

Polysaccharides from Cell Walls of *Aureobasidium* (*Pullularia*) *pullulans*

Part II. Heteropolysaccharide

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In addition to β -glucans,¹ the cell wall of *Aureobasidium* (*Pullularia*) *pullulans* contains a heteropolysaccharide composed of D-mannose, D-galactose, D-glucose, and D-glucuronic acid. Methylation analysis resulted in the detection of the following sugars; 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,5,6-tetra-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucuronic acid, 2,3,4-tri-*O*-methyl-D-mannose, 2,3,6-tri-*O*-methyl-D-galactose, 2,3,5-tri-*O*-methyl-D-galactose, and 2,4-di-*O*-methyl-D-mannose. The methylation analysis, in conjunction with periodate oxidation and partial hydrolysis studies indicate that the polysaccharide consists of a chain of (1 \rightarrow 6)-linked α -D-mannopyranose residues most of which are substituted in the 3-position with either a β -D-glucopyranose residue, a short chain of (1 \rightarrow 6)-linked β -D-galactofuranose residues, or an acidic unit attached to the main chain through a D-galactofuranosidic linkage.

The fractionation of the cell wall polysaccharides of *Aureobasidium* (*Pullularia*) *pullulans* into β -glucans and a crude heteropolysaccharide as well as structural studies on the β -glucans were reported in Part I of this series.¹ The present paper reports studies on the heteropolysaccharide. This was purified either by enzymic hydrolysis of contaminating β -glucan or by chromatography on DEAE-Sephadex column (Fig. 1). After one treatment with β -(1 \rightarrow 3)-glucanase, the ratio of mannose to glucose was 2:1, whereas, after two further treatments the ratio was 4:1. Tests later revealed that the enzyme preparation contained β -glucosidase activity,¹ therefore the polysaccharide was isolated by chromatography on DEAE-Sephadex. The polysaccharide thus obtained had a mannose:glucose ratio of 2:1, therefore, it appears that the enzyme treatments removed some glucose from the heteropolysaccharide. In most studies, however, the polysaccharide which had been subjected to enzymic treatment was used. Fractionation of the heteropolysaccharide with

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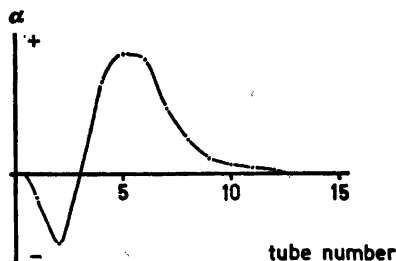


Fig. 1. Separation of the crude heteropolysaccharide on Sephadex DEAE. First peak is β -glucan. Second peak is heteropolysaccharide.

cetyl pyridinium chloride yielded fractions with similar compositions of neutral sugars but different uronic acid content. Before this fractionation the polysaccharide contained D-mannose, D-galactose, D-glucose, and D-glucuronic acid residues in the relative proportions 43:35:10:12 and had $[\alpha]_D + 12^\circ$.

Hydrolysis of the fully methylated polysaccharide yielded the methylated sugars listed in Table 1. The retention times on GLC of the derived methyl glycosides² or alditol acetates³ were the same as those of the corresponding authentic samples; however, an authentic sample of 2,4-di-O-methyl-D-mannose was not available for comparison. The identifications were confirmed

Table 1. Hydrolysis products from the methylated heteropolysaccharide.

Sugar ^a	Relative retention time ^b				Molar percentages	
	Methyl glycoside		Alditol acetate		Experi- mental	Calcu- lated ^c
	column a	column b	column b	column c		
2,3,4,6-Tetra-O-methyl-G	1.00 1.44	1.00 1.31	1.00	1.00	15	11
2,3,5,6-Tetra-O-methyl-Gal	1.44 1.88	1.23 1.55	1.09	1.16	4	3
2,3,4-Tri-O-methyl-GA	2.62 3.35	1.73 2.21	—	—	—	12
2,3,4-Tri-O-methyl-Man	3.21	1.61	1.93	2.48	12	9
2,3,6-Tri-O-methyl-Gal	3.21 4.68	1.61 2.12 2.30 2.62	1.74	2.48	11	20
2,3,5-Tri-O-methyl-Gal	4.56 6.12	1.89 2.60	2.35	3.30	16	12
2,4-Di-O-methyl-Man	9.43	2.61	3.52	5.28	42	32

^a G = glucose, GA = glucuronic acid, Man = mannose, and Gal = galactose.

