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

Part I. Glucans

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The cell wall of *Aureobasidium (Pullularia) pullulans* contains three types of β-glucan. One, extracted with dilute alkali, has a linear backbone of essentially (1→3)-linked glucose residues part of which are substituted by single glucose residues in the 6-position. The cell wall material insoluble in dilute alkali contains a highly crystalline, essentially linear (1→3)-linked glucan and an amorphous glucan containing (1→3) and (1→6)-linked glucose residues.

As part of a study of morphogenesis of *Aureobasidium (Pullularia) pullulans* [1] cell walls of the yeastlike and filamentous forms were prepared. Chemical analysis revealed that polysaccharides were the main constituent; consequently, knowledge of the structure of these polysaccharides became an important aspect of the biochemistry of morphogenesis of this organism and perhaps black yeastlike fungi in general. Considerable information on the structure of polysaccharides produced by *A. pullulans* has already been reported by Bender *et al.*[2] and Bouveng *et al.*[3]; however, only extracellular polysaccharides have been examined. Bernier [4] reports the isolation of a jellylike material containing glucose, glucuronic acid and small amounts of galactose and mannose which adhered strongly to the cell walls.

The present paper reports the structural features of glucans isolated from cell walls of the yeastlike form of this organism. Extraction of cell walls with water at 100° yielded a heteropolysaccharide, studies of which will be reported in a subsequent publication. Further successive extractions with 0.1, 0.25 and 1.0 M potassium hydroxide yielded β-glucans. Periodate oxidations of these, and a similar product obtained from cell walls of the filamentous form, indicated that their structures were similar (Table 1). Methylation analysis of the β-glucan extracted with 1.0 M potassium hydroxide yielded 2,3,4,6-tetra-*O*-methyl-D-glucose (7 %), 2,4,6-tri-*O*-methyl-D-glucose (82 %), 2,3,4-tri-*O*-

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Table 1. Periodate uptake and formic acid formation by alkali soluble glucans.

Type of glucan	$[\alpha]_D$	Moles $NaIO_4$ used per 162 g	Moles formic acid formed per 162 g
0.1 M KOH extracted	−3°	0.19	0.12
0.25 M » »	−5°	0.20	0.12
1.0 M » »	−2°	0.23	0.11
1.0 M » » [a]	—	0.18	—

[a] Extracted from cell walls of the filamentous form.

methyl-D-glucose (3 %), and 2,4-di-*O*-methyl-D-glucose (8 %). The results of periodate oxidation are in good agreement with these figures.

Enzymic degradation by a β-(1→3)-glucanase isolated from Basidiomycete QM 806 [5] resulted in the formation of glucose as the only reducing sugar. Tests revealed that the enzyme preparation contained a β-glucosidase which cleaved both gentiobiose and laminaribiose; consequently, D-glucono-1,5-lactone was added to inhibit this enzyme.[6] The enzymic digest then contained glucose, laminaribiose and two oligosaccharides. Partial acid hydrolysis of the oligosaccharide with the higher R'_F value gave gentiobiose. When material obtained after periodate oxidation, borohydride reduction, and mild acid hydrolysis (Smith degradation) of the original polysaccharide was subjected to the same treatment, only glucose and laminaribiose were found. These results imply that the side chains in the polysaccharide are single glucose residues.

The structural studies indicate that the glucan consists of a backbone of (1→3)-linked β-D-glucopyranose residues, with approximately one of every nine substituted in the 6-position by a β-D-glucopyranose residue. The structural significance of the low percentage of 2,3,4-tri-*O*-methyl-D-glucose found is difficult to evaluate. The linear portion of β-glucans isolated from different species of a related organism, studied by Bishop *et al.*,[7] contained (1→6)- and (1→3)-linkages in ratios varying from 1:0.38 to 1:4.1. It seems likely, therefore, that the linear portion of the *A. pullulans* glucan also contains some (1→6)-linkages.

The cell wall material remaining after extraction with potassium hydroxide was methylated and the chloroform soluble part of the product isolated. This material on hydrolysis yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, and 2,4-di-*O*-methyl-D-glucose as well as minor unidentified components. Because of the crude nature of this material the quantitative measurements were of limited value; however, they indicated that this material is similar to the alkali soluble glucan but contains a much higher percentage of linear (1→6)-linkages.

The highly crystalline fibrillar material obtained after acid treatment of the cell walls was also subjected to methylation analysis. The results indicated that the fibrils consisted of β-(1→3)-glucan having a low degree of branching and no (1→6)-linkages in the linear portion of the molecule.

Three β-glucans seem to be present in cell walls of *A. pullulans*. One is a crystalline essentially linear (1→3)-glucan. The other two glucans are amor-

phous, more branched but differ from one another in the relative proportion of (1→3)- and (1→6)-linked chain units. An extracellular highly branched β-glucan also containing (1→3)- and (1→6)-linkages is produced by the same organism.[3] Enzyme systems capable of synthesising β-glucans with (1→3)- and (1→6)-linkages and different degrees of branching seem to be characteristic for fungi. The present study illustrates how several such polysaccharides may be formed by one organism.

EXPERIMENTAL

Paper chromatograms were run on Whatman No. 1 paper with one of the following systems (v/v):

a) ethyl acetate-pyridine-water (8:2:1)
b) ethyl acetate-acetic acid-water (3:1:1)

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

TLC was on silica gel G using benzene-ethanol(5:1) as solvent.

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 180°.
c) 3 % ECNSS-M on Chromosorb G at 180°.

