for reducing-sugar analysis[2].

The rest was incubated for 24 h at 37°; a second 10-μL aliquot was then removed for reducing-sugar analysis. The incubation mixture was heated in a boiling-water bath for 3 min, and then cooled. A second aliquot of the same, or another, glycosidase was added, and the procedure was repeated. When no reducing sugar was released by the addition of any of the three glycosidases being used, the sample was divided into two parts. One part was directly analyzed by g.l.c., and the second was hydrolyzed prior to g.l.c. analysis.

RESULTS

Isolation. — Several polysaccharides were released into the chemically defined, liquid medium used for culturing *C. neoformans* serotype A. The polysaccharide mixture was resolved into two major fractions by treatment with CTAB: (*1*) CTAB-precipitable, GXM; and (*2*) CTAB-nonprecipitable, GalXM-N (see Scheme 1).

Chromatography of GalXM-N. — Chromatography of GalXM-N on DEAE-Sephadex A25, with a step gradient, gave a single, carbohydrate-containing peak at

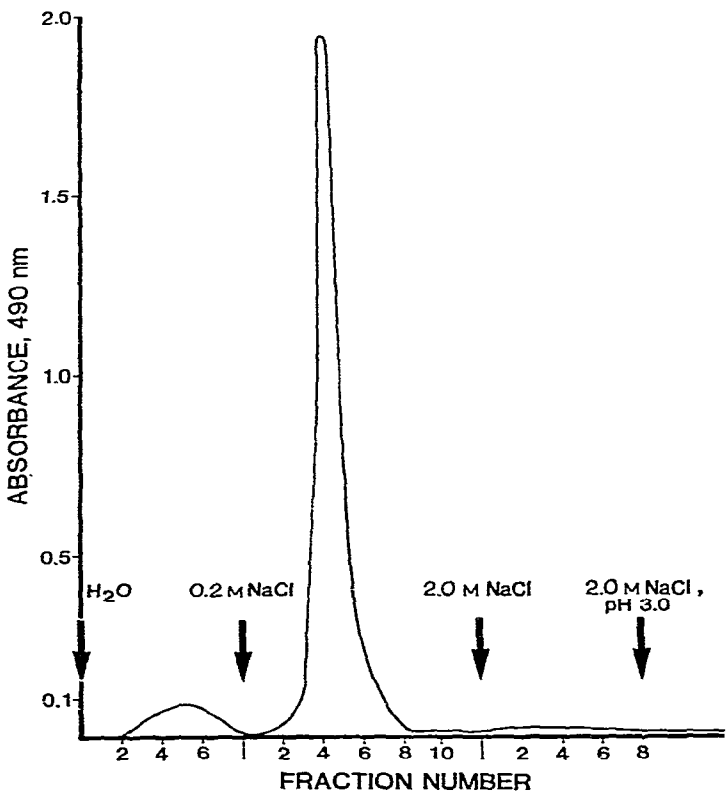


Fig. 1. Chromatography of GalXM-N on DEAE-Sephadex A25. (Each fraction contained 5 mL of eluate, collected at 20-min intervals.)