^b Relative to β -methyl-2,3,4,6-tetra-O-methyl glucoside for the methyl glycosides and 2,3,4,6-tetra-O-methyl 1,5-di-O-acetyl glucitol for the alditol acetates. Column b was used at 165° for the methyl glycosides and 200° for the alditol acetates.

^c Calculated assuming all glucuronic acid is attached to galactose through (1→5)-linkages and none of these linkages is cleaved during hydrolysis.

by mass spectrometry of the derived alditol acetates, a method which conclusively determines the substitution pattern but does not distinguish between different isomeric parent glycitols.⁴ Most of the methylated sugars were also obtained crystalline or transferred into crystalline derivatives.

The methylation analysis indicated that D-glucose and D-glucuronic acid occur solely as end groups, in addition, part of the D-galactofuranose residues are also terminal. The presence of a small amount of 2,3,5,6-tetra-*O*-methyl-D-mannose in the tetra-*O*-methyl hexose fraction is also suggested by GLC and indicates terminal D-mannofuranosidic residues. The identification of this compound is only tentative but gets some support from the fact that small amounts of mannose were released on mild acid hydrolysis of the polysaccharide.

2,3,4-Tri-*O*-methyl-D-mannose indicates D-mannopyranose residues linked through the 1- and 6-positions and 2,3,5-tri-*O*-methyl-D-galactose denotes D-galactofuranosidic chain residues also linked through the 1- and 6-positions. The isolation of 2,3,6-tri-*O*-methyl-D-galactose, which was also isolated from the methylated aldobiouronic acid obtained on hydrolysis of the fully methylated polysaccharide, indicates either D-galactopyranosidic residues linked through the 1- and 4-positions or D-galactofuranosidic residues linked through the 1- and 5-positions. Structural studies of the aldobiouronic acid obtained on partial acid hydrolysis of the polysaccharide demonstrate that the latter alternative is correct.

Only one dimethyl sugar, 2,4-di-*O*-methyl-D-mannose, was detected, therefore the polysaccharide appears to have only one mode of branching. This sugar, which does not seem to have been characterized before, gave a crystalline *p*-nitroaniline derivative, m.p. 221–223°, $[\alpha]_D -348^\circ$ (pyridine). The ratio of D-galactose:D-mannose:D-glucose calculated from the percentages of methylated sugars is 41:35:11, in good agreement with that found in the original polysaccharide, 43:35:10.

The polysaccharide consumed 0.86 moles periodate with the formation of 0.29 moles formic acid per anhydro sugar unit. The value for the formic acid is in good agreement with the results of methylation analysis, whereas the periodate consumption is somewhat low. *trans*-Hydroxyls in furanosidic rings are relatively resistant to periodate oxidation and it is significant that borohydride reduction and hydrolysis of the oxidized polysaccharide yielded galactose as well as glycerol, threitol, and mannose.

The polysaccharide isolated using DEAE-Sephadex was hydrolysed under mild conditions which should only cleave furanosidic linkages. The recovered polysaccharide showed $[\alpha]_D + 70^\circ$ and on hydrolysis yielded mannose, galactose, and glucose in the relative proportions 59:9:32. Not only most of the galactofuranose but also most of the glucuronic acid residues had been removed by this treatment. During fractionation on a DEAE-Sephadex column, 80 % of the modified polysaccharide was recovered as an essentially neutral fraction. Methylation analysis yielded 2,3,4,6-tetra-*O*-methyl-D-glucose (27 %), 2,3,4-tri-*O*-methyl-D-mannose (38 %), 2,3,6-tri-*O*-methyl-D-galactose (8 %), and 2,4-di-*O*-methyl-D-mannose (27 %).

