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STRUCTURAL STUDIES ON THE MAJOR, CAPSULAR POLYSACCHARIDE FROM *Cryptococcus bacillisporus* SEROTYPE B

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ABSTRACT

The capsular material from *Cryptococcus bacillisporus* serotype B has been separated into essentially two fractions. One of these (60% of the total) has been studied by the methods usual for the structural elucidation of polysaccharides. The results are consistent with a structure having nonreducing D-xylosyl as well as D-glucosyluronic acid groups attached to O-2 of D-mannosyl residues linked α-(1→3) in a linear backbone. Every third D-mannosyl residue is doubly substituted with a D-xylosyl group at O-2 and a D-glucosyluronic acid group at O-4.

INTRODUCTION

Cryptococcus neoformans is the etiologic agent for cryptococcosis, and it produces a large capsule of polysaccharide both *in vitro* and *in vivo*[1]. This polysaccharide contains the antigenic determinants for the various serotypes[2–4], and inhibits phagocytosis by leukocytes and macrophages[5–8], and it has therefore been the object of considerable attention.

We have recently reported on the structures of the capsular polysaccharides from *C. neoformans* serotypes C and D (refs. 9–11). Earlier, Blandamer and Danishefsky[12] described their work on the structure of a capsular polysaccharide from *C. neoformans* serotype B, but they reported only the sugar composition and the isolation of some oligosaccharides. Thus, a detailed, structural analysis of the capsular polysaccharide from serotype B remained to be performed. Some structural work on a capsular polysaccharide from *C. neoformans* serotype A has recently been reported by Merrifield *et al.*[13].

Since our last report[11], serotypes B and C have, on the basis of their biochemical, ecological, and genetical differences[14], been redefined as *Cryptococcus bacillisporus* serotype B and C, respectively. We now report our studies on the structure of the preponderant polysaccharide from the capsule of *C. bacillisporus* serotype B.

EXPERIMENTAL

General methods. — All evaporations were conducted in a rotary evaporator at or below 40° (bath temperature). Optical rotations were measured with a Perkin–Elmer 141 polarimeter at 20 ±1°, using sodium light (589 nm). ^{1}H-N.m.r. spectra were recorded with a Jeol JNM FX-100 spectrometer. The spectra of the polysaccharides in D_2O (2% solution) were recorded at 95° as described by Dutton *et al.*[15].

Carboxyl-reduced polysaccharide was prepared by the method of Taylor and Conrad[16].

Immunoelectrophoresis was performed in a 1% solution of agarose in 0.1M Tris acetate buffer, pH 8.3, at a constant voltage of ~6 V.cm^{-1} for 1.5 h. Rabbit antiserum raised to whole cells of *C. bacillisporus* serotype B was used for precipitation, which was allowed to proceed for 48 h at room temperature.

Double diffusion in agar gel was performed according to the method of Ouchterlony[17].

The *O*-acetyl content was estimated by the method of Bethge and Lindström[18].

Isolation of polysaccharide. — The polysaccharide was obtained from a type strain No. 444 isolated from the sputum of a patient with meningitidis. The culture was grown, and the crude polysaccharide isolated, as described[9]. The crude polysaccharide was fractionated on a column of DEAE-cellulose (Whatman DE-52) by using gradient elution with 0.01M phosphate buffer, pH 7.3, going from 0 to 1.0M in NaCl. Essentially two peaks were eluted (see Fig. 1). Peak I constituted ~60% of the load, and was eluted at 0.2 to 0.4M NaCl. Peak II was eluted at a salt concentration of 0.6 to 0.8M NaCl. The materials from both peaks were separately pooled, dialyzed, and lyophilized. The work reported here was performed on the material in Peak I.