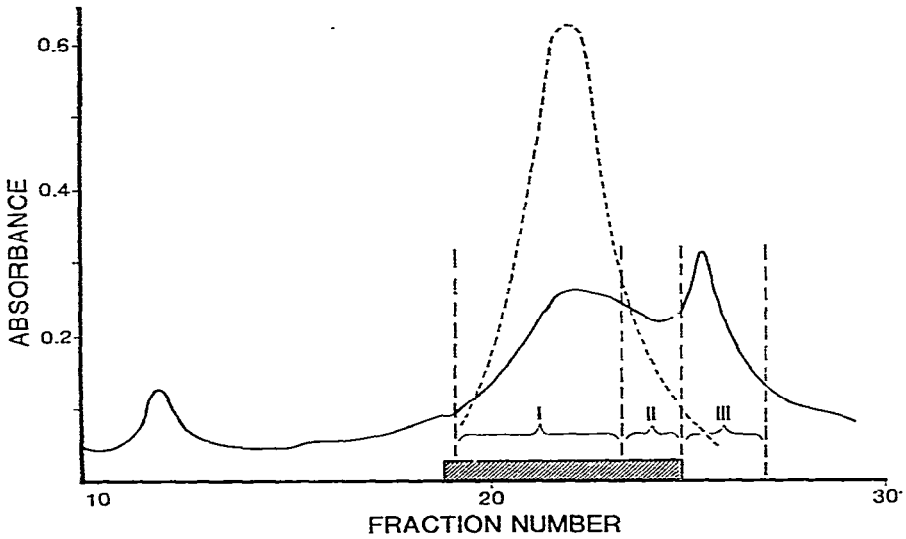
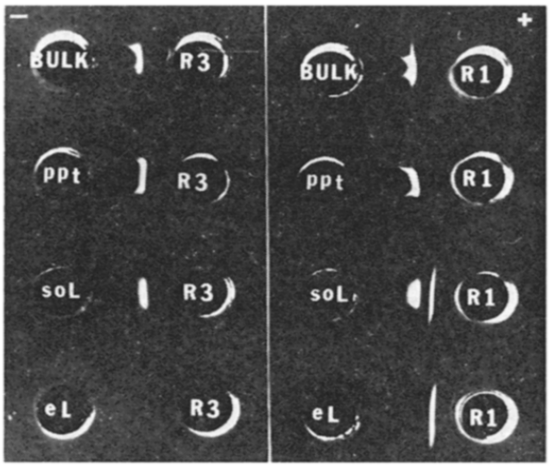


Fig. 2. Chromatography of GalXM-N on Sepharose CL-6B. (Each fraction contained 6 mL of eluate, collected at 20-min intervals. Key: ——, at 206 nm; – – – –, at 490 nm; and shaded, serologically active.)

0.2M NaCl (see Fig. 1). The carbohydrate-containing fractions were pooled in two portions, the ascending and the descending limbs of the peak. The total recovery of polysaccharide in this peak was 45% of the sample applied. No additional carbohydrate was eluted with 2M NaCl or 2M NaCl–1 mM HCl. In a subsequent experiment, a greater recovery (80%) of polysaccharide was realized when DE-52-cellulose was used as the ion exchanger; again, no additional material was recovered by elution at a high concentration of salt and a low pH. A small portion (3–5% of the load) was not bound to either of the ion exchangers.

GalXM-N was eluted from a column of Sepharose CL-6B in two overlapping, u.v. peaks absorbing at 206 nm (see Fig. 2); however, the carbohydrate was eluted as a symmetrical peak, associated with the first of these peaks. The fractions containing neutral carbohydrate, the first u.v.-absorbing area in Fig. 3, were combined, except for those tubes comprising the descending limb, which were pooled separately (see Peak I and Peak II in Fig. 2). Of the applied sample, 44 and 21% (by weight) were recovered in peak I and peak II, respectively. The remaining fractions (Peak III) accounted for 7%, giving a total recovery of 72%. The elution volume of Peak I (purified GalXM) indicated that purified GalXM had an apparent molecular weight of 275,000 ±25,000. No change in the apparent molecular weight of GalXM-N was observed after hydrolysis with 0.1M HCl.