The disaccharides 6-*O*- α -D-mannopyranosyl-D-mannose, 6-*O*- β -D-galactofuranosyl-D-galactose, and 5-*O*- α -D-glucuronopyranosyl-D-galactose were ob-

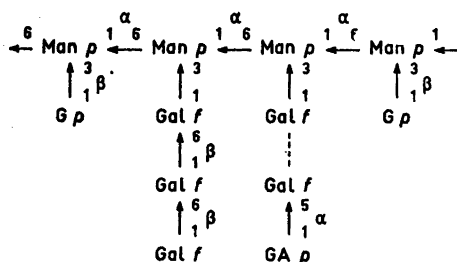


Fig. 2. Proposed structure for the heteropolysaccharide.

tained after partial acid hydrolysis of the polysaccharide. The first was obtained crystalline, identical with an authentic sample. The structures of the others were determined by methylation analysis and other methods as discussed in the experimental part. Methylation of 6-*O*- β -D-galactofuranosyl-D-galactose yielded 2,3,5-tri-*O*-methyl-D-galactose and not the expected 2,3,4-tri-*O*-methyl-D-galactose; similarly the same tri-*O*-methyl sugar was obtained on methylation of authentic 6-*O*- β -D-galactopyranosyl-D-galactose. Several other neutral and acidic fragments were formed on partial hydrolysis; however, they were obtained in an impure state or in insufficient quantities to permit further study. One of these fragments was an oligosaccharide which on further hydrolysis yielded galactose, mannose, and glucuronic acid.

The structural studies suggest the structure proposed in Fig. 2. The backbone of the polysaccharide is a chain of (1 \rightarrow 6)-linked α -D-mannopyranose residues, most of which are substituted in the 3-position. The substituents could be single D-glucopyranose residues (probably β -linked), short chains of (1 \rightarrow 6)-linked β -D-galactofuranose residues, or acidic units containing D-mannose, D-galactose, and D-glucuronic acid residues. The D-glucuronic acid is α -linked to a D-galactofuranose residue in the 5-position and the whole unit linked to the main chain by a furanosidic residue as revealed by the ease with which the unit is released on hydrolysis. The complete elucidation of the structure of this unit must await further investigation.

An extracellular polysaccharide from the same organism⁵ resembles the cell wall heteropolysaccharide in optical rotation, sugar composition and fragmentation pattern during acid hydrolysis and the two polysaccharides are probably closely related. The backbone of (1 \rightarrow 6)-linked α -D-mannopyranose residues and the presence of terminal galactofuranose residues are structural features common with the galactomannans isolated from a related genus, *Trichophyton*, by Bishop and coworkers.⁶

EXPERIMENTAL

Paper chromatograms were run on Whatman No. 1 or No. 3 paper with the following systems (v/v).

- ethyl acetate, pyridine, water (8:2:1).
- ethyl acetate, acetic acid, water (3:1:1).
- water saturated butanone.
- butanol, ethanol, water (5:3:2).

Paper electrophoresis was carried out on Whatman No. 3 paper using 0.05 M germanate buffer of pH 10.7.⁷

Components were detected with *p*-anisidine hydrochloride or silver nitrate-sodium hydroxide sprays.

TLC was on silica gel G using e) benzene, ethanol (5:1) or f) toluene, acetone (1:1) as developing solvents.

GLC was carried out on a Perkin Elmer model 881 instrument using the following columns:

- a) 15 % butane-1,4-diol succinate on Chromosorb G at 180°.
- b) 15 % polyphenyl ether (OS 138) on Chromosorb G at 165° or 200°.
- c) 3 % ECNSS-M on Chromosorb G at 180°.

