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Note

Preliminary study of a galactomannan extracted from *Conidiobolus coronatus*, a fungus pathogenic to man

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Conidiobolus coronatus is the etiological agent of entomophthoromycosis, a chronic, subcutaneous mycotic disease of humans and animals[1]. Histological studies of infected host-tissue indicate that the fungus is surrounded by a characteristic eosinophilic precipitate. At least some polysaccharide is present in this eosinophilic material, since a positive reaction is seen when the tissue is stained by the periodic acid–Schiff technique[1]. The precise nature of the precipitate is not yet known, but it is believed to be composed, in part, of an antigen–antibody complex[1].

The conditions of polysaccharide extraction and the sugar composition of *C. coronatus* have been previously reported[2]. The polysaccharides were found to be readily extracted by phosphate salt, alkaline, or 1,2-ethanediol solutions. From our initial results, it was postulated that the cell wall is composed of a thin, superficial layer of acidic polysaccharides. A much more important, second layer consists of glucans, mannans, and galactomannans. Other glucans associated with chitin are difficult to extract. Among the polysaccharides, a galactomannan was chosen for study as it is easily extracted and purified. Furthermore, mannan-containing polysaccharides frequently have antigenic properties, and so are useful in the serologic diagnosis of mycotic disease as well as in their epidemiologic studies.

RESULTS AND DISCUSSION

The galactomannan of *C. coronatus* was isolated from dried mycelium by extraction with 1,2-ethanediol and purified *via* its copper complex. A single spot was obtained on electrophoresis. The purified polysaccharide was water soluble and showed $[\alpha]_D^{20}$ +63° (water), which may indicate the presence of a majority of α-D linkages. Reducing properties were noted. A complete hydrolysis yielded mannose and galactose in a molar ratio of 17:3. The mol. wt. as calculated from the K_{av} value was found to be 140 000 by chromatography[3] on Sepharose 6B.

The methyl ethers obtained by hydrolysis after permethylation[4] were identified

TABLE I

IDENTIFICATION AND QUANTITATIVE DETERMINATION OF *O*-METHYL SUGARS[a]

O-Methyl derivatives (%)	*Polysaccharides*			
	Original	*After hydrolysis for (min)*		
		5	*10*	*30*
Di-*O*-methylmannose				
2,4-	6.8	14.7	15.3	16.2
3,4-	3.5	3.7	3.9	0
3,6-	3.5	1.7	1.2	0
Tri-*O*-methylmannose				
2,3,4-	9.5	21.4	22.6	16.0
2,4,6-	20.0	18.0	18.7	16.0
3,4,6-	33.0	15.1	16.0	35.2
Tetra-*O*-methylmannose				
2,3,4,6-	7.6	15.0	16.0	15.9
Tri-*O*-methylgalactose				
2,4,6-	7.3	3.6	0	0
Tetra-*O*-methylgalactose				
2,3,4,6-	7.6	6.0	6.3	0

[a]By g.l.c. of the per-*O*-(trimethylsilyl) derivatives.

by g.l.c. of the trimethylsilyl derivatives (see Table I and Fig. 1A). Methyl 3,4,6-, 2,4,6-, and 2,3,4-tri-*O*-methylmannoside indicated the presence of (1→2), (1→3), and (1→6) linkages; methyl 3,4-, 3,6-, and 2,4-di-*O*-methylmannoside indicated branching; and methyl 2,3,4,6-tetra-*O*-methylmannoside corresponds to end-groups. Methyl 2,4,6-tri-*O*-methylgalactoside indicated a (1→3) linkage, and methyl 2,3,4,6-tetra-*O*-methylgalactoside, which represents 50% of the total galactose content, corresponds to end-groups.

The partially degraded, nondialyzable polysaccharides obtained after short exposures to acid hydrolysis were studied by methylation analysis. After 5 min of hydrolysis, important changes were observed (see Table I and Fig. 1B). A significant decrease in the proportion of the methyl ethers corresponding to the (1→2)- and (1→3)-linked mannose residues was observed, as well as in the proportions of 3,6-di-*O*-methylmannose and of 2,4,6-tri-*O*-methylgalactose. The proportions of 2,3,4-tri- and 2,4-di-*O*-methylmannose were modified from 6.8 to 14.7%, and from 9.5 to 21.4%, respectively, but the ratios of 68:95 and 147:214 remained approximately unchanged.

