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Oligoglucuronide Production in *Mucor rouxii*: Evidence for a Role for Endohydrolases in Hyphal Extension

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Culture filtrates of *Mucor rouxii* contained oligomers of glucuronic acid which were labeled rapidly during pulses with D-[*U*-^{14}C]glucose. These oligomers were probably derived by enzymatic lysis of acidic polymers in the cell wall. The kinetics of the incorporation of label into oligouronides and cell wall polymers suggested that lysis of the wall was required for active hyphal extension. Experiments with cycloheximide, which inhibited hyphal extension, suggested that wall lysis was also required for the subapical cell wall synthesis which probably occurred under these conditions.

The apical extension of mycelial fungi is considered to involve a balance between the synthesis and lysis of wall polymers at the hyphal tip (1). A gradient of wall synthesis, with maximal activity at the apex, is readily demonstrable by the incorporation of radiolabeled precursors into both matrix and fibrillar phases of the wall, followed by autoradiography (3, 7, 9, 10). The evidence for the plasticization of the apical wall by lytic enzymes, however, is largely circumstantial (for reviews, see references 1, 8). In particular, the hyphal tips of *Mucor rouxii* burst when colonies grown on agar are flooded with dilute solution of acids and chelating agents (2). These treatments are considered to shift the putative balance of lysis and synthesis in the direction of lysis so that the wall can no longer withstand the internal turgor pressure (2). The antagonistic effects of calcium ions and acid on this bursting response prompted the suggestion that hyphal tip plasticity in this organism is determined by the physical state of the glucuronic acid-containing matrix polymers (as calcium salts or free acids) rather than by lysis, although this could not be ruled out (5). One effect of the action of lytic enzymes on the cell wall could be the release of soluble hydrolysis products into the external medium. This would depend both upon the mode (exo- or endo-) and extent of hydrolysis. In this paper we report the appearance in *M. rouxii* culture filtrates of radiolabeled oligoglucuronides during short labeling periods with glucose. These results suggests that *M. rouxii* has enzymes which can degrade wall matrix polymers and that their action may be required for hyphal extension.

MATERIALS AND METHODS

Growth of the organism and radioactive pulse-labeling. *M. rouxii* strain NRRL 1894 was grown in 1 liter of yeast extract-peptone-glycerol medium (6) in 2-liter Erlenmeyer flasks. The medium was inoculated with a washed sporangiospore suspension to a final concentration of 10^5 spores/ml and incubated at 28°C with orbital shaking. After 18 h, when the culture was in midlogarithmic phase of growth, the mycelium was harvested by filtration and washed with sterile medium; then 0.5 g was resuspended in 30 ml of medium supplemented with 20 μM D-glucose and 30 μCi of D-[*U*-^{14}C]glucose (New England Nuclear Corp; specific activity, 200 Ci/mol). After the appropriate time of incubation at 28°C, the culture was filtered and the mycelial mat and filtrate were stored at −20°C. Under these labeling conditions, only carbohydrates were labeled from D-[*U*-^{14}C]glucose (6) and almost all of the radioactive polymers were in the cell wall fraction.

Time course of radioactive labeling. The fungus was grown in 30 ml of yeast extract-peptone-glycerol medium inoculated at a spore density of 10^5/ml. After 18 h, each flask received 8 μCi of D-[*U*-^{14}C]glucose. A parallel set of flasks received, in addition, cycloheximide to a final concentration of 70 μg/ml. After the appropriate time, the cultures were filtered and the mycelium was plunged into boiling ethanol-water (70:30 by volume) for 20 min. After two further extractions with the ethanol-water mixture, the mycelium was extracted for 10 min with 5 ml of 1 M NaOH at 100°C to obtain the alkali-soluble (matrix-phase) materials of the wall. The residue (the fibrillar phase, chitin/chitosan) was collected on GF/A fibers, neutralized with 1 M HCl, and washed with water before counting. The NaOH extract was neutralized with HCl, and a sample was counted. The glass fiber disks were suspended in 5 ml of scintillation fluid consisting of 3.5 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2]-(5-phenyloxazolyl)benzene (POPOP) in 1 liter of a mixture of sulfur-free toluene and Triton X-100 (2:1, by volume). The radioactivity in aqueous samples and on chromatograms was determined as described previously (6). Radioactivity was measured in a Packard Tri-Carb 460 C scintillation counter. Counting efficiency was determined by the channels ratio method from a standard curve constructed with acetone and water as quenching agents. The distribution of radioactivity between neutral (glycoprotein) and acidic

