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Two Distinct Classes of Polyuronide from the Cell Walls of a Dimorphic Fungus, *Mucor rouxii*

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Polyuronides were extracted from purified yeast and mycelial walls of *Mucor rouxii* by sequential treatments with lithium chloride and potassium hydroxide and were fractionated by ion-exchange chromatography on DEAE-Sephadex. Two polymers (I and II) of different acidity were found in both wall types. Polymer I contained D-glucuronic acid, L-fucose, D-mannose, and much smaller amounts of D-galactose. Yeast and mycelial polymer I had similar uronic acid contents but differed in their neutral sugar compositions and molecular weights. Polymer II from both cell types contained largely D-glucuronic acid and had similar molecular weights. On partial acid hydrolysis, both polymers I and II gave rise to insoluble glucuronans which appeared to be homopolymeric. One-third of the total uronosyl residues of polymer I, and almost all of the uronosyl residues of polymer II, were present in homopolymeric segments. However, homopolymers derived from polymers I and II may not be identical.

Mucor rouxii is a dimorphic phycomycete which can develop in two radically different forms, depending on growth conditions. Under anaerobic conditions with a hexose carbon source, yeast-like cells develop; on the other hand, aerobic growth is mycelial (6). These morphological differences are associated with two different patterns of wall growth: spherically symmetrical growth in the yeast form and apical growth in the mycelial form (5). In addition, the yeast wall is 10 times thicker than the mycelial wall (7). There is great interest in the polymers of the walls of dimorphic fungi and in the role they play in determining cellular shape.

In *Mucor* species, the fibrillar component of the wall is composed of chitin and chitosan (partially deacetylated chitin) (7). The fibrils are embedded in matrix materials, which are mostly polymers containing large amounts of glucuronic acid (8). Two such polyuronides in *M. rouxii* have been described: mucoran (a heteropolymer of D-glucuronic acid, D-mannose, D-galactose, and L-fucose) and mucoric acid (a homopolymer of D-glucuronic acid). Some details of the structure of mucoran from yeast-phase cell walls have been described (4). Mucoric acid has been isolated from sporangiophore walls of *M. rouxii* by alkali extraction and partial acid hydrolysis (8) and its presence in mycelial walls as well has been inferred from X-ray analysis (15).

Barnicki-Garcia and Reyes (8) have suggested the mucoric acid and mucoran may be derived from a single heteropolymer rather than from distinct polymers. Indeed, the mycelial wall of *Mucor mucedo* does contain a single heteropolymeric polyuronide which can be fully extracted with 6 M lithium chloride (10). After partial acid hydrolysis, this polyuronide yields both insoluble polyglucuronic acid (mucoric acid) and soluble uronides.

Our work has focused on the characterization of polyuronides from the walls of *M. rouxii* as a contribution towards understanding cell wall architecture. In this paper we show the presence of two distinct polyuronides, in both yeast and mycelial walls, and compare their physicochemical properties.

MATERIALS AND METHODS

Growth of organisms. *M. rouxii* NRRL 1894 was grown at 28°C in yeast extract-peptone-glucose medium (6) in a fermentor gassed with air to obtain the mycelial phase and with a mixture of nitrogen and carbon dioxide (70:30, vol/vol) to obtain the yeast phase. After 24 h, the cells were harvested by filtration and washed with distilled water. For radioactive labeling, the organism was grown in 1 liter of yeast extract-peptone-glucose medium containing 100 μCi of D-[*U*-^{14}C]glucose.

Preparation of cell walls. The mats of washed cells were frozen and subjected to three passages through an Eaton pressure cell at 8,000 lb/in^2. The broken cells were thawed and then were washed 20 times with distilled water by centrifugation at 1,000 × *g* at 4°C. The final pellets consisting of cell wall pieces were lyophilized.

Extraction of walls. Walls were suspended in 5.6 M LiCl at a final concentration of 4 mg (dry weight) per ml for 18 h at 25°C with constant shaking. Insoluble material was removed by filtration and washed with 50

ml of 5.6 M LiCl. Initial extract and wash were combined. Subsequent extraction of the residue was carried out for 30 min at 25°C with 1 ml of 1 M KOH per 20 mg of wall material. This step was repeated five times, and the extracts were pooled. The residue was lyophilized. Depolymerization of chitosan with nitrous acid was carried out as described by Datema et al. (10). All fractions were individually and exhaustively dialyzed against glass-distilled water, then against 10 mM EDTA, and finally against 0.05 M potassium acetate.

Ion-exchange chromatography. Polysaccharides were chromatographed on a column (1 by 20 cm) of DEAE-Sephadex (Sigma Chemical Co., St. Louis, Mo.). The resin was equilibrated with 0.05 M potassium acetate, and the sample was introduced in the same solution. The chromatogram was developed with a linear gradient of 0.05 to 2.0 M potassium acetate (total volume, 1 liter), and 10-ml fractions were collected. The gradient was monitored with a refractometer. Polysaccharide elution profiles were constructed on the basis of the uronic acid assay (9).