Only slight differences were observed after a 10-min period of hydrolysis. The proportions of the methyl ethers were similar to those observed for the 5-min hydrolysis, except that the (1→3)-linked galactose residues were split off; only the 2,3,4,6-tetra-*O*-methylgalactose derived from the terminal units was observed. The existence of a side-chain containing (1→2)- and (1→3)-linked mannose residues was postulated,

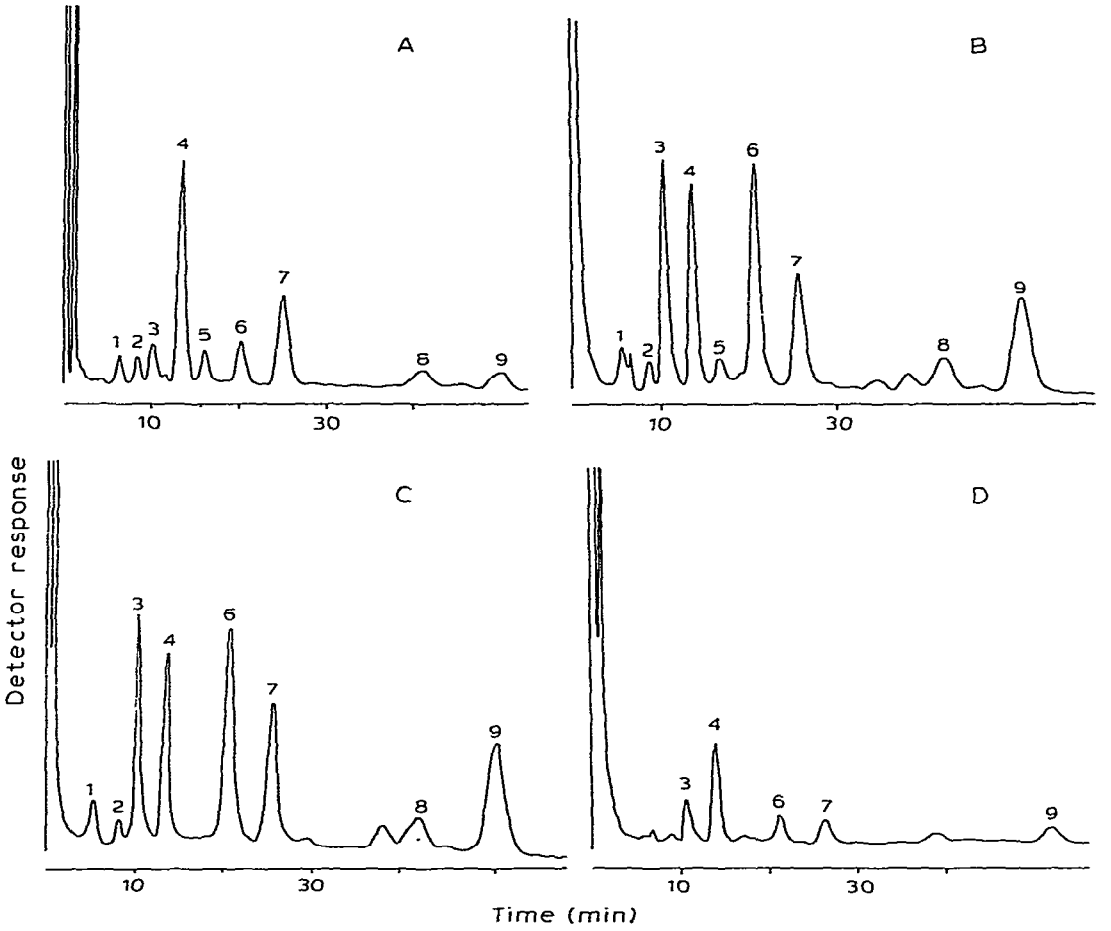

Fig. 1. Gas–liquid chromatogram of per-*O*-(trimethylsilyl) derivatives of *O*-methyl sugars from methylated galactomannan: (A) nondegradated polysaccharide; (B) polysaccharide obtained after 5 min of acid hydrolysis; (C) polysaccharide obtained after 10 min of acid hydrolysis; and (D) polysaccharide obtained after 30 min of acid hydrolysis. Identification of peaks: 1, 3,4-di-*O*-methylmannose; 2, 3,6-di-*O*-methylmannose; 3, 2,4-di-*O*-methylmannose; 4, 3,4,6-tri-*O*-methylmannose; 5, 2,4,6-tri-*O*-methylgalactose; 6, 2,3,4-tri-*O*-methylmannose; 7, 2,4,6-tri-*O*-methylmannose; 8, 2,3,4,6-tetra-*O*-methylgalactose; and 9, 2,3,4,6-tetra-*O*-methylmannose.

since these residues may be removed without changing the basic, nondialyzable structure.

Additional changes were observed after 30 min of hydrolysis (see Table I and Fig. 1D). No 3,6- and 3,4-di-*O*-methylmannose, nor 2,3,4,6-tetra-*O*-methylgalactose were observed, indicating a structure having (1→6)-, (1→3)-, and (1→2)-linked mannose residues. The branches afforded only 2,4-di-*O*-methylmannose. The relative proportion of 3,4,6-tri-*O*-methylmannose was increased, when compared to the values obtained after 5 and 10 min of acid hydrolysis; this higher value may be due

to the cleavage of branches of mannose residues linked at O-4 and O-6 of a mannose residue.

The results of the periodate oxidation and reduction by sodium borohydride, followed by mild acid hydrolysis, of the galactomannan indicated a cleavage of the main chain, thus excluding a main chain exclusively composed of (1→3)-linked mannose residue. It would consist of either (1→2)- or (1→6)-linked mannose residues. After 30 min of hydrolysis, only one type of branch point was present, either linked at O-3 or O-6, as shown by isolation of a dimethyl ether. According to these results, it is possible that the main chain is composed of two types of linkages. The stability of the native