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Polysaccharides Elaborated by *Pullularia pullulans*

Part III.* Polysaccharides Synthesised from Xylose Solutions

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The polysaccharides synthesised by *Pullularia pullulans* from xylose solutions have been isolated and fractionated to yield three components, *viz.* an α-linked glucan containing (1→4)- and (1→6)-linkages in the proportion 2.2:1, a β-linked glucan containing (1→3)- and (1→6)-linkages, and an acidic heteropolysaccharide containing galactose, glucose, mannose and hexuronic acid. A structure is proposed for the β-linked glucan.

It has already been reported[1,2] that *Pullularia pullulans* synthesises polysaccharides from a variety of sugar substrates and that the polysaccharide products appear to be a mixture of varying proportions of the same basic ingredients, two glucans, one α- and one β-linked, and an acidic polysaccharide or mixture of polysaccharides containing galactose, glucose, mannose and hexuronic acid[2]. The polysaccharides synthesised from solutions of xylose have been studied with a view to obtain some information concerning the components other than the α-glucan, since these components are formed from that substrate in relatively high proportions. The findings of this investigation are presented below.

The separation procedures required for the various batches of culture solution dealt with tended to vary to some extent from one instance to another but several generally attainable effects could be observed. The β-linked glucan was precipitated with cetyl trimethylammonium hydroxide (CTA-OH) in every instance. Treatment of the CTA-precipitate with dilute hydrochloric acid left the polysaccharide in a water-insoluble state and it could then be purified by aqueous extraction of impurities and reprecipitation from alkaline solution. The α-glucan was in some instances precipitated with CTA-OH or co-precipitated with the other two components but could be separated from

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the "acidic" β-glucan because of its solubility in water and from the acidic heteropolysaccharide either by preferential precipitation with ethanol or by the use of anionic cellulose ion-exchangers. One of the more complicated series of separations is described in the experimental section.

The samples of the α-linked glucan examined had specific rotations between + 180° and + 190° and appeared on oxidation with periodate to contain (1 → 4)- and (1 → 6)-like linkages in the ratio 2.2:1. The corresponding data for the α-glucan from sucrose-containing media were + 180° and 2.1:1 [2]. The present α-glucan thus appears to be similar to those isolated from cultures of the organism grown on glucose [1] and sucrose [2,3] at least in optical rotation and ratio of the component linkages. It is not synthesised, however, from xylose to the same extent of preponderance over the other products as from glucose and sucrose.

Samples of the "acidic" β-glucan were examined in some detail. The purified polysaccharide had $[\alpha]_D^{22}$ −3° (*c*, 1.0 in 1 M sodium hydroxide), less than 3 % uronic acid (by decarboxylation) and yielded glucose as the only reducing sugar on hydrolysis. On oxidation with periodate, the consumption of oxidant was 0.84 moles/162 g and the yield of formic acid 0.41 moles/162 g. The infra-red spectrum showed the characteristic absorption for a β-linked glucan at 890 cm^{-1} and a small carboxylate absorption at 1630 cm^{-1}. Enzymic degradation by a β-(1→3) glucanase isolated from Basidiomycete QM 806 [4] proceeded with efficiencies varying from 26 to 38 % with the formation of glucose and gentiobiose as the only products of low molecular weight in the molar ratio *ca.* 1:2. Gentiobiose was isolated from a digest and characterised as the β-octaacetate. The degradation of a sample of the glucan by the Smith degradation procedure [5] yielded glycerol, small amounts of glucose and higher saccharides, and a residual polysaccharide difficultly soluble in dilute sodium hydroxide. Partial hydrolysis of this product with dilute mineral acid yielded glucose, laminarobiose, laminarotriose and higher oligosaccharides of that series as identified by paper chromatography.

Methylation of the β-glucan yielded a product with $[\alpha]_D$ −41° and methoxyl content 44.0 %; the infra-red spectrum showed only minute hydroxyl absorption and no carboxylate absorption. Hydrolysis of the methylated polysaccharide yielded 2,3,4,6-tetra-*O*-methyl-D-glucose (40 mole %), 2,4,6-tri-*O*-methyl-D-glucose (20 mole %) and 2,4-di-*O*-methyl-D-glucose (41 mole %). The methylated sugars were identified by their melting points, optical rotations, chromatographic mobilities, and by comparing them with authentic specimens.

Although the β-glucan was isolated by precipitation with CTA-OH no definite evidence was obtained for the presence of acid groups as structural features in the purified product. The results obtained from methylation and hydrolysis, enzymic hydrolysis, and from periodate oxidation of the polysaccharide and degradation of the reduced oxidation product, suggest that the glucan consists of a backbone of (1→3)-linked β-D-glucopyranose residues, roughly two of every three substituted in the 6-position by a β-D-glucopyranose residue. Yeast glucan also consists of (1→3)- and (1→6)-linked β-D-glucopyranose residues but differs in the mode of distribution of the (1→6)-linkages in the molecules, these occurring not as terminal but as chain linkages in groups of up to three consecutive linkages [6]. Much more closely similar is

the corn gum glucan isolated by Smith and coworkers [7] in which single β-D-glucopyranose residues are linked to a $(1\rightarrow3)$-β-linked backbone through $(1\rightarrow6)$-linkages.