(mucoran) components in the NaOH extracts was determined by electrophoresis on glass fiber paper as described previously (6).

The radioactivity in the uronides of the culture filtrates was determined as follows: 3-ml samples were applied to columns of Dowex-1-acetate (1 by 4 cm). The columns were then washed with 30 ml of glass-distilled water. The uronides, which remained bound to the column, were subsequently eluted with 1 M HCl, and the radioactivity in a sample of the eluate was determined.

Analytical methods. Hydrolysis of preparations with 0.25 M sulfuric acid was performed as described previously (6). Neutral sugars were separated by descending paper chromatography on Whatman no. 1 paper in ethylacetate-pyridine-water (8:2:1 by volume) (solvent A). Uronides were separated by descending paper chromatography in 1-butanol-acetic acid-water (5:1:2 by volume) (solvent B). Electrophoresis was performed on a Savant flat-bed apparatus on Whatman 3MM paper (for uronides) or glass fiber paper (for polymers). The electrophoresis buffer was 0.05 M ammonium acetate (pH 6.5) containing 10 mM EDTA. Gel filtration was performed on a Sephadex G-25 column (1.4 by 41 cm). The column was eluted with glass-distilled water at a flow rate of 20 ml/h, and 2-ml fractions were collected. Radioactivity in column eluates and on paper chromatograms and electrophoretograms was determined as described previously (6). Uronides were identified by reduction to their parent sugars as described by Bartnicki-Garcia and Reyes (4). This method involved methylation to form the methyl ester, methyl glycoside, reduction with sodium borohydride, and hydrolysis to yield the neutral sugar(s) which was separated with the ethylacetate-pyridine-water solvent. The effects of cycloheximide on hyphal extension were determined by using 12-h germlings. Measurements of hyphal length were made from photomicrographs of cultures in the presence and absence of cycloheximide (70 μg/ml).

RESULTS

Labeled products in culture filtrates from pulse experiments. The culture filtrate from a 2-min pulse was fractionated by gel filtration on Sephadex G-25. Several peaks of radioactivity were seen (Fig. 1): a high-molecular-weight peak eluting behind the void volume of blue dextran, material of intermediate molecular weight (fractions 20 through 25), and a large peak with the same elution position as D-glucose. The high-molecular-weight material was not further characterized. The intermediate-molecular-weight material was pooled and subjected to electrophoresis on glass fiber paper at pH 6.5. Most (>85%) of the material was acidic and had a mobility similar to that of free D-glucuronic acid. The low-molecular-weight material (fractions 28 and 29) was neutral at pH 6.5 and had the same R_f value as D-glucose on paper chromatography in solvent A. It was therefore identified as D-glucose.

Characterization of oligosaccharides in culture filtrates. The filtrate after a 60-min pulse was dialyzed for 6 h to remove labeled glucose and was then fractionated on Sephadex G-25. Two peaks of discrete molecular weight were seen (Fig. 2). Their elution positions suggested that the material in peak II was a trisaccharide and that the material in peak I was a penta- or hexasaccharide. These materials were