polysaccharide during acid hydrolysis, its cleavage by periodate treatment, and the increase in the proportion of the 2,3,4-tri-*O*-methylmannose after 5 and 10 min of hydrolysis are in favor of (1→6) linkages. The proportion of 2,4,6-tri-*O*-methylmannose (20%) derived from the original product is in favor of some (1→3) linkages. A main chain consisting exclusively of (1→2)-linked mannose residues was excluded.

Some structural features can be postulated from the foregoing results. The main chain has either (1→6) linkages, or alternating (1→6) and (1→3) linkages. The side chains attached to O-3 or O-6 have terminal (1→2)- and (1→3)-linked mannose residues, which are easily removed. Short chains of galactose are attached to O-6 and O-4 of the (1→2)-linked mannose residues. These findings are in agreement with the decrease and then disappearance of the galactose units, and of 3,4- and 3,6-di-*O*-methylmannose as products of methylation.

The structure of the galactomannan seems not very different from the basic structure of mannan-containing polysaccharides present in yeasts and filamentous fungi. These polysaccharides frequently have (1→2)-, (1→3)-, and (1→6)-linked mannose residues. When present, the galactose residues are quite often in terminal position[4-6]. Mannans have been often reported to occur in the cell wall of zygomycetous fungi, more or less related to *C. coronatus*. However, a galactomannan has not thus far been reported to occur in this group of fungal organisms.

EXPERIMENTAL

Preparation of cultures. — *C. coronatus* strain 951–72 (ATCC 32867), originally isolated from a human case of entomophthoromycosis, was used throughout this study. Mycelial suspensions were prepared by inoculating Sabouraud dextrose broth (400 mL) contained in 1-L flasks and shaking the cultures for 5 days at 30°.

Extraction and preparation of the polysaccharides. — The mycelium was harvested by centrifugation and dried by several treatments with acetone. The dried powder was extracted with 10 parts (w/w) of freshly distilled 1,2-ethanediol at room temperature. After 5 days, the suspension was centrifuged, and the clear, yellow solution was dialyzed against distilled water to remove 1,2-ethanediol. The polysaccharides were precipitated by addition of ethanol (3 vol.), washed, and dried with ethanol. Proteins and nucleic acids were largely removed by this procedure[7].

