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Polysaccharides Elaborated by *Pullularia pullulans*

Part I. The Neutral Glucan Synthesised from Sucrose Solutions

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Dedicated to Professor *Holger Erdtman* on his 60th birthday

From the crude polysaccharide isolated from cultures of the fungus *Pullularia pullulans* in solutions of sucrose, a neutral glucan and an acidic polysaccharide, containing largely glucose, have been obtained. Structural investigation of the former yielded evidence of an essentially linear molecule of more than 250 α-D-glucopyranose residues linked (1 → 6) and (1 → 4) in the ratio 1:2.

The data compiled up to the present would seem to indicate that the polysaccharides of moulds are based almost without exception on the sugars galactose, glucose and mannose, with the addition of glucosamine in the case of intracellular material [1]. Glucuronic acid and fucose are the most common additives to this basic composition. The distribution of this relatively simple list of ingredients among the occurring molecular species is, however, somewhat varied, both homo- and heteropolymers having been characterised in the limited number of structural investigations reported [2]. More recently, a complex acidic polysaccharide material has been isolated from *Gibberella fujikuroi* [3], containing glucose, galactose, mannose and glucuronic acid, but there is some uncertainty concerning the homogeneity of the preparation. The evidence as it stands does confirm the presence of a polymer in which mannopyranose, galactofuranose and glucuronopyranose residues are combined, the latter forming both terminal and chain units.

Pullularia pullulans has been found to produce two physically distinct polysaccharide components, *viz.* a water-soluble glucan, and a difficultly soluble, jelly-like material, strongly adherent to the cells, and containing glucose, glucuronic acid and small amounts of galactose and mannose [4]. Wallenfels and co-workers [5], in a series of communications, report the isolation of "pullulan", an unbranched glucan of chain length *ca.* 300 glucopyranose units, with α-(1→4)- and α-(1→6)-linkages occurring in the ratio

3:2, from cultures of *P. pullulans* grown on glucose as the carbon source. A brief treatment of this "pullulan" with hot aqueous formic acid yielded a residual polysaccharide which could be converted completely to maltotriose by the action of an enzymic preparation isolated from *Aerobacter aerogenes*.

The present work is directed towards the examination of the polysaccharides elaborated extracellularly by *P. pullulans* from various sugar substrates. Polymer formation has been observed to occur from sucrose, glucose, maltose, mannose, xylose and arabinose as carbon sources but not from either galactose or mannitol. As this list of substrates is traversed from sucrose to arabinose, the associated efficiency of polysaccharide production falls, and the relative amounts of galactose, mannose and glucuronic acid in the polysaccharide mixtures increase. Fractionation of these mixtures with cetyl trimethylammonium hydroxide [6] (CTA—OH), into neutral and acidic components, has shown a clearly defined pattern through the series, *viz.* soluble acidic fractions, rich in galactose and mannose, insoluble acidic fractions, rich in glucose, both types having low specific rotations and containing phosphate and uronic acid; and neutral soluble fractions, rich in glucose, with high specific rotations and free from phosphate and uronic acid. The efficiency of the fractionation is governed by the composition of the crude material to a large extent; both neutral and acidic glucans have been isolated readily from the crude mixtures of polysaccharides obtained from sucrose and maltose cultures, which contained little mannose and galactose initially.

The crude polysaccharide isolated from cultures grown on sucrose yielded 97 % of reducing sugar on hydrolysis, namely glucose, with very small amounts of galactose, mannose and fructose and had specific rotation *ca.* + 190°, 0.44 % nitrogen, and 2.42 % phosphate. On oxidation with periodate, the material consumed 1.41 mole of periodate and released 0.45 mole of formic acid per mole of combined anhydroglucose, indicating the presence of 45 % (1→6)-like and 51 % (1→4)-like linkages.

The purified neutral glucan obtained by fractionation with CTA—OH contained only glucose, was free from phosphate, and showed a single immobile component on paper electrophoresis in borate buffer (pH 10). It had a specific rotation of + 190°, 0.11 % ash, showed no colour reaction with iodine solution and was unattacked by α-amylase. Oxidation with periodate led to the consumption of 1.31 mole of periodate and the liberation of 0.32 mole of formic acid per mole of combined anhydroglucose indicating the presence of 32 % (1→6)-like and 67 % (1→4)-like linkages. Reduction and hydrolysis of the polyaldehyde thus formed yielded 0.63 mole of erythritol, 0.35 mole of glycerol and 0.02 mole of glucose per anhydroglucose residue in the original polysaccharide. Methylation and subsequent hydrolysis yielded 2,3,4-tri-*O*-methyl-D-glucopyranose (33 %), 2,3,6-tri-*O*-methyl-D-glucopyranose (66 %) and 2,3,4,6-tetra-*O*-methyl-glucopyranose (0.4 %). The partial acetolysate of a sample yielded, on deacetylation, glucose (26 %), isomaltose (3 %), maltose (23 %) and higher saccharides including two trisaccharides. These trisaccharides, although not characterised by the formation of crystalline derivatives, appeared, from their chromatographic and electrophoretic mobilities, optical rotations, and behaviour on partial hydrolysis of the reduced and unreduced sugars, to be isomaltotriose (4 %) and maltotriose (> 8 %), respectively.