Isolation of the polysaccharide. The method used to extract the heteropolysaccharide from the cell walls has already been reported.¹ Two methods to isolate the polysaccharide were used. Firstly, the crude heteropolysaccharide was treated successively with β -1,3-glucanase until no further reduction in glucose content was effected. Quantitative analysis by GLC (column c) of the acetylated polyols formed on reduction of a hydrolysate showed 43 % mannose, 35 % galactose and, 10 % glucose. An independent measurement by the carbazole method⁸ indicated 12 % glucuronic acid. Further enzyme treatments with pullulanase and snail enzyme failed to reduce the glucose content. The polysaccharide (5 g) was dissolved in water (100 ml) and 100 ml 1 % cetyl pyridinium chloride (CPCI) added. Sodium sulphate was added until 0.003 M. The resulting precipitate was collected by centrifugation and the CPCI removed by dissolving the precipitate in water containing sufficient sodium bromide to effect solution and precipitating from ethanol. The precipitate was redissolved in water containing sodium bromide and reprecipitated with ethanol yielding 1.1 g polysaccharide. The supernatant was concentrated and the polysaccharide collected by precipitation with ethanol (yield, 3.8 g). The precipitable and non-precipitable fractions contained glucose, galactose, mannose, and 17 % and 10 % glucuronic acid, respectively. Column chromatography on Sephadex DEAE (A-25) was the second method of isolating the polysaccharide (Fig. 1). The polysaccharide (1.0 g) was placed on top of the column (40 × 2 cm) and the column was eluted with a gradient of potassium acetate (0–2 M, 400 ml) with collection of 20 ml fractions. The fractionation was followed polarimetrically; tubes 4 to 13 were pooled. A heteropolysaccharide with more glucose but the same sugar composition and optical rotation as the material remaining after successive β -1,3-glucanase treatments was isolated.

Methylation analysis

The polysaccharide (1.5 g), $[\alpha]_D + 12^\circ$ (c 1.0, water), was methylated as described by Srivastava *et al.*,⁹ followed by treatments with methyl iodide and silver oxide. The product (1.3 g), $[\alpha]_D + 33^\circ$ (c 1.3, chloroform), separated from chloroform when light petroleum was added as a thick syrup which dried as a transparent glass. The methoxyl content was 39 % which is lower than theoretical; however, the polysaccharide showed only a small amount of hydroxyl absorption in the I.R. The methylated polysaccharide (1.0 g) was hydrolysed with 90 % formic acid at 100° for 1 h followed by 0.25 M sulphuric acid at 100° for 18 h. The resulting methylated sugars were separated into tetra-, tri-, di-*O*-methyl, and acidic sugars by preparative TLC (solvent c). The substitution pattern of each sugar listed below, with the exception of 2,3,4-tri-*O*-methyl glucuronic acid, was determined by mass spectrometry of the alditol acetate derivatives.⁴

*The tetra-*O*-methyl fraction.* Demethylation revealed glucose, galactose, and a smaller quantity of mannose as the parent sugars. The two main components were separated by TLC (solvent f) and identified as follows; 2,3,5,6-tetra-*O*-methyl-D-galactose was reduced to the corresponding galactitol, m.p. and mixed m.p. 82–83°, $[\alpha]_D - 26^\circ$ (c 0.1, water).

2,3,4,6-Tetra-*O*-methyl-D-glucose crystallized from light petroleum. The m.p., 80–83°, was low; however, the I.R. spectrum was identical to that of authentic 2,3,4,6-tetra-*O*-methyl-D-glucose.

*The tri-*O*-methyl fraction.* Demethylation gave galactose and mannose as the parent sugars. The methyl sugars were separated by paper chromatography (solvent c) and characterized as follows; 2,3,4-tri-*O*-methyl-D-mannose was indistinguishable from an authentic sample by GLC. 2,3,6-Tri-*O*-methyl-D-galactose was indistinguishable from an

authentic sample by GLC; oxidation with bromine yielded the γ -lactone, m.p. 98–99°, $[\alpha]_D -25^\circ$ (c 1.1, water).

2,3,5-Tri-O-methyl-D-galactose was indistinguishable from an authentic sample by GLC.