The acidic heteropolysaccharide was examined briefly. Samples of the material isolated from different cultures contained similar proportions of the constituent sugars, *i.e.* galactose, glucose, mannose roughly in the proportion 4:1:5 and *ca.* 10 % uronic acid. The infra-red spectrum showed absorptions for carboxylate at 1610 and 1405 cm^{-1} and for amide at 1640 and 1520 cm^{-1}, indicating the presence of protein as contamination. Fractionation on columns of diethylaminoethyl (DEAE) cellulose [8] by elution with aqueous potassium acetate of increasing concentration yielded fractions of increasing uronic acid content and of slightly differing contents of neutral sugars. The polysaccharide was extensively degraded by hydrolysis with 0.01 N sulphuric acid at 100° for 2.5 h, the principal products being galactose, acids and a residue of high molecular weight, *i.e.* not passing the dialysis membrane in an LKB ultrafilter. This high molecular weight residue was completely eluted from a DEAE cellulose column at half the concentration of potassium acetate that had to be used to elute the original polysaccharide. The presence of glucuronic acid was indicated among the acidic components in a hydrolysate.

The results obtained from the examination of the acidic heteropolysaccharide do not permit any conclusions to be drawn concerning the structure but do suggest that the material is a mixture of closely similar molecular species with only small differences in acid content and sugar composition. There is some superficial resemblance to the polysaccharide isolated by Siddiqui and Adams from *Gibberella fujikuroi* [9] but the latter did not yield free sugars on heating with very dilute mineral acid.

EXPERIMENTAL

Culture of the organism and isolation of the polysaccharides. The mould was grown at 27° in batches of nutrient solution containing 50 g xylose per litre and the following salts (same as for the sucrose cultures [2]): 1 g KH_2PO_4, 0.2 g $CaCl_2 \cdot 6H_2O$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.02 g $FeCl_3 \cdot 6H_2O$ and 2.5 g $NaNO_3$ per litre. After 4—7 days the cultures were cooled to 5° and the cells separated by supercentrifugation. The cell-free solutions were added to 1.5 volumes of ethanol and the precipitated polysaccharide collected, dissolved in water, reprecipitated with ethanol, washed and dried. Depending on the duration of the culturing, the polysaccharide yield varied from 3 to 5 g crude material per litre, with heavy contamination by a dark pigment in the cases of the older cultures. A crude sample of a pigment-free isolate had the following properties: $[\alpha]_D^{20}$ + 60°, uronic acid 2.2 %, neutral sugar composition in a hydrolysate galactose 9.5 %, glucose 77.5 %, mannose 13.1 %.

Fractionation and purification of the polysaccharide. A solution of crude polysaccharide (19 g) in water (220 ml), previously clarified by supercentrifugation, was treated with 0.23 M CTA-OH (170 ml), the pH thereby being raised from 4.5 to 8.0. The precipitate formed was separated by centrifugation and washed with water. It was dispersed in 0.2 M hydrochloric acid (1000 ml) and the regenerated polysaccharide precipitated with ethanol from the neutralised solution, washed and dried to give fraction A (9.7 g). The supernatant and washings from the CTA precipitate were combined, deionised, concentrated and lyophilised to give fraction B (3.1 g).

Both A and B yielded mainly glucose on hydrolysis with small amounts of galactose, mannose and acid, the contribution of the minor products being markedly greater in A. B had $[\alpha]_D^{22}$ + 128°. A and B were further fractionated by use of the differing solubilities of the component polysaccharides as shown in Table 1.

Table 1. Fractionation of *P. pullulans* polysaccharides and properties of the fractions.

Fract.	Weight (g)	$[\alpha]_D$	Constituents	Soluble in	Insoluble in
A_1	3.95	+ 10°	Gl (main), Gal, Man, Acid	1 M NaOH	Water
A_2	2.00	+ 144°	Gl (main), Gal, Man, Acid	Water	70 % EtOH
A_3	2.62	+ 69°	Gl, Gal, Man, Acid	70 % EtOH	
B_1	1.40	+ 186°	Gl	Water	70 % EtOH
B_2	1.00	+ 36°	Gl, Gal, Man, Acid	70 % EtOH	

A_1 was extracted with water and then reprecipitated from alkaline solution to give A_{11} (2.5 g) with $[\alpha]_D^{22}$ −3° (*c*, 1.0 in M NaOH) and yielding on hydrolysis glucose as the only reducing sugar. It contained 2.6 % uronic acid by decarboxylation and the infrared spectrum showed the characteristic absorption for a β-linked glucan at 890 cm^{-1} and a small carboxylate absorption at 1620 cm^{-1}.