The acidic glucan was isolated from the polysaccharide mixtures produced in sucrose cultures by means of CTA—OH and copper ethylenediamine. It yielded glucose and uronic acid, mainly as the copper salt, on hydrolysis, was insoluble in water and had $[\alpha]_D$ 0 ± 4° (*c* 0.65 in N NaOH). It contained 66.1 % of reducing sugars (not corrected for combination with uronic acid), 9.9 % of uronic anhydride (decarboxylation), copper (7.8 %) and nitrogen (0.4 %). It consumed 0.74 mole of periodate per mole of anhydrosugar with release of 0.37 mole of formic acid. Reduction and hydrolysis of the periodate-oxidised polysaccharide yielded glycerol and glucose; no erythritol was detected. The structure of this glucan is now being studied.

It is clear that the neutral glucan described above is very closely akin to the material studied by Wallenfels *et al.* and is possibly identical to their "restpullulan" in all but fine structure (Table 1). It should be stated, however,

Table 1.

	$[\alpha]_D$	Ratio (1→6) to (1→4)-linkages By periodate oxidation	By methylation
Crude glucan	+ 194°	0.82	—
Purified glucan	+ 190°	0.48	0.50
"Pullulan" [5]	+ 192°	0.64	0.60
"Restpullulan" [5]	—	0.50	—

that the neutral glucan may well contain a small amount of (1→3)-linkage (1—2 %) for, although no 2,3,6-tri-*O*-methyl glucose was detected in the methylation study, small amounts of unoxidised glucose were detected in experiments based on periodate oxidation.

The polysaccharides produced from the other sugars as sources of carbon have not yet been examined in detail. A neutral glucan has been isolated from cultures grown on maltose, using the CTA—OH fractionation technique, and a preliminary examination shows properties similar to that synthesised from sucrose solutions. The polysaccharide yields only glucose on hydrolysis, has a specific rotation of + 198°, and shows no electrophoretic mobility in borate buffer (pH 10). Periodate oxidation indicates the presence of *ca.* 32 % of (1→6)-linkages, the remainder being ostensibly (1→4). Reduction and hydrolysis of the oxidised polymer yields erythritol, glycerol, and a little glucose. The products of partial acid hydrolysis include components identified chromatographically as maltose, isomaltose and possibly nigerose.

EXPERIMENTAL

Evaporations were carried out under reduced pressure at temperatures not exceeding 40°.

Paper chromatograms were run on Whatman No. 1 and 3MM papers, using the following systems;

A. butanol, ethanol, water, 10:3:5;
B. butanol, pyridine, water, 2:7:2;
C. ethyl acetate, pyridine, water, 10:4:3;
D. ethyl acetate, acetic acid, water, 3:1:3;
E. benzene, dimethylsulphoxide, 20:1, with papers impregnated with dimethyl sulphoxide.

Table 2. Fractionation of crude polysaccharide from *P. pullulans* grown on sucrose-containing cultures.

Fraction	ml CTA—OH added	wt. (g)	$[\alpha]_D$ (NaOH)	Sugars Gal	Gl	Man	Phosphate
1	4	0.163	0 ± 4°	tr.	+	tr.	+
2	4	minute					
3	4	0.510	+ 51°	tr.	+	tr.	4.6 %
4	40	6.645	+ 160°	—	+	—	—
5	40	2.060	+ 170°	—	+	—	—
6	residue	0.293	+ 170°	—	+	—	—

Paper electrophoresis was carried out on Schleicher and Schüll glass fibre papers and on Whatman No. 3 and 3 MM papers in 0.1 M borate buffer of pH 10 at 0.8 kV.

Components were detected by the use of anisidine hydrochloride, α-naphthol-sulphuric acid in butanol, and silver nitrate-sodium hydroxide.

Sugar determinations were made with hypoiodite and Somogyi [8] reagents, those of erythritol and glycerol with periodate-chromotropic [9] acid. Periodate and formic acid were determined iodometrically.