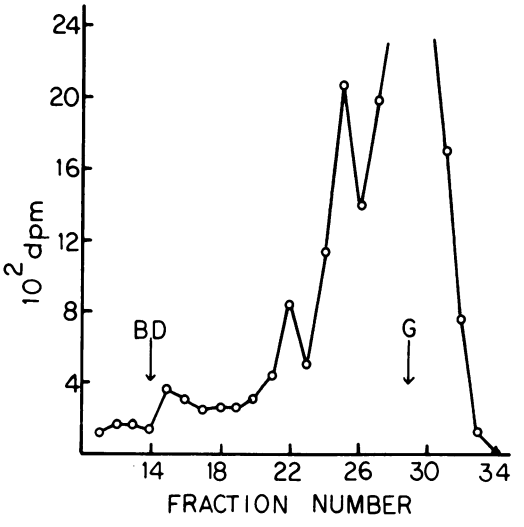

FIG. 1. *Sephadex G-25 gel filtration of culture filtrate material from a 2-min pulse experiment with D-[U-^{14}C]glucose as described in Materials and Methods. The elution positions of blue dextran (BD) and glucose (G) are shown.*

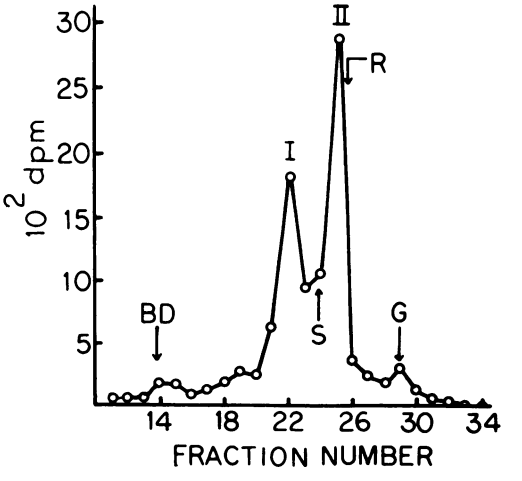

FIG. 2. *Sephadex G-25 gel filtration of culture filtrate material from a 60-min pulse experiment with D-[U-^{14}C]glucose after dialysis to remove labeled glucose as described in Materials and Methods. The elution positions of glucose (G), raffinose (R), stachyose (S), and blue dextran (BD) are shown.*

not retained in the dialysis bag on further dialysis for 24 h. The material in each peak was pooled and subjected to electrophoresis at pH 6.5. Over 95% of the applied radioactivity was acidic, with a mobility identical to that of D-glucuronic acid (Fig. 3). This suggested that the oligosaccharides were uronides and possibly oligomers of glucuronic acid; aldobiouronic acids would have a mobility less than that of D-glucuronic acid at this pH (13). Hydrolysis of the material in both peaks followed by paper chromatography of the hydrolysate in solvent A yielded traces of the neutral sugars D-galactose, D-mannose, and L-fucose, but most of the material remained at the origin of the chromatogram, the position of uronic acids. Chromatography of the hydrolysate in solvent B showed a large peak with the mobility of D-glucuronic acid (Fig. 4). Methylation, reduction, and hydrolysis of the material in peak I yielded D-glucose as the major neutral product, with traces of D-mannose and D-galactose. The remaining radioactivity was in D-glucuronic acid. The same treatment of the material in peak II (Fig. 2) also gave glucose as the major neutral product, with much smaller amounts of D-galactose, D-mannose, L-fucose, and D-ribose. The radioactivity in glucose represented a 28% conversion from the original material for peak I and 23% for peak II. Although the yields of glucose were low, the combined results suggest that the oligosaccharides are oligomers of D-glucuronic acid.