A_2 was purified by passing its aqueous solution through a column of Whatman DEAE cellulose to give A_{21} containing only glucose and having $[\alpha]_D^{22}$ + 180° (*c*, 0.9 in water).

A_3 was resolved into three fractions by chromatography on a DEAE cellulose column. The fractionation and the properties of the fractions are summarised in Table 2. The composition of the crude heteropolysaccharide fractions from two other cultures is included in the Table.

Enzymic degradation of A_{11}. Unstable aqueous solutions of the material were obtained by dissolving in 0.5 M sodium hydroxide and removing the sodium ions with Dowex 50W ion exchange resin. Solutions thus prepared were buffered with citrate (0.05 M, pH 4.6) and treated with a β-(1→3)-glucanase isolated from Basidiomycete QM 806 [4]. Polysaccharide concentration was *ca.* 2 mg/ml (hypoiodite oxidation of a hydrolysed sample) and enzyme concentration 0.5 mg/ml. The increase in reducing power was estimated by hypoiodite oxidation or with the Somogyi reagent. The degree of hydrolysis was 26 % at 50° and 38 % at 60−65°. This relatively low efficiency of the enzyme might be a result of retrogradation of the polysaccharide.

Chromatographic examination of the deionised digests showed the only products of low molecular weight to be glucose and gentiobiose. Fractionation of the products of a digest (60 mg) on a column of Sephadex G-25 [10] (4.5 × 140 cm) yielded pure gentiobiose (12 mg) which was characterised as the β-octaacetate, m.p. and mixed m.p. 193.5−195°, $[\alpha]_D^{22}$ −4.9° (*c*, 0.7 in chloroform). The molecular ratio of glucose to gentiobiose determined by hypoiodite oxidation was 1:2.02.

Periodate oxidation of A_{11}. Aqueous solutions of A_{11} obtained as described above were treated with sodium metaperiodate. The observed consumption of periodate and liberation of formic acid were 0.84 mole/162 g and 0.41 mole/162 g, respectively. Total hydrolysis of a sample of the polyalcohol, obtained by reducing the fully oxidised poly-

Table 2. Fractionation of A_3 and properties of the fractions.

Fraction	Weight (g)	Eluant (M KOAc)	$[\alpha]_D$	Neutral sugars % Gl	Gal	Man	Uronic acid %
A_{31}	0.828	0.01	+ 180°	100	—	—	—
A_{32}	0.350	0.20	+ 9°	9.1	39.5	47.5*	7.7
A_{33}	0.420	0.50	+ 26°	5.2	44.0	50.8	13.8
Crude mtrl			+ 16°	10.7	40.1	49.2	9.8
(see text)			—	10.2	39.5	50.3	—

* Contained also 4.0 % arabinose.

saccharide with sodium borohydride,[11] with sulphuric acid yielded glycerol and glucose. Hydrolysis of the polyalcohol with 0.25 N sulphuric acid at room temperature over night [5] liberated principally glycerol along with a little glucose and traces of di- and trisaccharides. The bulk of the material (70 %) remained insoluble. It could be dissolved in 2 M sodium hydroxide but precipitated on neutralisation of the alkaline solution. Partial hydrolysis (0.25 N sulphuric acid at 100° for 5 h) yielded a series of saccharides which was fractionated on a Sephadex column. The di- and trisaccharide fractions were examined by paper chromatography and found to consist of laminarobiose and laminarotriose. Neither gentiobiose nor any other di- or trisaccharide could be detected.

Methylation of A_{11}. The polysaccharide (2.0 g) was dissolved by stirring under a nitrogen atmosphere in 42.5 % aqueous sodium hydroxide (80 ml) containing 0.05 g sodium borohydride. The solution was cooled in an ice-bath and dimethyl sulphate (10 ml) was added over a period of 8 h. Dioxan (160 ml) was added and over 3 days three further 10 ml portions of dimethyl sulphate. The solution was diluted with water, neutralised with sulphuric acid and taken to dryness. The solids were extracted exhaustively with pyridine. The extracted material was treated with acetic anhydride (15 ml) in pyridine (70 ml) at room temperature over a period of 24 h. The acetylated product (2.1 g) was dissolved in dioxan (80 ml), and dimethyl sulphate (24 ml) and powdered sodium hydroxide (24 g) were added over 48 h with vigorous stirring. The reaction was conducted at room temperature during the first 8 h and subsequently at 50°. The product (2.0 g) was further methylated with methyl iodide (6 ml) and silver oxide (5 g) in dimethyl formamide (50 ml). The final product had 44 % methoxyl and $[\alpha]_D^{22}$ $-41°$ (*c*, 0.9 in chloroform). The infra-red spectrum showed only a minute hydroxyl absorption and no carboxylate or carbonyl absorption. Hydrolysis of a sample yielded di-, tri- and tetramethylglucoses in the molar percentages 40, 23, and 37, respectively, measured by hypoiodite oxidation of the components after separation on filter sheets.

