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Composition of Regenerated Cell Walls of Reverting *Aureobasidium pullulans* Protoplasts

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Protoplasts of *Aureobasidium pullulans*, formed by treating normal blastospores with polysaccharide-hydrolyzing enzymes, synthesized glycans when incubated in shallow culture. The polysaccharides formed a loose, thick coating on the cells. The glycans that were formed are similar to the glycans in normal cell walls; however, they lack the branching that is normally found. This result is consistent with the findings of others who have studied the cell wall composition of reverting yeast protoplasts.

When *Aureobasidium pullulans* grows on painted surfaces, it produces an undesirable black pigment. Since the cell walls of fungi are unique, the prevention of cell wall biosynthesis seems to be a logical method for controlling the growth of these organisms. However, little is known about the processes involved in the biosynthesis of cell wall polysaccharides. To learn more about these processes, we have studied the regeneration of cell walls by protoplasts of *A. pullulans*.

Others (3, 6, 7) who have studied the regeneration of cell walls in fungal protoplasts have based their characterization of the newly formed polysaccharides on solubility and X-ray analysis. Our results are based on the chemical analysis of the polymers and are similar to those reported earlier.

A. pullulans NRRL Y-2562 was obtained from the U.S. Department of Agriculture Northern Regional Research Laboratory, Peoria, Il. The organism was maintained on YM agar (12). The cells were incubated in liquid culture for 16 h at 25°C on a rotary shaker (120 rpm) and isolated by centrifugation at 12,000 × *g* for 10 min at 4°C. The cell mass was washed with distilled water. Protoplasts were produced by incubating the cells in a 0.75 M $MgSO_4$ solution containing 2.5 mg of Driselase (Plenum Scientific Co.) per ml for 4 h at 37°C in a reciprocating water bath-shaker (4, 12). The protoplasts regenerated cell walls 100 to 150 μm thick (as measured from electron micrographs) during incubation for 2 h at 30°C in 2 ml of modified Winge medium (9) in a 250-ml culture flask (shallow culture). Electron micrographs did not reveal evidence of cell wall formation by protoplasts incubated for up to 16 h in 2 ml of medium in a culture tube (13 by 1.3 cm) (deep culture). The partially reverted protoplasts were isolated, washed, and sonicated for three 30-s intervals at 4°C with a Branson S-75 sonicator set for maximum output. The regenerated cell wall (RCW) was washed by suspending it in water and centrifuging. This washing procedure was repeated twice with water and twice with 95% alcohol. Cell wall material from normal cells was isolated in a similar manner.

Dehydrated RCW was stored in a desiccator over silica gel. A 30-mg sample of RCW was extracted three times with 60°C water. The combined hot-water-soluble extracts (HWS) were freeze-dried (6 mg). HWS was washed and dried as described above for RCW.

The HWS residue was extracted three times with 1 N NaOH at 4°C. The combined NaOH extract was centrifuged to remove a small quantity of insoluble material (0.6 mg), and the alkaline solution was dialyzed for 16 h against frequent changes of distilled water. A residue (AS; 18.5 mg) was recovered by freeze-drying. The small amount of alkali-insoluble material was not investigated further.

The HWS fraction had a molecular mass of less than 1.2×10^4 daltons, since it passed through a dialysis bag with a cutoff of 1.2×10^4 daltons. Glycan AS had a molecular mass greater than 1.2×10^4 daltons, since it was retained by a similar dialysis bag.

Gas-liquid chromatographic separations were made on a Bendix 2500 gas chromatograph with helium as a carrier gas at a flow rate of 40 ml/min. Butane boronic ester derivatives (5) of sugar alcohols were separated on a 6-ft (ca. 183-cm) glass column containing 3% OV-17 on Gas-Chrom Q (100/200 mesh; Alltech Associates, Inc., Applied Sciences Div., State College, Pa.). The column temperature was 225°C, and the inlet and transfer temperatures were both 240°C. Peaks were identified by comparing their retention times with those of authentic samples. The acetylated *O*-methyl-alditol ethers were analyzed on a 6-ft (ca. 183-cm) glass column containing 3% OV-225 on Gas-Chrom Q (100/200 mesh). The column temperature was programmed from 160 to 200°C at 2°C per min. The inlet and transfer temperatures were both maintained at 215°C. Peaks were identified by comparing their retention times with those of authentic samples. Peak areas were determined by the half-height method (8).