Time course of production of radiolabeled oligouronides. The time courses of the incorporation of radioactivity from D-[*U*-^{14}C]-glucose into the acidic culture filtrate material and different fractions of the wall are shown in Fig. 5 through 7. The time course of the production of uronides (Fig. 5) closely paralleled that of the incorporation of radioactivity into both the matrix (NaOH soluble) (Fig. 6) and fibrillar (NaOH insoluble) (Fig. 7) wall components. The decrease in the rate of wall labeling was probably due to the depletion of radiolabeled glucose in

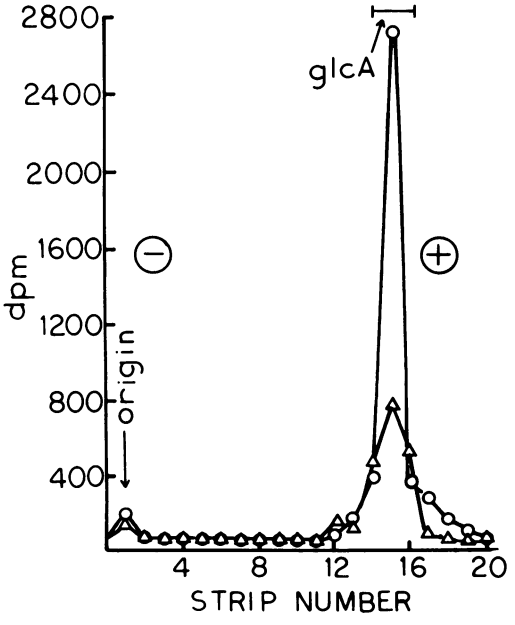

FIG. 3. *High-voltage paper electrophoresis of oligosaccharides derived by Sephadex G-25 gel chromatography from a 60-min pulse experiment. Symbols: △, peak I; ○, peak II. The position of the glucuronic acid marker is shown (glcA).*

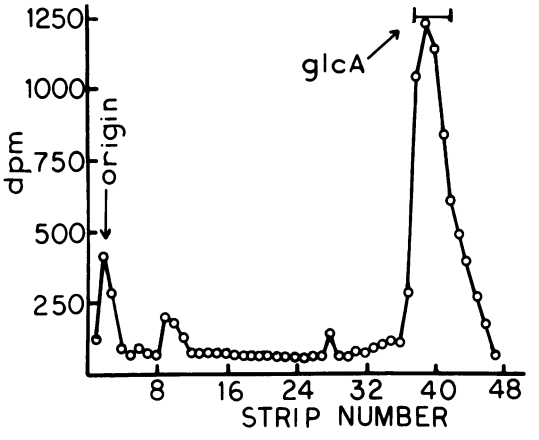

FIG. 4. *Descending paper chromatography of a hydrolysate of the material from peak II as shown in Fig. 2. The position of the glucuronic acid marker is shown (glcA).*

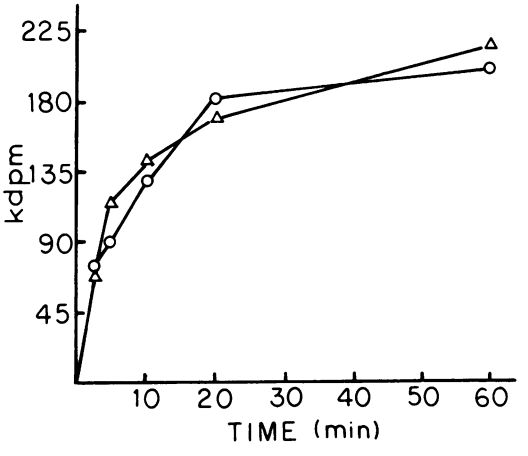

FIG. 5. *Time course of incorporation of radioactivity into culture filtrate uronides. Uronides were extracted by ion-exchange chromatography as described in Materials and Methods. Symbols: △, plus cycloheximide, 70 μg/ml; ○, no cycloheximide.*