Culture of the organism and the isolation of the polysaccharides. The mould was grown in solutions of the following composition: 0.1 g KH_2PO_4, 0.2 g $CaCl_2.6H_2O$, 0.3 g $MgSO_4.7H_2O$. 0.1 g NaCl, 0.02 g $FeCl_3.6H_2O$, and 2.5 g $NaNO_3$ per litre. As carbon source, 20—50 g of appropriate sugar was used per litre. The inoculated solutions were shaken at 25° for 5—6 days. Usually the time of growth was contrived to be less than that required for the development of the dark colour of the mature organism.

The viscous cultures were heated above 90° for 30 min, cooled and the cells separated by supercentrifugation. The supernatant solutions were de-ionised, concentrated and added dropwise to an excess of ethanol (3—4 volumes). The precipitated polysaccharides were separated by centrifugation or decantation, washed with ethanol and ether and dried *in vacuo* over phosphorus pentoxide.

Polysaccharides synthesised from sucrose. From 3—5 litre batches of culture grown on sucrose solutions (30 g/l) the crude polysaccharides were obtained in quantities of 16—25 g. The white fibrous products proved extremely difficult to redissolve completely in water. Acid hydrolysis yielded glucose along with traces of galactose, mannose and fructose. Paper electrophoresis showed the presence of a major immobile component and a minor mobile component which appeared as an elongated streak on glass fibre papers.

Table 3. Properties of polysaccharide materials from *P. pullulans.*

	Crude polysaccharide	Acidic polysaccharide	Neutral glucan
Specific rotation	+ 190° ± 5	0 ± 4°	+ 190°
Reducing sugars	97 %	66 %	—
Phosphate	2.4 %	—	0
Nitrogen	0.4 %	0.4 %	—
Copper	—	7.8 %	—
Uronic anhydride	—	9.9 %	—
Periodate consumption (moles/mole anhydrosugar)	1.41	0.74	1.31
Formic acid release (same basis)	0.45	0.37	0.32

The polysaccharide (12 g) was dissolved in water (1 000 ml) and fractions were obtained by the addition of 0.23 N CTA—OH as shown in Table 2.

The first three precipitates were washed with water and treated with aqueous acetic acid-hydrochloric acid. The treatment with acid failed to dissolve fractions 1 and 2. The weights mentioned in Table 2 refer to the total material then soluble in N NaOH and precipitable from the neutralised solution with ethanol. Fractions 3, 4 and 5 were easily dissolved in 10 % aqueous acetic acid and the regenerated polysaccharides were precipitated with excess ethanol. Fraction 6 was obtained by passing the solution through cation-exchange resin, concentrating, and adding dropwise to a large excess of ethanol.

Fractions 1 and 3 were combined and dissolved in copper ethylenediamine. The solution on acidification with acetic acid, deposited a blue copper complex which, on dissolving in water and treating with hydrochloric acid, gave a greyish insoluble precipitate. This material was separated and washed with ethanolic hydrochloric acid, ethanol, ether and dried *in vacuo* over phosphorus pentoxide to a grey powder (0.3 g). Hydrolysis of this acidic component yielded glucose and uronic acid. Fractions 4, 5 and 6 were combined to reconstitute the neutral glucan component. Some analytical data are given in Table 3 for the crude polysaccharide, and the components subsequently separated.

Applying the degradative method of Smith *et al.*[7] to the preparations mentioned in Table 3 it was found that both the crude mixture and the neutral glucan produced erythritol and glycerol, along with small amounts of glucose, whereas the acidic polysaccharide yielded only glucose and glycerol.

Degradation of the neutral glucan. The polysaccharide (200 mg) was oxidised at room temperature with 0.16 M periodate solution (50 ml) over a period of 10 days. The solution was neutralised with barium carbonate and the filtrate treated with sodium hydridoborate [7] (200 mg). After 4 h the solution was treated with acetic acid, to destroy excess hydridoborate, de-ionised, and the boric acid removed by repeated distillation with methanol, to leave the polyalcohol (176 mg). The hydrolysate (7 h at 100° with N sulphuric acid) was separated on paper chromatograms (solvent A) and the components estimated, giving glucose (1.88 mg), glycerol (21.9 mg) and erythritol (52.6 mg), the molecular proportions being 1.6, 35.0 and 63.4 %, respectively. Glycerol was characterised as the tri-*O*-benzoate, m.p. and mixed m.p. with an authentic sample 70—71°. The erythritol, m.p. and mixed m.p. 118—119°, was converted to the tetra-acetate, m.p. and mixed m.p. 83—84°.