The di-O-methyl fraction. Demethylation gave mannose. GLC indicated a single component. Reduction with sodium borohydride followed by oxidation with periodate yielded a pentose which was indistinguishable from 2,4-di-O-methyl-L-arabinose by GLC. The *p*-nitroaniline derivative of di-O-methyl-D-mannose was prepared with m.p. 221–223°, $[\alpha]_D -348^\circ$ (c 0.05, pyridine). (Found: N 8.2. $C_{14}H_{20}O_5N_2$, requires: N 8.5).

The acidic fraction. Paper chromatography (solvent d) indicated three components which were separated by preparative paper chromatography (Whatman No. 3) using the same solvent. The three components corresponding to glucuronic acid, aldobiouronic acid and an oligosaccharide were recovered and characterized as follows; *2,3,4-tri-O-methyl-D-glucuronic acid* was indistinguishable from an authentic sample by GLC. Reduction of the methyl glycoside mixture with lithium aluminium hydride in ethyl ether yielded the corresponding methyl glucosides which were indistinguishable by GLC from those obtained from 2,3,4-tri-O-methyl-D-glucose. The aldobiouronic acid was hydrolysed (4% methanolic hydrogen chloride, 100°, 12 h) and examined by GLC. Equal amounts of 2,3,4-tri-O-methyl-D-glucuronic acid and 2,3,6-tri-O-methyl-D-galactose were detected. The oligosaccharide which constituted two-thirds of the weight of the acidic fraction was hydrolysed; 2,3,4-tri-O-methyl glucuronic acid, a smaller quantity of 2,3,6-tri-O-methyl galactose and tetra-O-methyl sugars were detected by GLC.

Methylation analysis of the partially degraded heteropolysaccharide. The heteropolysaccharide (427 mg) isolated by chromatography on DEAE-Sephadex was hydrolysed with 0.01 M HCl (100 ml) at 100° for 3 h. After concentrating at 40°, the solution was added dropwise to ethanol. The hydrolysis was repeated twice with isolation of the alcohol insoluble material each time. The final alcohol insoluble fraction (202 mg), $[\alpha]_D +70^\circ$ (c 1.0, water) contained 59% mannose, 32% glucose, and 9% galactose. Chromatography on Sephadex DEAE (A-25) showed that 80% of this fraction was neutral, that is glucuronic acid was no longer present. The polysaccharide (103 mg) was methylated as previously described yielding a product (135 mg) with $[\alpha]_D +79^\circ$ (c 1.0, chloroform). Analysis by GLC indicated 27% 2,3,4,6-tetra-O-methyl-D-glucose, 38% 2,3,4-tri-O-methyl-D-mannose, 8% 2,3,6-tri-O-methyl-D-galactose, and 27% 2,4-di-O-methyl-D-mannose.

Quantitative analysis of the methylated heteropolysaccharides. The quantitative analyses reported in Table 1 and above were determined by GLC of alditol acetates (column b, 200°).

Periodate oxidation. The heteropolysaccharide (209 mg) was dissolved in water (8 ml) and 0.06 M sodium periodate (40 ml) was added. The volume was adjusted to 50 ml with water and the oxidation allowed to proceed in the dark at room temperature for 48 h. Periodate consumption was measured at intervals by the optical method¹⁰ and formic acid was measured by titration after reduction of excess periodate with ethylene glycol. The periodate consumption was 0.86 moles and 0.29 moles formic acid were produced per 162 g polysaccharide. The resulting polyaldehyde was reduced with sodium borohydride and precipitated with ethanol. Hydrolysis yielded glycerol, threitol, galactose, and mannose.