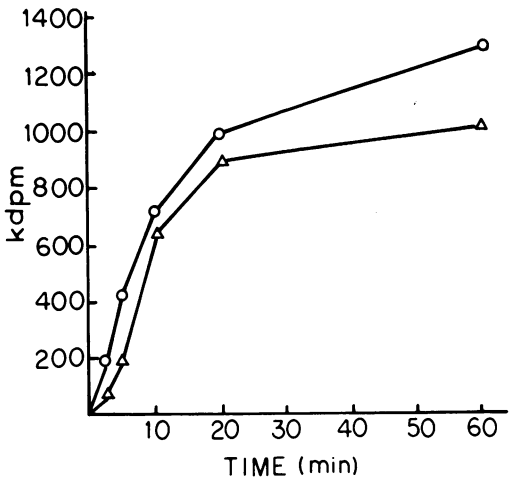

FIG. 6. *Time course of incorporation of radioactivity from D-[U-^{14}C]glucose into matrix-phase materials of the wall. Matrix materials were extracted with NaOH and counted as described in Materials and Methods. Symbols: △, plus cycloheximide, 70 μg/ml; ○, no cycloheximide.*

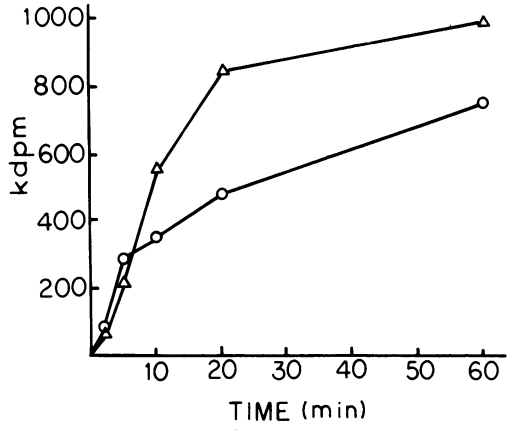

FIG. 7. *Time course of incorporation of radioactivity from D-[U-^{14}C]glucose into fibrillar-phase (NaOH insoluble) materials of the wall. Symbols: △, plus cycloheximide, 70 μg/ml; ○, no cycloheximide.*

the medium. Uronides could be detected with 2 min of labeling, suggesting that the lytic events leading to their release into the medium may be required for active growth of the organism.

Effects of cycloheximide on apical growth and the production of oligouronides. Previous work demonstrated that the protein synthesis inhibitor cycloheximide halts the apical elongation of *Aspergillus nidulans* but in the short term does not inhibit polysaccharide synthesis. Under these conditions, new wall is deposited subapically, prolonged incubation leading to walls with up to five times the normal thickness (10, 12). The effects of cycloheximide on apical extension, wall synthesis, and uronide production in *M. rouxii* were therefore investigated to determine whether the lytic processes leading to uronide formation were only associated with wall deposition under conditions of apical growth. At the concentration used, cycloheximide rapidly and completely inhibited hyphal extension of *M. rouxii* germlings (Fig. 8), but the incorporation of radioactivity into the wall was largely unaffected (Table 1). Although cycloheximide changed the distribution of radioactivity between different wall fractions, uronide production was unaffected. Similarly, preincu-

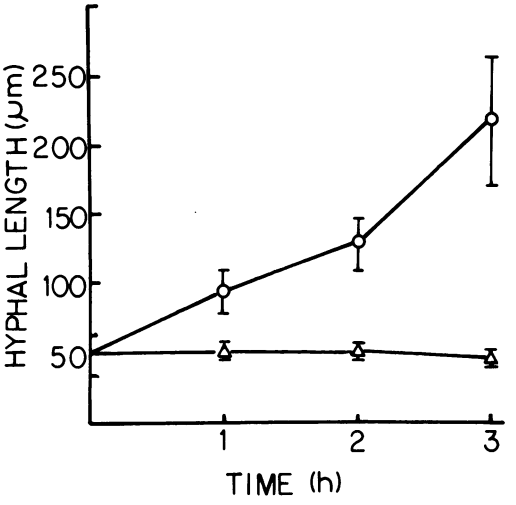

FIG. 8. *Effects of cycloheximide on hyphal extension of 12-h germlings of M. rouxii. Hyphal lengths were determined from photomicrographs. Symbols: △, plus cycloheximide, 70 μg/ml; ○, no cycloheximide. Error bars represent ± 2 standard error.*

TABLE 1. *Incorporation of radioactivity into different wall fractions and uronides in the presence and absence of cycloheximide*[a]

Wall component[b]	% Total incorporated radioactivity	
	+Cycloheximide, 70 μg/ml	Control
Mucoran	46.0	49.2
Glycoprotein	1.0	9.5
Chitin/chitosan	48.1	36.3
Uronides	4.9	5.0
Total radioactivity incorporated (kdpm)	3,037	3,096

[a] Labeling period was 1 h.