Isolation of polysaccharides. Cell walls (50 g) from the yeastlike form of *A. pullulans* [1] were extracted with water at 100° for one hour and successively with 0.1, 0.25, and 1.0 M potassium hydroxide at room temperature for 24 h. The cell walls were dispersed at the beginning of each extraction using an ultrasonic bath (Philips L 368). Material which did not precipitate on neutralization of the 0.1 M potassium hydroxide extract was combined with the aqueous extract. This combined fraction was added to ethanol (5 parts) and the precipitate collected by centrifugation. A hydrolysate contained glucose, galactose, mannose, and glucuronic acid.

Material which precipitated on neutralization of the alkaline extracts was purified by redissolving them in 1.0 M potassium hydroxide and precipitating with acetic acid to yield pure glucans. The cell wall material remaining after the extractions on hydrolysis yielded glucose and traces of galactose and mannose. In a typical extraction 16 g cell wall material yielded water soluble polysaccharides (1.9 g), glucans (1.1, 1.5, and 1.0 g) and a residue of 9 g.

A small quantity of cell walls from the filamentous form (110 mg) was extracted in same manner except the 0.25 M potassium hydroxide extraction was omitted.

Periodate oxidations. The glucans were dissolved in water containing minimum amounts of potassium hydroxide to effect solution. The solutions were adjusted to pH 7 by addition of 5 M hydrochloric acid and excess sodium metaperiodate added. The periodate consumption was determined at intervals by the arsenite or optical method.[8] After 78 h, when a constant value was reached, excess periodate was reduced by addition of ethylene glycol and the formic acid released determined by titration (Table 1). Borohydride reduction of the oxidised polymer followed by acid hydrolysis yielded glucose and glycerol.

Methylation analysis of the alkali soluble glucan. The glucan (1.0 g) extracted by 1.0 M potassium hydroxide was methylated as described by Srivastava *et al.*[9], followed by treatments with methyl iodide and silver oxide. The methylated polysaccharide (1.26 g), $[\alpha]_D^{20}$ −17° (*c* 1.0, chloroform) showed only a weak hydroxyl absorption in the I.R. A portion (500 mg) was treated with 90 % formic acid (25 ml) at 100° for 1 h followed by 0.25 M sulphuric acid (50 ml) at 100° for 18 h. The resulting methylated sugars were separated by TLC and characterised as follows: *2,3,4,6-tetra*-O-*methyl*-D-*glucose* crystallized from light petroleum.[5] The m.p. 83—85° was low; however, the 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* crystallized from ethyl ether. M.p. and mixed m.p. 121—124°, $[\alpha]_D^{20}$ + 111° (*c* 0.4, methanol). *2,3,4-Tri*-O-*methyl*-D-*glucose* was enriched in the mother liquors of the

2,4,6-isomer. It was indistinguishable from an authentic sample by GLC of the methyl glucoside mixture or the acetate of the glucitol derivative (column c).[10] *2,4-Di-O-methyl-D-glucose* was converted to the *p*-nitroaniline derivative, m.p. 250–251°. Further, it was indistinguishable from an authentic sample by GLC.

Quantitative GLC analysis of a methanolysate (3 % methanolic hydrogen chloride at 100° for 12 h) was performed on columns a and b. Response factors for the ionization detector were determined using synthetic mixtures.

Methylation analysis of residue remaining after extraction and fibrils. The residual material not extracted during the successive alkali extractions (0.4 g) was dissolved, under nitrogen, in 45 % sodium hydroxide (50 ml) containing sodium borohydride (25 mg). The solution was cooled with ice and dimethyl sulphate (20 ml) was added during 8 h. The solution was stirred for an additional 12 h, neutralized with 5 M sulphuric acid and dialysed. The solution was concentrated to a small volume, dimethyl sulphoxide (50 ml) was added and water removed by repeated codistillations with benzene at 40°. The partially methylated polysaccharide was then further methylated as described above for the alkali extracted glucan. The product was then dissolved in chloroform and separated from undissolved material to yield the fully methylated polysaccharide (201 mg), $[\alpha]_D$ −5° (*c* 1.2, chloroform). Analysis indicated 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, and 2,4-di-*O*-methyl-D-glucose as well as unidentified sugars.

Cell wall fibrils were prepared by treatment of the cell wall material (1.26 g) with 0.5 M sulphuric acid at 100° for 6 h as previously reported.[1] The black insoluble product was methylated as described above. The grey coloured product (267 mg), $[\alpha]_D$ −20° (*c* 1.5, chloroform) was subjected to methanolysis and examined by GLC. The proportion of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, and 2,4-di-*O*-methyl-D-glucose was 2:96:2.

Enzymic hydrolysis. The glucan extracted by 0.25 M potassium hydroxide (200 mg) was dissolved in the same solution (5 ml) and acetic acid was added to pH 4.2. The volume was made to 10 ml with water; D-glucono-1,5-lactone (20 mg) and β-(1→3)-glucanase (5 mg) were added. The mixture was incubated for 18 h at 40°, deionized and examined by paper chromatography (solvents a and b). Glucose, laminaribiose, and two other reducing sugars with lower R_F-values were detected. The more mobile of the latter was isolated by preparative paper chromatography (solvent b). Treatment of this substance with 0.025 M sulphuric acid at 100° for 5 h and subsequent paper chromatography revealed the presence of gentiobiose. Similar enzymic hydrolysis of glucan which had been subjected to periodate oxidation, borohydride reduction, and mild acid hydrolysis resulted in the liberation of glucose and laminaribiose only.

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