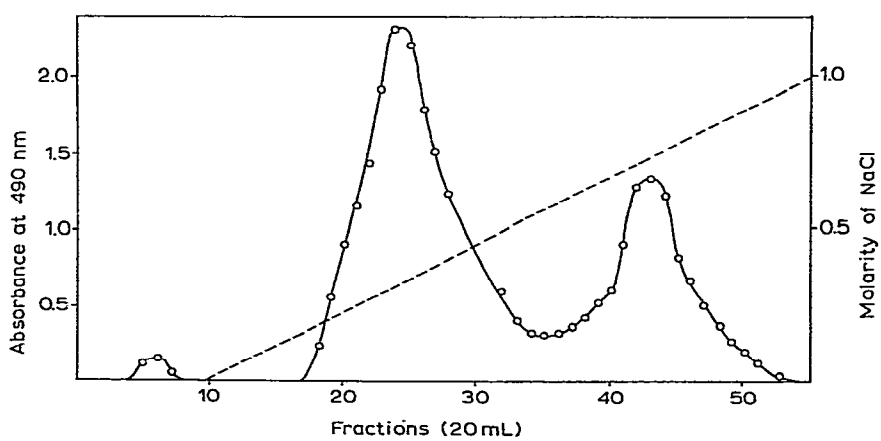


Fig. 1. Chromatography of the capsular material of *C. bacillisporus* serotype B on a column (2.5 × 30 cm) of DEAE-cellulose, using 0.01M phosphate buffer, pH 7.3, and a linear gradient of 0 to 1.0M NaCl as indicated. Peak I is that eluted at 0.2 to 0.4M, and Peak II, that eluted at 0.6 to 0.8M NaCl.

Chromatography. — Paper chromatography was performed by the descending method, using Whatman Nos. 1 and 3 MM papers. The solvent systems used were (*A*) 10:4:3 (v/v) ethyl acetate–pyridine–water, (*B*) 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water, and (*C*) 6:4:3 (v/v) 1-butanol–pyridine–water. The sugars were detected either by the alkaline, silver nitrate reagent[19] or by spraying with a solution of *p*-anisidine hydrochloride in 1-butanol[20].

Column chromatography was performed by using a column (2.5 × 30 cm) of DEAE-cellulose (Whatman DE-52) in the phosphate form. The column was washed with 0.01M phosphate buffer, pH 7.3, before loading the sample. The eluant was the same buffer with a linear gradient of 0 to 1.0M NaCl. Gel-permeation chromatography was performed on either a column (1.6 × 70 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, New Jersey) or a column (2.5 × 75 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden). The eluant in both cases was 0.01M phosphate buffer, pH 7.3, containing 0.15M NaCl (phosphate-buffered saline, PBS). Column fractions were tested for content of reducing sugar by means of the phenol–sulfuric acid reagent[21].

Gas–liquid chromatography (g.l.c.) was performed in a Finnigan-9500 gas–liquid chromatograph equipped with a flame-ionization detector. The glass columns used were respectively packed with (*a*) 3% of ECNSS-M, (*b*) 3% of NPGS (Hi-Eff-3BP), and (*c*) 3% of OV-225, each on Gas Chrom Q (100–120 mesh). Gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) was performed with an LKB-Bromma 2091 instrument operated at 70 eV.

Hydrolysis. — To effect complete hydrolysis, the polysaccharide was hydrolyzed in sealed tubes with 0.5M aqueous sulfuric acid for 16 h at 100°. In some experiments, M sulfuric acid was used for comparison purposes. Partial hydrolysis with acid was performed by using 0.5M hydrochloric acid for 4 h at 80°, a condition found to be optimal for oligosaccharide formation. In one experiment, the purified polysaccharide (100 mg) was dissolved in 0.5M hydrochloric acid (50 mL), and the solution was heated for 4 h at 80°. The solution was cooled, made neutral with powdered silver carbonate, de-ionized with a cation-exchange resin, and concentrated to a small volume. This was fractionated by chromatography on a column (1.5 × 15 cm) of Dowex 1-X4 (formate) anion-exchange resin. The neutral sugars were eluted with water; the acidic sugars were then eluted with M formic acid, and the eluate was evaporated to a syrup. Paper chromatography in solvents *B* and *C* showed the presence of one major oligosaccharide component, which was obtained in pure form by preparative paper-chromatography in solvent *B*.

The molar ratios of the sugars in the hydrolyzed polysaccharides were determined by g.l.c. of the derived alditol acetates, and by dividing the respective peak-areas by the molar response-factors for the individual sugars[22].

Autohydrolysis of the polysaccharide was performed by heating the polysaccharide solution [de-ionized by passage through a column of Dowex 50 (H^+) resin] in a sealed tube for 16 h at 100°. The solution was cooled and lyophilized.

Periodate oxidation. — A solution of the polysaccharide (1 mg/mL) in 0.02M

sodium metaperiodate was kept in the dark at room temperature. Aliquots were withdrawn at intervals, and the consumption of periodate was measured by recording the optical absorbance[23] at 223 nm. Smith degradation of the polysaccharide (34 mg) was performed as described previously[11].