Hydrolysis of methylated A_{11}. The methylated polysaccharide (1.6 g) was dissolved in 90 % formic acid (50 ml) and heated at 100° for 1 h. After removal of the acid by evaporation the residue was dissolved in 0.5 N sulphuric acid (50 ml) and heated at 95° for 16 h. After neutralisation (Dowex 50W) and concentration 1.5 g of methylated sugars were obtained.

The mixture of methylated sugars was resolved by chromatography on columns of cellulose (solvent: butanol:ethanol:water 10:3:5) and silica gel [12] (solvents: chloroform and chloroform-butanol) to give 2,4-di-*O*-methyl-D-glucose (0.56 g), 2,4,6-tri-*O*-methyl-D-glucose (0.29 g) and 2,3,4,6-tetra-*O*-methyl-D-glucose (0.62 g), the molar percentages being 41, 20, and 40, respectively.

*Characterisation of the methylated sugars. 2,3,4,6-Tetra-*O*-methyl-D-glucose.* The sugar had m.p. and mixed m.p. 93–96° and $[\alpha]_D^{22}$ $+ 80°$ (equil.; *c*, 1 in water). Its infra-red spectrum was identical to that of authentic 2,3,4,6-tetra-*O*-methyl-D-glucose.

*2,4,6-Tri-*O*-methyl-D-glucose.* The sugar had m.p. and mixed m.p. 119–123° and $[\alpha]_D^{22}$ $+ 70°$ (equil.; *c*, 0.33 in aqueous methanol). The infra-red spectrum was identical to that of an authentic sample of 2,4,6-tri-*O*-methyl-D-glucose.

*2,4-Di-*O*-methyl-D-glucose.* The sugar had m.p. 123–125° and $[\alpha]_D^{22}$ $+ 42 \rightarrow + 72°$ (12 h; *c*, 0.5 in water). Bell and Manners [13] give m.p. 125–129° and $[\alpha]_D$ $+ 43.3 \rightarrow + 73.7°$. The mobility on paper electrophoresis in borate buffer of pH 10 was lower than for the 2,3-, 2,6-, 3,6-, and 4,6-di-*O*-methyl-D-glucoses. Acetylation with sodium acetate and

Table 3. Properties of fractions A_{31} and B_1.

Fraction	Sugar content	$[\alpha]_D$	IO_4 consumed mole/162 g	HCOOH released mole/162 g
A_{31}	Glucose	+ 180°	1.33	0.31
B_1	Glucose	+ 186°	1.32	0.31
Glucan fr. sucrose cultures	Glucose	+ 190°	1.31	0.32

acetic anhydride yielded an acetate with m.p. 106—107° and $[\alpha]_D^{22}$ + 11° (*c*, 1.5 in chloroform). 1,3,6-Tri-*O*-acetyl-2,4-di-*O*-methyl-β-D-glucose is reported to have m.p. 105—106° and $[\alpha]_D^{22}$ + 11.5° [13].

Examination of the a-linked glucan. Fractions A_{31} and B_1 had the properties summarised in Table 3. The corresponding data for the α-linked glucan isolated from sucrose cultures are included for comparison.[2]

Examination of the heteropolysaccharide. The properties of some heteropolysaccharide fractions are given in Table 2. The infra-red spectrum of the unfractionated polysaccharide showed carboxylate absorptions at 1610 and 1405 cm^{-1} and amide bands at 1640 and 1520 cm^{-1}.

On heating with 0.01 N sulphuric acid at 100° for 2.5 h the polysaccharide was substantially degraded, *ca.* 60 % of the material passing through the membrane of an LKB ultrafilter. The low molecular weight fraction consisted of galactose and acidic and neutral oligosaccharides. The non-filterable moiety had an uronic acid content of 6.3 % compared with 9.8 % in the undegraded material.

A sample of the crude polysaccharide was fractionated on a DEAE cellulose column by stepwise elution with aqueous potassium acetate increasing in molarity from 0 to 0.50 in steps of 0.05. A sample of the fraction eluted by 0.20 M acetate (152 mg) was degraded with 0.01 N sulphuric acid as described above and separated into high and low molecular weight fractions by ultrafiltration. The non-filterable moiety (72 mg) was absorbed on a DEAE cellulose column and the stepwise elution repeated. All the material was eluted by 0.10 M potassium acetate, with nothing appearing in the 0.20 M eluate, the concentration required for the undegraded material.

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