Methylation of the glucan. The polysaccharide (4 g) was dissolved in dimethyl formamide (75 ml) and the solution was cooled. A mixture of acetic anhydride (25 ml) and pyridine (40 ml) was added slowly with stirring and, after the addition was complete, the solution was was stirred for a further 15 h at room temperature, and finally at 45° for 2 h. The cooled solution was added dropwise with stirring to cold water and the precipitated acetate was collected, dissolved in chloroform, and reprecipitated with light petroleum. The white fibrous solid (6 g) had $[\alpha]_D^{26}$ + 180° (*c* 0.45 in chloroform).

The acetate (6 g) was dissolved in dioxan (100 ml) and dimethyl sulphate (84 ml) and finely powdered sodium hydroxide (70 g) were added with stirring in portions of 12 ml and 10 g over a period of 48 h. The product (4.0 g) was remethylated with methyl iodide (12 ml) and silver oxide (10 g) in dimethyl formamide (100 ml) to give a methyl ether (3.18 g) with a methoxyl content of 44.2 %. Two further methylations gave a product (2.90 g) with 45.9 % methoxyl and $[\alpha]_D^{23}$ + 220° (*c* 1.38 in chloroform). Hydrolysis

Table 4. Fractionation of hydrolysed methylated glucan (2.0 g) from *P. pullulans.*

Fraction	Sugars	Weight	Mole %
32—100	2,3,6-trimethylglucose	1.17 g	65.6
101—275	2,3,4-trimethylglucose	0.58 g	32.5
Residue	trimethyl- and		0.5
	tetramethyl-		0.4
	glucoses	0.033 g	

Table 5. Fractionation of deacetylated partial acetolysate of *P. pullulans* glucan.

Fraction	Sugars	Weight (g)	Weight % of total
25—72	glucose	1.228	25.5
90—108	isomaltose, trace glucose	0.129	2.7
109—140	maltose, trace isomaltose	1.085	22.6
141—153	isomaltotriose, traces maltose, maltotriose	0.200	4.2
154—159	maltotriose, traces maltose, isomaltotriose	0.400	8.3
160—240	maltotriose, higher saccharides, traces acetates	1.769	36.8

of a small sample and examination on a paper chromatogram (solvent A) showed the presence of trimethyl glucose and a very faint trace of tetramethyl glucose.

Hydrolysis of the methylated glucan. Hydrolysis of a portion (2.0 g) of the methyl ether with 90 % formic acid (100 ml; 1 h at 100°) and 0.5 N sulphuric acid (100 ml; 14 h at 100°) yielded a syrupy mixture of sugars (2.04 g) which was adsorbed on a carbon-Celite column (44 × 3.5 cm). Elution was carried out, first, with a gradient of 2.5—5.5 % aqueous methyl ethyl ketone (5 l) and subsequently with 10 % aqueous methyl ethyl ketone to give the sequence of fractions (25 ml) shown in Table 4.

*Characterisation of the methyl ethers. 2,3,6-Tri-*O*-methyl-*D*-glucose.* The ether had m.p. 112—116° on recrystallisation from chloroform-light petroleum and $[\alpha]_D^{24}$ + 92° → + 68° (*c* 0.84 in water). The melting-point was undepressed on admixture with an authentic sample. The derived aldonic acid formed a phenyl hydrazide with m.p. 144—145°, undepressed on admixture with an authentic sample of 2,3,6-tri-*O*-methyl-D-gluconic acid phenyl hydrazide.

*2,3,4-Tri-*O*-methyl-*D*-glucose.* The syrup had $[\alpha]_D^{24}$ + 82° (*c* 1.95 in water). Reaction with *p*-phenylazobenzoyl chloride yielded a product, m.p. 164—165°, undepressed on admixture with authentic 1,6-di-*O*-*p*-phenylazobenzoyl-2,3,4-tri-*O*-methyl-D-glucose.

The residue gave on partition between water and chloroform trimethyl glucose and tetramethyl glucose, 26.4 % and 23.0 %, respectively (by hypoiodite oxidation). The latter component was identified as 2,3,4,6-tetra-*O*-methyl-glucose by its chromatographic mobility (solvents A and E) and colour reaction with *p*-anisidine hydrochloride spray reagent.