[b] Fractions were derived as described in Materials and Methods.

TABLE 2. *Effect of preincubation with cycloheximide on the incorporation of radioactivity into wall fractions and uronides*[a]

Wall component[b]	Control	% Total incorporated radioactivity			
		0[c] min	15 min	30 min	60 min
Matrix phase	63.3	50.9	57.2	64.5	61.8
Fibrillar phase	29.0	41.8	35.4	27.5	30.3
Uronides	7.7	7.3	7.4	8.0	7.9
Total radioactivity incorporated (kdpm)	6,753	6,608	6,869	6,359	

[a, b] See Table 1.
[c] Time of preincubation (minutes).

bation with cycloheximide before labeling did not change the degree of incorporation into the wall or the production of uronide (Table 2). Although the site of wall deposition in *M. rouxii* under the influence of cycloheximide is not known, by analogy with the work of Sternlicht et al. (12) it is likely to be subapical. These results thus imply that newly synthesized wall material is susceptible to lysis irrespective of the site of incorporation.

DISCUSSION

Isolated mycelial walls of *M. rouxii* contain, in addition to the fibrillar chitin and chitosan, readily extractable acidic heteropolymers (mucoran) and nonextractable material containing D-glucuronic acid (6). The nonextractable uronide may be equivalent to the mucoric acid (poly-D-glucuronic acid) of sporangiophores, as has been suggested for the walls of the yeastlike form of this organism (4). Our data suggest that the fungus degrades poly-D-glucuronic acid, allowing the release of oligouronides into the medium.

The absence of aldobiouronic acids or neutral oligosaccharides from culture filtrates implies that enzymatic lysis was restricted to domains of the acidic polymers containing poly-D-glucuronic acid or to a separate D-glucuronic acid homopolymer. Alternatively, lysis in other regions or polymers may not lead to the release of soluble fragments, or these fragments may be quickly reassimilated by the fungus. The release of the oligomeric radioactive fragments in the 2-min pulse experiment suggests that endohydrolysis of newly synthesized polymers was occurring. The rapid production of uronides in the time course experiment suggests a role for lysis of acidic polymers in hyphal extension. However, uronides were also found when, on treatment with cycloheximide, hyphal extension ceased and new wall was presumably deposited subapically. Thus, intususseption of new wall material at any site may require lysis of matrix-phase polymers. Our results are very similar to those of Polachek and Rosenberger (11), who observed that newly synthesized wall is more susceptible to autolysis than older wall whether formed apically or subapically in the presence of cycloheximide.

Dow and Rubery compared the effects of calcium ions and acid on hyphal tip bursting with the effects of these ions on the rheological properties of higher-plant pectin (5). It was suggested that plasticity during extension growth is determined by the acidic polymers of the wall through their gel-forming properties. Enzymatic lysis by endohydrolases would afford another, although irreversible, method of increasing the plasticity of the wall at the extreme apex by a reduction of the ability to form a rigid gel, decreasing the interaction between microfibrils. This might allow wall expansion under the influence of the internal turgor pressure. The mechanism of subsequent wall rigidification is unknown, but could involve increased microfibril length or thickness, interactions with ions, or deacetylation of chitin to give the positively charged chitosan which may be capable of a stronger interaction with the matrix uronides. Our data do not exclude a role for the lysis of the fibrillar-phase polymers, but emphasize a role for the matrix polymers in hyphal morphogenesis.

ACKNOWLEDGMENTS

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