Alkaline β-elimination. — The procedure of Lindberg *et al.*[24] was followed. A suspension of the methylated polysaccharide (8 mg) in 1:2:15 (v/v) 2,2-dimethoxypropane–dichloromethane–methanol (18 mL) was heated under reflux until dissolution was complete. Freshly cut sodium metal (240 mg) was added to the cooled solution, followed by a trace of *p*-toluenesulfonic acid, and the mixture was heated under reflux for 2 h. The turbid suspension was cooled, the base neutralized with 1:1 water–acetic acid, the organic solvents were removed under diminished pressure, and the aqueous solution remaining (12 mL) was dialyzed against distilled water, and then lyophilized (6.9 mg). This material was subjected to mild hydrolysis with 10% (aqueous) acetic acid for 1 h at 100°, and the solution was cooled, lyophilized, and the residue remethylated by the Hakomori method[25].

Methylation. — Polysaccharides were methylated according to Hakomori[25], and were recovered by extensive dialysis against distilled water, followed by lyophilization. The products were hydrolyzed, converted into their alditol acetates, and analyzed by g.l.c. and g.l.c.–m.s.[25–28]. Molar ratios of methylated sugars were determined by the method of Sweet *et al.*[29].

Oxidation with chromium trioxide. — The method of Lindberg *et al.*[30] was used. The oxidized polysaccharide was recovered as described[11].

RESULTS AND DISCUSSION

The purified polysaccharide of *C. bacillisporus* serotype B was obtained by ion-exchange chromatography on DEAE-cellulose using gradient elution (see Fig. 1). The major component from the column proved to be homogeneous when examined by gel chromatography on Sepharose 6B (see Fig. 2), and it constituted ~60% of the crude, capsular material. The K_{av} was 0.13, indicating a molecular weight of $\sim 7 \times 10^5$. This fraction was totally excluded from a column of Sephadex G-200, showing the absence of any contaminant of low molecular weight. The purified polysaccharide appeared homogeneous in immunoelectrophoresis in agar gels *versus* rabbit anti-type B serum (kindly donated by Dr. J. E. Bennett). The material had $[\alpha]_{589}^{20}$ 0° (*c* 0.25, 1:1 Me_2SO–water), and an *O*-acetyl content of 10.4%. The ^1H-n.m.r. spectrum of the polysaccharide showed a sharp *O*-acetyl peak at δ 2.0 and a broad, single, anomeric peak at δ 5.0. The acetate groups are quite labile, as autohydrolysis of the polysaccharide diminished the intensity of the *O*-acetyl peak almost to zero.

Complete hydrolysis of the polysaccharide, followed by chromatography in solvents *A*, *B*, and *C*, respectively, showed the presence of three sugars, having mobilities identical to those of mannose, xylose, and glucuronic acid. This was confirmed by conversion of the sugars into their alditol acetates, followed by g.l.c.

on column (*a*) with inositol hexaacetate as the internal standard. The presence of glucuronic acid in the polysaccharide was confirmed by carboxyl-reduction of the polymer, following which, glucose was detected instead of glucuronic acid. Quantitative analysis showed the presence of mannose, xylose, and glucuronic acid in the ratios of ~3:3:1. Glucuronic acid was quantitated by a colorimetric method[31], and also by g.l.c. (as a derivative of glucose, following hydrolysis of the carboxyl-reduced polysaccharide). Measurement of the optical rotation of the component sugars showed that all three had the D configuration.

Partial hydrolysis of the polysaccharide yielded one major oligosaccharide; this had a mobility of 0.45 relative to that of D-glucose in solvent *B*, and was identified

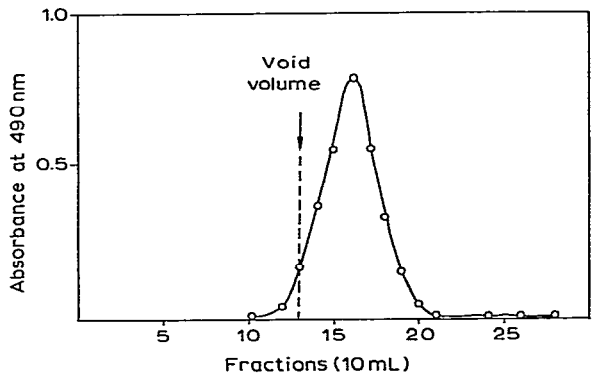


Fig. 2. Chromatography of the major polysaccharide fraction (I) (from the DEAE-cellulose column) on a column (2.5 × 75 cm) of Sepharose 6B, using phosphate (0.01M) buffered saline, pH 7.3, as the eluant.