Enzymatic hydrolysis of the polysaccharide material was carried out at 37°C in 0.1 M phosphate buffer (pH 7.4) for 4 h with 2.5 mg of Driselase per ml. The mixture was boiled for 5

TABLE 1. Sugar composition of the cell wall of normal *A. pullulans* cells and RCW

Cell type	Sugar composition (%)			
	Glucose	Mannose	Galactose	Hexosamine
Normal				
Chlamydospore[a]	69	16	14	2
Blastospore[a]	34	41	24	3
Blastospore	77	21	2	T
Blastospore	76	20	4	T
Regenerated protoplasts	75	25	T	

[a] These values were obtained from Brown et al. (2) and are added for comparison.

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TABLE 2. Methylation analysis of the polysaccharides of 2-h RCW isolated from reverting *A. pullulans* protoplasts

Sugar (as alditolacetate)	% Sugar in cell wall[a]	Retention time (h)
2,3,4,6-Tetra-*O*-methyl-mannose[b]	4.5	0.97
2,3,4-Tri-*O*-methyl-mannose[b]	82.2	1.76
2,3,4,6-Tetra-*O*-methyl-glucose[c]	1.5	0.98
2,4,5-Tri-*O*-methyl-glucose[c]	91.4	1.78

[a] Small quantities of three unidentified materials were present in both samples. These did not have retention times corresponding to any known methyl-alditol acetates.
[b] Derivatives prepared from HWS.
[c] Derivatives prepared from AS.

min to stop the enzymatic reaction and centrifuged at 2,000 × *g* for 5 min to remove the denatured protein. The hydrolysates were converted to butane boronic acid esters and separated by gas-liquid chromatography.

The results from several determinations of the sugar composition of the normal cell wall of *A. pullulans* blastospores and of the regenerated protoplast polysaccharides are listed in Table 1.

These results are from the hydrolysis of the cell wall polysaccharides by the enzyme mixture in Driselase. A number of combinations of time, temperature, and acid concentrations gave various yields of sugars, always less than that released by enzymatic hydrolysis (data not shown).

Samples of HWS and AS were methylated by the method of Sanford and Conrad (11). Several methylations were required to completely methylate the polysaccharides. Methylation was considered complete when no -OH stretching was observed at 3,400 to 3,500 cm^{-1} in the infrared spectrum of $CHCl_3$ films of the methylated polysaccharide.

Permethylation of HWS was followed by hydrolysis and conversion of the hydrolysate to *O*-methyl-alditol acetates (10). Separation of the mixture by gas-liquid chromatography gave two peaks which were identified by their retention times (Table 2). The ratio of 2,3,4-tri-*O*-methyl-mannose to 2,3,4,6-tetra-*O*-methyl-mannose was 18:1. It appears that the HWS fraction of the regenerated cell wall is a 1,6-linked mannan.

Similarly, AS was an unbranched polymer containing only 1,3-linked glucose residues (Table 2). The ratio of 2,4,6-tri-*O*-methyl-glucose to 2,3,4,6-tetra-*O*-methyl-glucose was 61:1. The hydrolysis of AS with the β-1,3-glucanase from *Sporotrichum dimorphosporum* (10) suggests that AS is a linear β-1,3-glucan.

The cell wall glycans of the reverting protoplasts of *A. pullulans* lack the branching seen in the cell walls of normal cells. Similar results have been observed in the reverting protoplasts of other fungi (6, 7). Cell wall polysaccharides synthesized by cell-free preparations from *Saccharomyces cerevisiae* (1) contained the branching found in normal cell walls. It may be, on the basis of these results, that the enzymes that synthesize the branch points are located in the space between the cell membrane and the cell wall and are lost when protoplasts are formed.

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