Partial hydrolysis. The heteropolysaccharide (5 g) was dissolved in 0.01 M HCl (500 ml) and the solution heated to 100° for 3 h. After concentrating at 40°, the solution was added dropwise to ethanol. The precipitate was collected and rehydrolysed as above; this was repeated twice with isolation of the ethanol soluble and insoluble fractions each time. The alcohol soluble fraction was placed on top of a Dowex 1 (acetate form) column and the column eluted with water until the eluate no longer contained sugars, then the column was eluted with 0.5 M H_2SO_4 yielding the acidic fraction. Paper chromatography (solvent b) of the neutral fraction indicated mainly galactose; however, small amounts of mannose, a disaccharide and trisaccharide were also present. The components were separated by preparative paper chromatography and identified as follows; galactose was reduced to galactitol, m.p. 187–188°. The disaccharide, $[\alpha]_D -18^\circ$ (c 1.8, water), M_G 1.1, gave galactose with a trace of mannose on hydrolysis. Mild hydrolysis (0.02 M HCl for 3 h at 100°) cleaved the disaccharide completely. Methylation and subsequent analysis by GLC revealed 2,3,5,6-tetra-O-methyl-D-galactose and 2,3,5-tri-O-methyl-D-galactose. Methylation and hydrolysis of authentic 6-O- β -D-galactopyranosyl-D-galactose yielded

the same tri-*O*-methyl sugar. The trisaccharide had M_G 1.22 and gave galactose only on hydrolysis.

The acidic fraction was neutralized with barium carbonate, filtered, concentrated, treated with Dowex 50 (H^+) and examined by paper chromatography (solvent b). The bulk of the material had very low mobility although traces of more mobile components could be detected. These were removed by column chromatography on Sephadex G-15. The purified material, $[\alpha]_D + 62^\circ$ (*c* 1.4, water), gave glucuronic acid, mannose, and galactose on hydrolysis. Part of this material (40 mg) was acetylated in formamide with pyridine-acetic anhydride, then methylated in dioxane with dimethyl sulphate and sodium hydroxide followed by treatments with methyl iodide and silver oxide. 2,3,4-Tri-*O*-methyl-D-glucuronic acid with smaller amounts of 2,3,4,6-tetra-*O*-methyl mannose and 2,3,6-tri-*O*-methyl-D-galactose were detected by hydrolysis and analysis by GLC. The remaining material was hydrolysed (0.1 M HCl, 100° , 3 h) and although most of the material still did not migrate, monosaccharides and two additional components were detected by paper chromatography (solvent b). The latter were separated by preparative paper chromatography using the same solvent. The more mobile component, corresponding in mobility to an aldobiouronic acid, had $[\alpha]_D + 67^\circ$ (*c* 0.5, water); hydrolysis revealed galactose and glucuronic acid. The disaccharide was reduced with sodium borohydride and oxidized with sodium periodate; 0.96 moles formaldehyde were produced per mole of reduced aldobiouronic acid. Since 2,3,6-tri-*O*-methyl-D-galactose was detected in the aldobiouronic acid from the methylated polysaccharide, the above results show that the aldobiouronic acid is 5-*O*- α -D-glucuronosyl-D-galactose.

The alcohol insoluble fraction was hydrolysed with 0.04 M HCl at 100° for 5 h and the alcohol soluble products isolated. This was repeated until all the material was alcohol soluble; then the material was separated on a carbon column using a gradient of ethanol as eluent. The monosaccharides were separated by paper chromatography (solvent a) and identified as follows; mannose was converted to the *p*-nitroaniline derivative with m.p. $217-218^\circ$, $[\alpha]_D - 338^\circ$ (*c* 0.2, pyridine). Glucose was also converted to the *p*-nitroaniline derivative, m.p. $180-181^\circ$, $[\alpha]_D - 180^\circ$ (*c* 0.08, pyridine). Paper chromatography of the disaccharide containing fraction (solvent b) indicated several components. These were separated by paper chromatography (Whatman No. 3) using the same solvent. The component with the highest R_F was identified as 6-*O*- α -D-mannopyranosyl-D-mannose as follows; hydrolysis indicated mainly mannose with traces of glucose and galactose, therefore, the separation by paper was repeated. The disaccharide then crystallized; recrystallisation from ethanol gave the pure compound, m.p. and mixed m.p. $186-187^\circ$, $[\alpha]_D + 61^\circ$ (*c* 0.4, water), M_G 1.44.

Because of the complexity of the other fractions they were not further investigated.

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