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Note

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

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INTRODUCTION

Cryptococcus neoformans consists of four antigenic types designated as serotypes A, B, C, and D. The occurrence of a fifth type, A-D, has been postulated recently[1–3]. This classification is based on the serologic properties of the soluble capsular polysaccharides of yeast. The major capsular antigens obtained from all the serotypes of *C. neoformans* identified to date consist of a closely related set of high mol. wt., partially *O*-acetylated, D-glucurono-D-xylo-D-mannans (GXMs). Current models of GXM structure are based on data obtained from no more than two isolates of each serotype. These models depict a general structure consisting of an α-(1→3) linear mannopyranan bearing β-D-xylopyranosyl (Xyl*p*), β-D-glucopyranosyluronic acid (Glc*p*A), and 6-*O*-acetyl substituents[1,2]. The mannopyranan of serotypes A and D was described as being substituted at C-2 only, whereas the mannopyranan of serotypes B and C was described as having additional Xyl*p* moieties linked to C-4. A simple structural relationship between the four serotypes was proposed based on the existence of a core structure, containing three D-mannopyranosyl (Man*p*) units and one Glc*p*A unit, to which Xyl*p* units are added to the mannopyranan in increments of one unit. In this way, precise molar ratios of D-xylose:D-mannose:D-glucuronic acid of serotypes D, A, B, and C were assigned as 1:3:1, 2:3:1, 3:3:1, and 4:3:1, respectively[1]. Additional analytical data[3,4], obtained recently from a study of a larger number of isolates of each serotype, show that the precise molar ratio and substitution patterns as proposed in the original models of GXM structure are an oversimplification except in the case of serotype B[5]. In addition, substituent dispositions characteristic of one serotype have been identified in heterologous isolates[3,4]. Herein, we present evidence that shows the presence of structural

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heterogeneity within serotype D GXM obtained from six isolates of *C. neoformans* selected from different laboratories.

EXPERIMENTAL

Native and modified O-*acetyl*-D-*glucuronoxylomannan (GXM).* — Isolates of *C. neoformans* used in this study were as follows: F12 and 127 (E. S. Jacobson, Veterans Administration Hospital, Richmond, VA); 430, 1254, and 3168 (E. Reiss, The Centers for Disease Control, Atlanta, GA); 9375 (H. J. Shadomy, The Medical College of Virginia, Richmond, VA). All isolates were grown in a chemically defined medium, and GXM was isolated and purified as described previously[5,6]. The mol. wt. of all the purified GXM samples was reduced with the aid of ultrasonic irradiation (GXM-S), and then a portion of each GXM-S was chemically *O*-deacetylated at pH 11 (NH_4OH) for 24 h at 23° (refs. 4 and 5).

Analytical methods. — (The experimental details of the following methods are described or are appropriately cited in ref. 5). Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen[7]. *O*-Acetyl content was quantified by the procedure of Hestrin[8] using D-mannitol hexaacetate as the standard. GXM-S was analyzed by ion-exchange column chromatography using DEAE Sepharose CL-6B (Pharmacia) and a linear elution gradient of 0.01M Na_2HPO_4 to 0.01M Na_2HPO_4–01.0M NaCl, pH 7.1. The constituent monosaccharides of GXM-S were identified and quantified as their per-*O*-acetylated aldononitrile (PAAN) derivatives by gas-liquid chromatography after hydrolysis of the polysaccharide in 2M trifluoroacetic acid for 1 h at 120°. Per-*O*-methylation of GXM-S was done by the method of Hakomori[9] as modified by Darvill *et al.*[10]. G.l.c.–m.s. of per-*O*-methyl PAAN derivatives was done with a capillary gas-liquid chromatograph equipped with an ion-trap detector (Perkin–Elmer GC/ITD) as described previously[5], except g.l.c. was done using an SPB-5 0.25-μm capillary column (30 m × 0.25 mm, Supelco). ^{13}C-N.m.r. spectra were recorded at 70° with a Varian VXR-400 spectrometer, equipped with a 10-mm multinuclear probe, operated at 100.58 MHz (^{13}C). All other parameters were exactly as described previously[5].