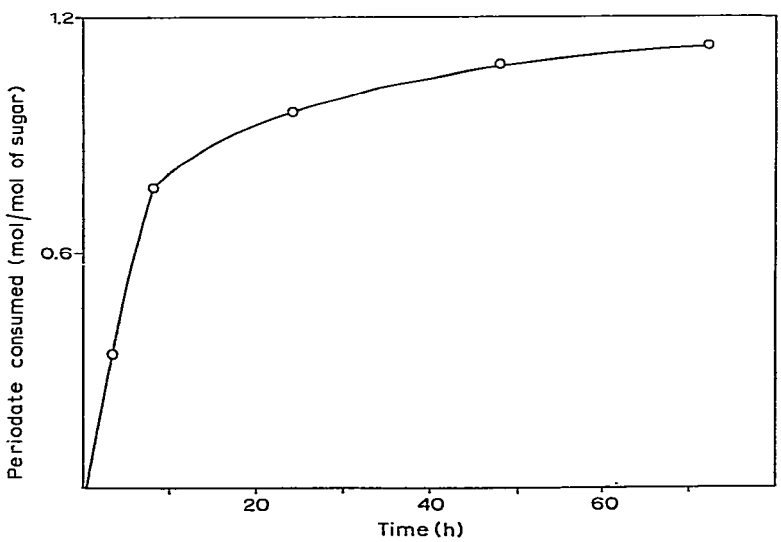


Fig. 3. Periodate oxidation of the capsular polysaccharide of *C. bacillisporus* serotype B. (The consumption of periodate is based on an average molecular weight of 154.)

TABLE I

METHYLATION ANALYSIS OF THE MAJOR, CAPSULAR POLYSACCHARIDE FROM *C. bacillisporus* SEROTYPE B

Methylated sugars[a] *(alditol acetates)*	T[b]	*Sample*[c] *I*	*II*	*III*	*IV*
2,3,4-Me_3-Xyl	0.65	2.5	2.0	2.5	—
2,3,4,6-Me_4-Glc	1.00	—	1.0	—	—
2,4,6-Me_3-Man	2.10	—	—	—	+
4,6-Me_2-Man[d]	3.34	2.0	1.9	3.0	—
2,6-Me_2-Man[d]	3.38	—	—		—
6-Me-Man	4.50	1.0	1.0	—	—

[a]2,3,4-Me_3-Xyl = 2,3,4-tri-*O*-methyl-D-xylose, etc. [b]Retention times are relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on column (*a*) at 170°. [c](I) Methylated polysaccharide; (II) methylated, carboxyl-reduced polysaccharide; (III) methylated, and remethylated, after alkaline degradation; (IV) methylated, Smith-degraded polysaccharide. [d]These two sugars came together as a single peak, and were identified from mixed mass-spectra.

as 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose by hydrolysis, by borohydride reduction followed by hydrolysis, and by methylation analysis. The optical rotation, $[\alpha]_{589}^{20}$ −28° (*c* 0.9, water) indicated a β-linkage, and this was confirmed by the release of D-glucuronic acid from the disaccharide on treatment with β-D-glucosiduronase (EC 3.2.1.31; Boehringer–Mannheim) in 0.01M phosphate buffer, pH 7.3, for 20 h at room temperature.

Oxidation of the acetylated polysaccharide with chromium trioxide[30] for 3 h resulted in the loss of xylose, but the mannose residues appeared to be unaffected, indicating that the xylosyl groups were β-linked, and that the mannose residues were α-linked.

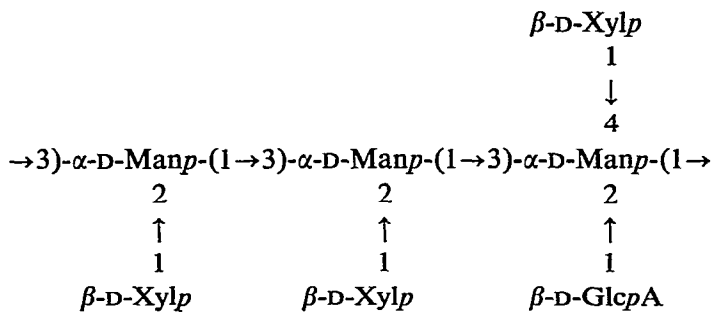
Periodate oxidation of the polysaccharide resulted in the uptake of 1.12 mol of periodate per mol of sugar (see Fig. 3) based on an average molecular weight of 154. Smith degradation[32] of the native polysaccharide resulted in the total loss of all of the glucosyluronic acid and xylosyl groups. The periodate-resistant polysaccharide obtained had $[\alpha]_{589}^{20}$ +120° (*c* 0.2, 1:1 Me_2SO–H_2O), and gave only mannose on hydrolysis. Methylation analysis of the Smith-degraded polysaccharide showed the presence of 2,4,6-tri-*O*-methylmannose as the only hydrolytic product. Therefore, the periodate-resistant core was a (1→3)-linked mannopyranan. The optical rotation, +120°, indicates α-intersaccharidic linkages. Methyl α-D-mannopyranoside and methyl β-D-mannopyranoside, respectively, have $[\alpha]_{589}^{20}$ +82° and −70° (*c* 0.9 and 1.4, 1:1 Me_2SO–H_2O).