The polysaccharides were dissolved in distilled water (500 mg/10 mL) and an equal volume of Fehling's solution was added. The mixture was kept overnight at 4°, and then centrifuged. The insoluble copper complex was washed with 4% potassium hydroxide, then with ethanol, and decomposed with Amberlite IR-120 (H^+) cation-exchange resin. After 1 h of stirring, the resin was removed by filtration. The mannan-containing polysaccharide was purified twice by ethanol precipitation. This fraction was a white powder representing 0.05–1% of the total dried mycelium. The same fraction could be obtained by extraction with 4% potassium hydroxyde.

Sugar composition. — Sugar components were determined after hydrolysis with M hydrochloric acid for 2 h at 100°. The acid was neutralized with silver carbonate and the sample dried *in vacuo*. The sugars were identified as their acetate and per-*O*-trimethylsilyl derivatives by g.l.c.

Partial hydrolysis of polysaccharides. — The hydrolysis was performed in sealed tubes with Amberlite IR-120 (H^+) cation-exchange resin at 100°, for 5, 10, and 30 min; the resin was filtered off, and the solution was dialyzed against distilled water for 2 days. The undialyzable fraction was precipitated with ethanol.

Periodate oxidation. — Purified galactomannan (20 mg) in distilled water (10 mL) was added to 50mM sodium metaperiodate (10 mL), and the mixture was kept in the dark for 24 h at room temperature. The excess of periodate was removed with 1,2-ethanediol. Sodium borohydride (20 mg) was added and, after 7 h at room temperature, the pH of the solution was adjusted to 5–6 with acetic acid, and the solution was dialyzed against distilled water. The retentate was concentrated *in vacuo*, and hydrochloric acid added to a concentration of 0.5M. After 7 h at room temperature, the solution was dialyzed and the polysaccharide was recovered from the solution by freeze-drying[8].

Electrophoresis. — Electrophoresis was performed on Whatman No. 1 paper in 25 or 50mM borate buffer, at a constant field-strength of 6 $V \cdot cm^{-1}$ for 2 h.

Molecular weight determinations. — Fractions (1 mL) were collected from a Sepharose 6B (Pharmacia) column eluted with 0.15M sodium chloride. The fractions were tested for sugar content with the phenol–sulfuric acid reagent. The molecular weight was calculated from the K_{av} values.

Methylation. — The polysaccharides were methylated by the method of Hakomori[9]. The methylated polysaccharides were hydrolyzed with 90% formic acid for 2 h, and afterwards with 125mM sulfuric acid for 16 h. The methyl ethers were converted to methyl glycosides by heating for 2 h with methanolic hydrogen chloride. The products were per-*O*-(trimethylsilyl)ated according to the procedure of Sweeley *et al.*[10] by treatment with 5:2:1 (v/v) pyridine–hexamethyldisilizane–chlorotrimethylsilane (100 μL for each 10 mg of initial polysaccharide). The methyl ethers of sugars used as standards were either prepared from the natural polysaccharides or synthesized by chemical methods[11–13].

Gas–liquid chromatography. — These studies were performed with a Girdel apparatus, model 300. A column (3 mm i.d. × 2 m) of 15% of DEGS on Gas Chrom Q operating at 130° with 20 mL/min of N_2 was used for the study of the mannose and

galactose derivatives obtained by the methylation technique. The sugar composition was determined by the separation of the trimethylsilyl derivatives on a column (3 mm i.d. × 2 m) of 3% of GE-SE 30 silicone on Gas Chrom Q at 165°, with 25 mL/min of N_2 as the carrier gas, and of the acetate derivatives on a column (3 mm i.d. × 2 m) of 3% of ECNSS-M on Gas Chrom Q operated at 185° with 30 mL/min of N_2 as the carrier gas. The DEGS column was conditioned during 12 h at 180°, and the GE-SE 30 column during 12 h at 200°.

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