RESULTS AND DISCUSSION

The yield of GXM from the serotype D isolates was generally only about 15% of that observed in comparable studies with other serotypes[4,5]. The viscosity of the medium did not increase appreciably during the growing period. The viscosity remained low even after concentrating the medium 10-fold; this observation portended the low yield of polysaccharide obtained by ethanol precipitation. In some instances the GXM was recovered directly from concentrated, dialyzed medium by lyophilization, and the ethanol precipitation step was omitted. Purification was done by selective precipitation with hexadecyltrimethylammonium bromide in the usual manner[5,6]. GXM-S from isolate 9375 eluted as a single peak by ion-exchange column chromatography using

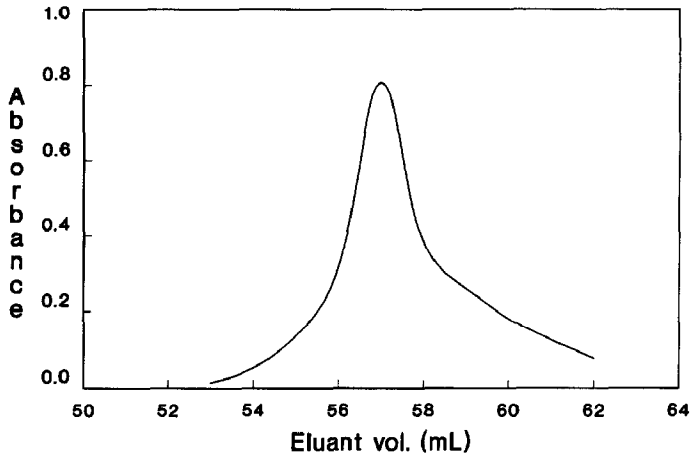


Fig. 1. Ion-exchange column chromatography of GXM-S 9375 on DEAE-Sepharose CL-6B using a linear gradient of 0.01 M Na_2HPO_4 to 0.01 M Na_2HPO_4–1.0 M NaCl. The eluant was assayed for neutral carbohydrate by the phenol–sulfuric acid method, and absorbance measurements were obtained at 490 nm.

DEAE CL-6B (Fig. 1). Structural analysis of GXM-S and GXM-S further purified by ion-exchange chromatography and gel-filtration chromatography did not produce significantly different data. This was true for 21 isolates studied in our laboratory. Therefore, in this study all the data were derived from the analysis of GXM-S and *O*-deacetylated GXM-S. The molar ratio of the substituents, Man, Xyl, and GlcA, and *O*-acetyl, calculated relative to Man taken as 3.00, is presented in Table I. A first impression of the data, rounded to the nearest integer, suggests that this group of serotype D isolates have identical GXMs. Interpretation of the methylation data presented in Table II, rounded to the nearest integer, also suggests the presence of a single GXM chemical species in these serotype D isolates. The molar ratio for 9375

TABLE I

Molar ratios of GXM from *C. neoformans* serotype D

Strain	D-*Man*	D-*Xyl*	D-*GlcA*	O-*Acetyl*
F12[a]	3.00	0.96	0.88	
127	3.00	0.80	0.81	1.73 (9.2%)
430	3.00	0.79	0.78	1.71 (9.2%)
1254[a]	3.00	0.89	0.68	
9375	3.00	0.85	0.75	1.74 (9.3%)
3168[b]	3.00	1.02	0.57	1.76 (9.5%)

[a] Molar ratios determined on *O*-deacetylated polysaccharides. [b] Logged in as a serotype A isolate. Chemical and n.m.r. evidence identifies it as a serotype D, but the serotype has not been confirmed by serological methods.

TABLE II

G.l.c.–m.s. methylation analysis of GXM from *C. neoformans* serotype D

Strain	*Methylated PAAN derivatives (molar ratios)*			
	Tri-O-*Me* 2,3,4-D-*Xyl*[a]	2,4,6-D-*Man*	*Di*-O-*Me* 4,6-D-*Man*	O-*Me* 6-D-*Man*
127[b]	0.88	1.38	1.56	0.06
430	1.04	1.23	1.71	0.06
1254	1.36	1.39	1.57	0.04
9375	1.30	0.99	1.95	0.05

[a] Calculated values based on the degree of substitution of D-mannose. [b] Isolates 127 and F12 have nearly identical structural features as determined by ^{13}C-n.m.r. spectroscopy.

appears to be the idealized situation since the methylation data (Table II) show that two of every three Man units were monosubstituted with either Xyl or GlcA.