Serology. — The GXM and the GalXM-N fractions were found to be serologically reactive (examined by i.d. and c.i.e.). In addition, GalXM-N and purified GalXM formed a sharp, precipitin line with con A, whereas GXM did not. The high molecular weight and viscosity of GXM retarded its diffusion through the agarose gel, and its precipitin line was usually contiguous to the antigen well. GalXM-N behaved nor-



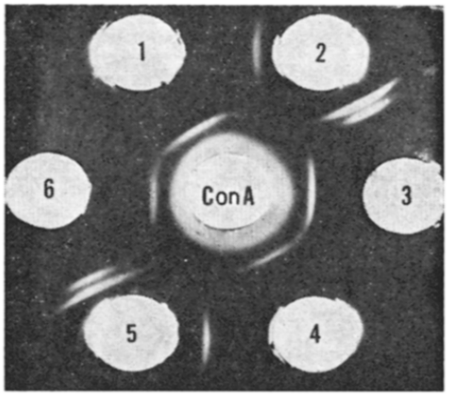


Fig. 3. C.i.e. analysis of *C. neoformans* polysaccharide fractions: ethanol precipitate from the culture media (bulk), CTAB-precipitate (ppt), CTAB-supernatant (sol), and DEAE-Sephadex eluate (eL). (R1 and R3 refer to rabbits from which specific antisera against *C. neoformans* serotype A were obtained.)

Fig. 4. I.d. in gel analysis of *C. neoformans* polysaccharide fractions. (Wells 1 and 4 contained purified GalXM; wells 2 and 5, specific antisera to *C. neoformans* serotype A; and wells 3 and 6, the unfractionated polysaccharide from the mutant strain J-67.)

mally in i.d., and formed one major and one minor precipitin line, the former being the closer to the antiserum well. The resolution and sensitivity of c.i.e., facilitated the demonstration of the serological differences between the GXM and the GalXM fractions, and of the effectiveness of the fractionation procedures. In c.i.e. the ethanol precipitate and GXM gave single precipitin lines having the same relative mobility. GalXM-N gave two precipitin lines in c.i.e.; the line farther from the anode corresponded to GXM, and the second sharp precipitin line had not previously been observed by others (see Fig. 3). The c.i.e. pattern obtained with rabbit No. 1 differed from that of rabbit No. 3, in that, No. 3 produced antibodies only to the major GXM, whereas No. 1 produced antibodies to both GXM and GalXM. Both rabbits were immunized with formaldehyde-killed, whole *C. neoformans*. Fig. 4 shows the difference in immunodiffusion patterns between purified GalXM (resulting in one immunoprecipitin arc) and the unfractionated, culture supernatant liquor obtained from *C. neoformans* acapsular mutant *J-67*, which contained two antigens reactive with anti-*C. neoformans*. Analysis by i.d. of the pooled fractions from the column of Sepharose CL-6B showed that Peak I gave a single precipitin line, characteristic of GalXM, whereas Peak II gave the two precipitin lines observed for GalXM-N. *O*-Deacetylated GalXM was indistinguishable from the native polysaccharide by i.d. Reduced GalXM-N formed, in i.d., a diffuse precipitin line that appeared after 48 h.

Smith degradation. — GalXM-N was extremely sensitive to oxidation with sodium periodate, the integrity of most of the carbohydrate residues being destroyed. Xylose was completely eliminated, and two-thirds of both the galactose and mannose

TABLE I

SUMMARY COMPARISON OF PROPERTIES OF EXOPOLYSACCHARIDES OF *Cryptococcus neoformans CDC B2550* SEROTYPE A

Property	*GalXM*	*GXM*[a]
Percent of total exopolysaccharide	12	88
Molar ratios:		
Xylose	1	2
Mannose	1.8	5
Glucuronic acid	0.2	1
Galactose	1.9	0
O-Acetyl	2	6.5
Phosphate	0.4	n.d.[b]
Peptide, %	3	1.6
Molecular weight	275,000 ±25,000[c]	[d]
Periodate susceptibility (Smith degradation)	90% susceptible	38%
Viscosity	low	high
Con A affinity	reactive	nonreactive
Complexation with CTAB	no	yes

[a]Ref. 1. [b]n.d., not determined. [c]Determined by gel-permeation chromatography on Sepharose CL-6B. [d]Determined by Bhattacharjee *et al.*[23] for GXM of serotype D, $\sim 8 \times 10^5$.