Methylation[25] of the purified, native polysaccharide, followed by hydrolysis, and analysis of the sugars as their alditol acetates by g.l.c. and g.l.c.–m.s., showed the presence of 2,3,4-tri-*O*-methylxylose, 4,6-di-*O*-methylmannose, and 6-*O*-methylmannose in the molar ratios of ~5:4:2. (The acidic sugar was not eluted from the column.) Methylation analysis of the carboxyl-reduced polysaccharide showed the

presence of 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-tri-*O*-methylxylose, 4,6-di-*O*-methylmannose, and 6-*O*-methylmannose in the molar ratios of ~1:2:2:1 (see Table I).

Alkaline β-elimination of the methylated polysaccharide, followed by remethylation, and analysis of the alditol acetates, showed the presence of 2,3,4-tri-*O*-methylxylose, 4,6-di-*O*-methylmannose, and 2,6-di-*O*-methylmannose. The absence of 6-*O*-methylmannose, and the appearance of an equivalent amount of 2,6-di-*O*-methylmannose, showed that the glucosyluronic acid groups had been linked to O-2 of the mannosyl units already bearing a substituent on O-4.

From these data, the following structural formula is suggested for the major capsular polysaccharide here investigated.



The only structural feature not depicted is the location of the *O*-acetyl groups. Estimation of the *O*-acetyl content by g.l.c. as benzyl acetate[18] showed a value of 10.4%, and this was supported by the ^{1}H-n.m.r. data. This represents ~3 *O*-acetyl groups per repeating unit of seven sugars. We were unsuccessful in an attempt to locate the *O*-acetyl groups by the method of de Belder and Norrman[33], but, from the results of the periodate oxidation, it would appear unlikely that they occur on the terminal xylosyl or glucosyluronic groups, and they are therefore probably situated on the mannose core of the polysaccharide, a conclusion that agrees with the immunochemical data (see later). Immunodiffusion in agar gels showed that the polysaccharide of serotype B cross-reacts with both anti-type C and anti-type D sera. The homologous reaction with the anti-type B serum available to us was weak, but this precipitation appeared to be undiminished by carboxyl reduction, or carboxyl reduction followed by *O*-deacetylation of the type B polysaccharide. It thus appears that the immunodeterminants are groups other than carboxyl or acetate.

The work reported here was performed on the major fraction (60% of the load) eluted from the DEAE-cellulose column. Cursory examination of the minor fraction showed it also to contain mannose, xylose, and glucuronic acid, but to have a higher content (21.4%) of glucuronic acid. It appears that the production of two polysaccharides is not a general characteristic for *C. bacillisporus* serotype B. A different strain of serotype B (No. 409) was grown, and its capsular polysaccharide examined by ion-exchange chromatography on DEAE-cellulose. A single, broad band was eluted at the same position as the major peak, yielding the material here reported on for strain No. 444.

The structure of the polysaccharide from *Cryptococcus bacillisporus* serotype B bears a strong resemblance to that of the capsular polysaccharide from *C. bacillisporus* serotype C (refs. 9 and 10) (previously called *C. neoformans*) in having an α-(1→3)-linked mannose backbone bearing xylosyl and glucosyluronic acid groups as (non-reducing) end-groups. All of the mannose residues in both polysaccharides are substituted, and both polymers are partially acetylated. There are, however, structural differences, in that the mannose:xylose:glucuronic acid ratios for type B are 3:3:1, compared to 4:3:1 for the type C polysaccharide. One of every three mannosyl residues in the type B polysaccharide is doubly branched, whereas two of every three mannosyl residues in the type C polysaccharide are doubly substituted.

The polysaccharide from serotype B differs substantially from that of *C. neoformans* serotype D (ref. 11). In the latter polysaccharide, one of every three mannose residues in the backbone is unsubstituted, and it does not appear likely that any are doubly branched. Thus, this appears to support the separate[14] species-classification of type D (with type A).

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