Total composition analyses show the identity and the relative proportion of the substituents present. Methylation analysis confirms the composition and, in addition, identifies the linkages of the various substituents; this allows the formulation of an average structure for GXM. N.m.r. spectroscopy takes the resolution of the GXM structure a step further, since these measurements reflect not only the types and number of linkages of the substituents, but also their anomeric configuration and their sequential relationships. This is particularly apparent upon examination of the anomeric

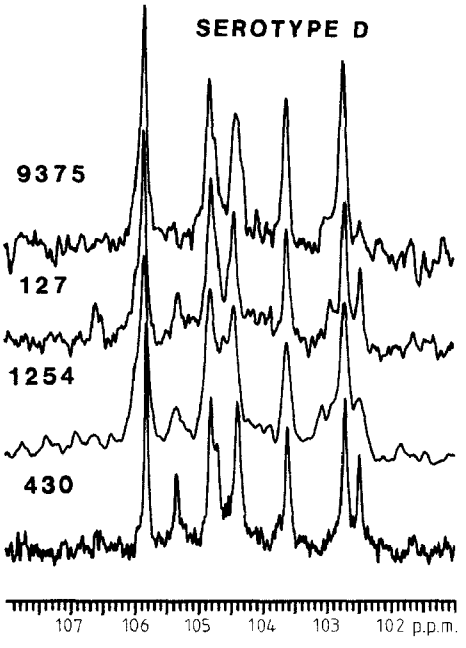
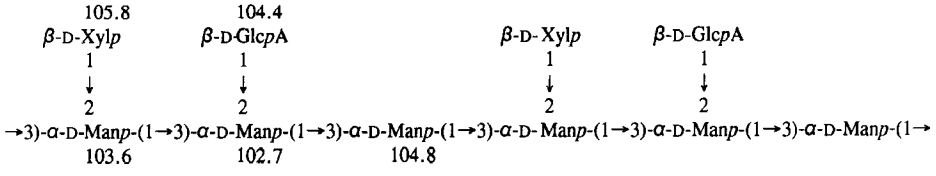


Fig. 2. Anomeric region of proton-decoupled ^{13}C-n.m.r. spectra (70°, 100 MHz) of *O*-deacetylated GXM-S.

resonances depicted in Fig. 2. The serotype D GXM with the greatest percentage of repetitive sequence, demonstrated by the occurrence of the least number of anomeric carbon resonances, is that of isolate 9375. Structure **1** illustrates the probable disposition of the sugar residues for this isolate. This structure is identical to the model presented previously by Bhattacharjee *et al.*[1]; however, the model as originally presented is a representative structure of many possible alternatives that fit the data available at the time. The current ^{13}C-n.m.r. data allow the firm assignment of **1**. The chemical shift assignments of the anomeric carbon atoms, as shown in **1** and **2**, are based on studies of related polysaccharides and model compounds[4,5,11,12]. However, the respective chemical shift assignments for unsubstituted Man*p* in the mannopyranan and that for 2-*O*-β-D-Glc*p*A, as given in ref. 11, are reversed in **1**. The reassignment of these two resonances is based on the data obtained in an experiment in which only the anomeric carbon atoms of Glc*p*A were selectively proton decoupled. The complete assignment of the ^{1}H- and ^{13}C-n.m.r. spectra and the structure of the *O*-deacetylated GXM-S, from 9375, was recently determined by 2-D n.m.r. spectroscopy[13]; the results of this study confirm the assignments given in **1**. All serotype D isolates, except 9375, contain major resonances at 105.3 and 102.5 p.p.m. It is obvious from the n.m.r. spectra that other dispositions of the side-chain residues are present as shown in **2**. The anomeric carbon resonance at 105.3 p.p.m. is due to an unsubstituted Man*p* residue which preceded a Man*p* (102.5 p.p.m.) substituted with Glc*p*A[12]. The structure of 9375 is predominately **1**, whereas GXM-S of the other isolates are mixtures of **1** and **2** and small amounts of yet undefined sequences.