residues were oxidized. Approximately 50% of the oxidized polysaccharide was recovered as the polyalcohol after reduction, dialysis, and lyophilization. The dialyzate mainly contained glycerol, although mannose and galactose were also detected. Mild hydrolysis with acid rendered 90% of the polyalcohol dialyzable. The retentate contained mannose and galactose which were sensitive to a second oxidation with sodium periodate. The quantity of the retentate was insufficient to permit performing a detailed analysis. The dialyzate was lyophilized, and then desalted by gel-permeation chromatography on a column (1.0 × 50 cm) of Bio-Gel P-2. The carbohydrate, eluted as a single, symmetrical peak, was composed of mannose, galactose, and glycerol. Sequential treatment of the P-2 eluate with specific glycosidases showed that most of the sample was composed of *O*-α-D-galactopyranosyl-*O*-α-D-mannopyranosyl-*O*-β-D-galactopyranosylglycerol (30%) and *O*-β-D-galactopyranosyl-*O*-α-D-mannopyranosyl-*O*-β-D-galactopyranosylglycerol (70%). Glycerol was the major sugar alcohol produced. Erythritol was barely detectable, and threitol was absent.

Polysaccharide composition. — The GalXM chromatographed on Sepharose CL-6B, referred to as purified GalXM, was further characterized. The monosaccharide content was 78%, and the molar ratios of xylose:mannose:galactose:glucuronic acid were 1:1.8:1.9:0.2. Compositional comparisons with other polysaccharide fractions are given in Table I. Purified GalXM contained 2% of *O*-acetyl group, as determined colorimetrically and by g.l.c. Peptide was present in the purified GalXM at a level of 3%. The amino acids serine, threonine, aspartic acid, glutamic acid, glycine, and alanine comprise 78% of the peptide material (see Table II).

TABLE II

AMINO ACID ANALYSIS OF GalXM CHROMATOGRAPHED ON SEPHAROSE CL-6B[a]

Amino Acid	*Micrograms*	*Nanomoles*	*Weight % in peptide*
Aspartic Acid	0.443	3.85	10.2
Threonine	0.531	5.26	14.0
Serine	0.683	7.86	21.0
Glutamic Acid	0.390	3.02	8.0
Glycine	0.174	3.06	8.1
Alanine	0.129	6.27	16.6
Valine	0.195	1.97	5.2
Methionine	0.030	0.23	0.61
Isoleucine	0.112	0.99	2.6
Leucine	0.170	1.50	4.0
Tyrosine	0.101	0.62	1.6
Phenylalanine	0.113	0.77	2.0
Glucosamine	0.117	0.63	1.7
Histidine	0.039	0.29	0.77
Lysine	0.097	0.76	2.0
Arginine	0.094	0.60	1.6
Total	3.42	37.68	100%

[a]Sample size, 112.5 μg.

The second, u.v.-absorbing peak from the column of Sepharose CL-6B (see Fig. 2, area III) accounted for only 7% of the polysaccharide applied. Analysis of this fraction revealed 19% of a carbohydrate composed of xylose:mannose:glucose:galactose:uronic acid in the molar ratios of 1:4:0.4:1.8:0.1. It contained 4% of phosphate and 10% of protein. This fraction was not characterized further, as it constituted only a relatively small fraction of the total carbohydrate of GalXM-N.

Reduction of purified GalXM gave glucose, which was absent from the native polysaccharide, indicating that glucuronic acid was present. The neutral-sugar composition of the polysaccharide obtained from the ascending and descending limbs of the DEAE-Sephadex A25 column differed neither from each other nor from the GalXM purified on Sepharose CL-6B.