Partial acetolysis of the glucan[10]. The polysaccharide (8 g) was dissolved in a mixture (50 ml) of 100 parts acetic anhydride and 9 parts sulphuric acid and kept at 40° for 48 h. The cooled solution was poured into ice-water and the aqueous mixture was extracted with chloroform to give a syrupy mixture of acetates (12.6 g). Deacetylation of this mixture with sodium in methanol-chloroform at —5° for 60 min[11] yielded a mixture of sugars (5.4 g) which was adsorbed on a carbon-Celite column (55 × 6.5 cm). Gradient elution was carried out in the following stages:

(1) 0 —10 % aqueous ethanol (5 l)
(2) 10—35 % aqueous ethanol (5 l)
(3) 35 % aqueous ethanol with a methyl ethyl ketone gradient of 0—10 % (5 l). Fractions (50 ml) were obtained as shown in Table 5.

Characterisation of the sugars. Fractions 25—72. The sugar, on recrystallisation from methanol, had m.p. 140—142° and $[\alpha]_D^{23}$ + 93° → + 52°, and was converted by acetylation with acetic anhydride and sodium acetate to *β*-penta-acetylglucose, m.p. 130—131°, and $[\alpha]_D^{23}$ + 0° (*c* 0.123 in chloroform).

Fractions 90—108. After purification on filter sheets (solvent C) the sugar was chromatographically and electrophoretically indistinguishable from isomaltose.

Table 6. Fractionation of polysaccharides from *P. pullulans* grown on maltose-containing cultures.

Fraction	ml CTA–OH	ml NaOH	Wt. (mg)	Neutral sugars		
				Gal	Gl	Man
1A	5	2	100	+	+	+
1B			40	–	+	–
2A	10	4	124	+	+	+
2B			50	+	+	+
3	residue		766	–	+	–

Fractions 109–140. The sugar on recrystallisation from methanol-ethanol had m.p. 118–120° and $[\alpha]_D^{24}$ + 109° → + 129°. Acetylation gave maltose β-octa-acetate, m.p. 158.5–159.5° and $[\alpha]_D^{22}$ + 62° (*c* 1.30 in chloroform).

Fractions 141–153. The principal component was purified on filter sheets by chromatography and electrophoresis to give an amorphous solid (98 mg) with $[\alpha]_D^{24}$ + 124° (*c* 0.98 in water), R_G 0.37 (solvent C) and M_G 0.53. Partial acid hydrolysis gave products chromatographically identical to glucose and isomaltose. Partial hydrolysis of the reduced trisaccharide yielded the same sugars as the only reducing products.

Fraction 154–159. The main component was purified on filter sheets by chromatography and electrophoresis to give an amorphous solid (240 mg) with $[\alpha]_D^{24}$ + 144° (*c* 0.52 in water), R_G 0.42 (solvent C), and M_G 0.30.

Partial hydrolysis of the trisaccharide and its reduction product yielded only glucose and maltose. Acetylation afforded a crystalline derivative, m.p. 95–100° and $[\alpha]_D^{25}$ + 81°. Attempts to purify the acetate further by chromatography on Magnesol-Celite columns failed to raise the melting-point.

The mixture of higher saccharides was not examined further.

Polysaccharides synthesised from maltose. The crude polysaccharide mixture resembled those obtained from sucrose cultures but contained rather more combined galactose and mannose. The material (1.5 g) was dissolved in 100 ml aqueous boric acid (0.01 M) and stepwise additions were made alternatively of 0.09 N CTA–OH and 0.01 N NaOH. Fractions were obtained as shown in Table 6.

The precipitates were stirred with 10 % aqueous acetic acid (containing a little HCl), centrifuged, and the soluble matter precipitated by the addition of ethanol (A fractions). The insoluble material was washed with ethanol, ether and dried (B fractions). The supernatant of fraction 2 was de-ionised, the boric acid removed as the volatile methyl ester, and the concentrated aqueous solution added to several volumes of ethanol to precipitate fraction 3.

Fractions 1A and 2A, on paper electrophoresis, moved as elongated streaks while fraction 3 was immobile. Refractionation of the combined fractions 1A, 2A and 2B gave only fractions containing galactose, glucose and mannose. The principal component, fraction 3, gave only glucose on total hydrolysis while the products of partial acid hydrolysis included saccharides corresponding in chromatographic mobility (solvent C) to maltose, isomaltose and possibly nigerose. Periodate oxidation led to the consumption of 1.3 mole of periodate and the liberation of 0.3 mole of formic acid per mole of combined anhydroglucose. Reduction and hydrolysis of the polyaldehyde thus formed showed the presence of erythritol, glycerol and a little glucose, when the products were examined on paper chromatograms (solvent A). The polysaccharide had $[\alpha]_D^{20}$ + 198° (*c* 0.81 in water).

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