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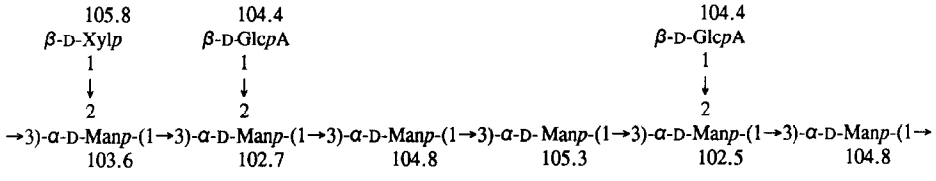

2

Three isolates of *C. neoformans*, originally logged in the laboratory as serotype A, have carbohydrate molar ratios that fit serotype D models. Two of the three isolates, although deficient in Xyl, have anomeric resonances characteristic of serotype A (98, Fig. 3) and were confirmed as serotype A in another study[4]. The third isolate, 3168 (Table I), gave a ^{13}C-n.m.r. spectrum with the characteristics of serotype D GXM (Fig.

3), and it did not show the typical anomeric resonances of serotype A GXM (Fig. 4). The serotype of isolate 3168 has not been reconfirmed serologically, but we predict that it will be identified as serotype D.

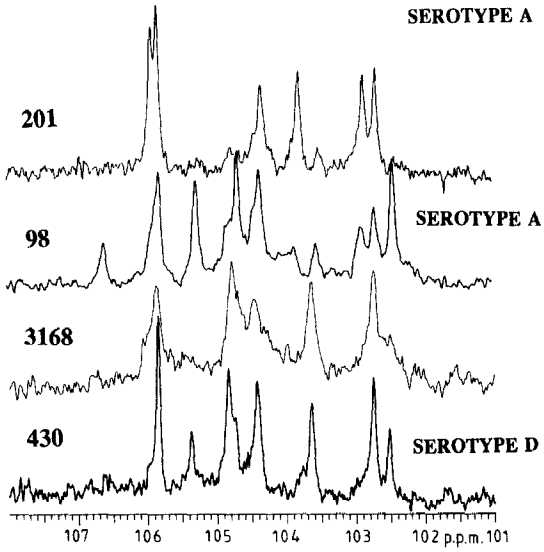

Fig. 3. Anomeric region of proton-decoupled ^{13}C-n.m.r. spectra (70°, 100 MHz) of *O*-deacetylated GXM-S.

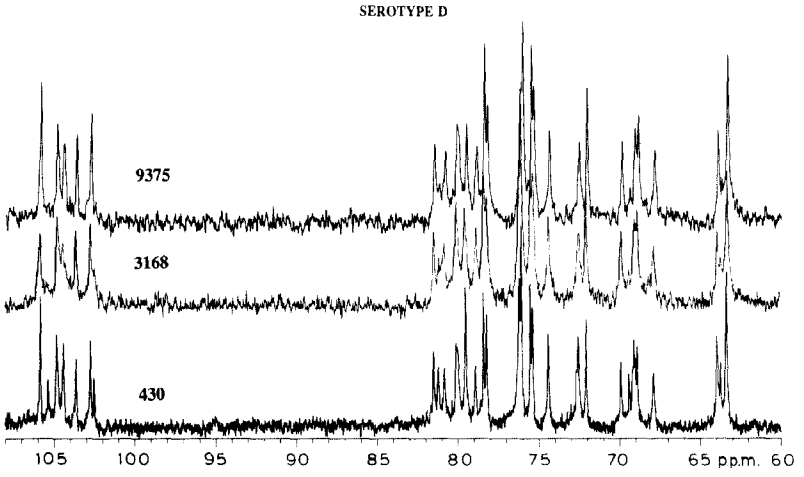

Fig. 4. Proton-decoupled ^{13}C-n.m.r. spectra (70°, 100 MHz) of *O*-deacetylated GXM-S.

CONCLUSIONS

The glucuronoxylomannan of 9375 gives the simplest n.m.r. spectrum of all the isolates studied to date. The anomeric region for *O*-deacetylated GXM-S from 9375 contains only five resonances. These resonances are assigned in **1**. All other serotype D isolates have two resonances for unsubstituted Man*p* and two resonances for Man*p* substituted at C-2 with Glc*p*A (**2**). Therefore, in isolates other than 9375, glc*p*A is present in two different major environments. The proportion of glc*p*A in each environment varies with the isolate studied. Particular substituent dispositions appear to reoccur within each serotype, and these are observable by ^{13}C-n.m.r. spectroscopy. In the future, it will be possible to use n.m.r. spectroscopy to predict the serotype of a particular isolate of *C. neoformans* prior to using serological technics.

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