DISCUSSION

The chemical basis for the classification of *C. neoformans* into four serotypes is the elaboration of four closely related, but chemically distinct, glucuronoxylomannans of high molecular weight[1,22–24]. Earlier researchers reported galactose as a component of impure polysaccharide preparations[9–11]. However, recent studies concerning the chemical characterization of the capsular polysaccharides of *C. neoformans* serotypes B, C, and D did not verify its presence[22–24]. We observed that the ethanol precipitate of the unpurified, major, viscous polysaccharide of *C. neo-*

formans type A (GXM) contained a trace of galactose[1], but, after precipitation of GXM as the CTAB complex, no galactose was present. This fact, and the observed unaccountability for 10% of the original polysaccharide, prompted recovery and analysis of the material (GalXM-N) not precipitated by CTAB. GalXM contained galactose, xylose, and mannose (see Table I) and was serologically distinct from GXM by i.d. and c.i.e. The GXM (ref. 1) and the GalXM may be related to the two immunoprecipitin lines previously observed[6] for an unfractionated, antigen preparation of *C. neoformans* type A.

Chromatography of GalXM-N on a column of DEAE-Sephadex A25 resulted in a product that gave a single precipitin line by i.d. and c.i.e., but that differed little in its overall chemical analysis when compared to GalXM-N. The low recovery (45%) of GalXM-N by chromatography on DEAE-Sephadex A25 was a disadvantage compared with the greater recovery (73%) obtained after chromatography on Sepharose CL-6B. In a subsequent experiment, the recovery was improved to 80% by using DE-52-cellulose instead of DEAE-Sephadex.

The single, symmetrical peak obtained by chromatography on Sepharose CL-6B was coincident with the first absorbance peak, at 206 nm. This absorbance was probably due to the *O*-acetyl substituents of the polysaccharide and the content of peptide in purified GalXM. The elution pattern of GalXM, pretreated with 0.1M HCl for 15 min, did not deviate from that observed for the native polysaccharide, indicating that the small proportion of phosphate present did not link major segments of GalXM together.

The amino acid composition of the 3% of peptide in purified GalXM was dominated by serine (21%), threonine (14%), aspartic acid (10%), and glutamic acid (8%). A covalent linkage may exist between the peptide and GalXM.

In addition to differences in monosaccharide composition and serological behavior, two other functional differences between the major, viscous GXM and GalXM indicate that there are two distinct heteroglycans: (*1*) two-thirds of the mannose of GalXM was decomposed by treatment with $NaIO_4$, and (*2*) the GalXM polysaccharide formed a sharp line of precipitation with con A in gel. The affinity for con A indicates that some of the mannose residues are disposed as nonreducing termini. GalXM has an apparent molecular weight of 275,000 $\pm$25,000, contains relatively little uronic acid, and does not form viscous solutions. In contrast, all of the mannose residues of GXM occur in $NaIO_4$-resistant, (1$\rightarrow$3) linkages constituting the linear backbone of the polysaccharide; GXM does not interact with con A. GXM is a polysaccharide of high molecular weight[11] ($\sim 8 \times 10^5$) that is rich in glucuronic acid and that forms highly viscous solutions. The specific interaction of GalXM with con A should provide a means for its purification by affinity-column chromatography.

The origin of the galactose that was ubiquitous in preparations of the cryptococcal polysaccharides described by past workers[9-11,25], but not detected in more-recent reports[22-24], only to re-emerge in this study, must be investigated by extending a search for galactoxylomannan to additional isolates of serotype A, and to other

serotypes. Acapsular mutants derived from two serotype D isolates, *C. neoformans* 602 (ref. 7) and *C. neoformans* 67, produce heteroglycans having properties chemically and serologically similar, if not identical, to those of GalXM. Therefore, GalXM may also be found in one or more of the other serotypes of *C. neoformans*.

The exact origin of GalXM is at present obscure. It is not known whether the heteroglycan is a readily soluble, cell-wall polysaccharide, or if it is an actively secreted, storage polymer. Moreover its role in human infection, if any, is unknown. It may provide an antigenic stimulus different from that of the low zone tolerance exhibited by the major, viscous GXM, and it may thus prove more useful in monitoring immune responses in infection.

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