Gel chromatography. A column (1.5 by 42 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, N.J.) was equilibrated with 0.05 M phosphate buffer (pH 7.0) containing 0.2 M NaCl. The flow rate was 18 ml/h, and 1.8-ml fractions were collected. Alternatively, a column (1.5 by 42 cm) of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) was used with 0.05 M phosphate buffer (pH 7.0), and 1.8-ml fractions were collected at a flow rate of 10 ml/h.

Reduction and acid hydrolysis of polymers. Polysaccharides were treated with EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] at pH 4.75 and then were reduced with sodium borohydride at pH 7.0 (17). After reduction, the polymers were extensively dialyzed against water, concentrated by rotary evaporation at 30°C, and then hydrolyzed in 1 M trifluoroacetic acid (TFA) for 1 h at 120°C (1).

Sugar analysis. Descending paper chromatography was used with ethylacetate-pyridine-water (8:2:1). Neutral sugars were converted to their alditol acetates (1) and analyzed by gas chromatography.

Molecular weight determination. A Spinco model E analytical centrifuge was used to determine weight average molecular weights by the meniscus depletion sedimentation equilibrium technique. Samples of polysaccharide were dissolved in 20 mM (2-*N*-morpholino) propanesulfonic acid–KOH buffer (pH 7.0) containing 0.1 M potassium chloride (final concentration, 2 mg/ml of polysaccharide) and subjected to equilibrium sedimentation. The rotor speed was 24,000 rpm, and the temperature was 20°C. Interference patterns were recorded after equilibrium was reached (24 to 28 h). Weight average molecular weights were calculated with a value of 0.58 for the partial specific volume of the polysaccharide, an average value for polysaccharides computed from data given in Horton et al. (14).

Other analytical methods. Protein was measured by the method of Lowry et al. (16) with bovine serum albumin as the standard. Phosphate was measured after ashing with magnesium nitrate by the method of Ames (2). Uronic acid was measured by the carbazole method as described by Bitter and Muir (9) with glucuronolactone as the standard. Neutral sugars and uronic acids were also measured by the method of Dubois et al. (13) with glucose and glucuronolactone as the standards. Scintillation counting was performed as described previously (12). Sugars on paper chromatography were detected by the alkaline silver nitrate method (18).

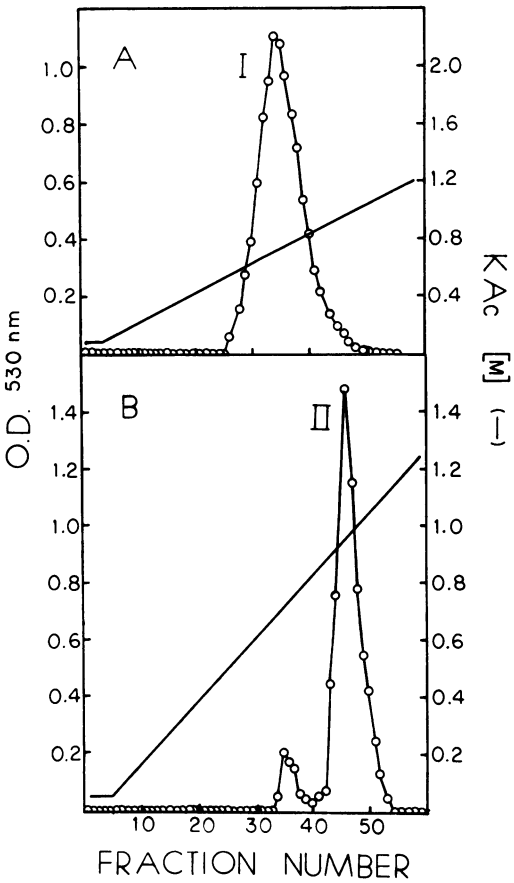

FIG. 1. Ion-exchange chromatography on DEAE-Sephadex of polyuronides extracted from mycelial walls by sequential treatments with lithium chloride (A) and potassium hydroxide (B). Chromatograms were developed with an acetate (KAc) gradient. OD, Optical density.

RESULTS

Ion-exchange chromatography. The LiCl-extractable material was subjected to ion-exchange chromatography on DEAE-Sephadex. The elution profiles are shown in Fig. 1A and 2A. Carbazole-positive material from both yeast and mycelial walls eluted as a single broad peak with maximum concentration of polymer at 0.72 M potassium acetate (polymer I). Some phenol, sulfuric acid-positive material (representing about 20% of the total) eluted almost immediately from the column (data not shown); otherwise, the elution profiles determined with the two reagents were identical. This early-eluting material is probably glycoprotein in nature (11).

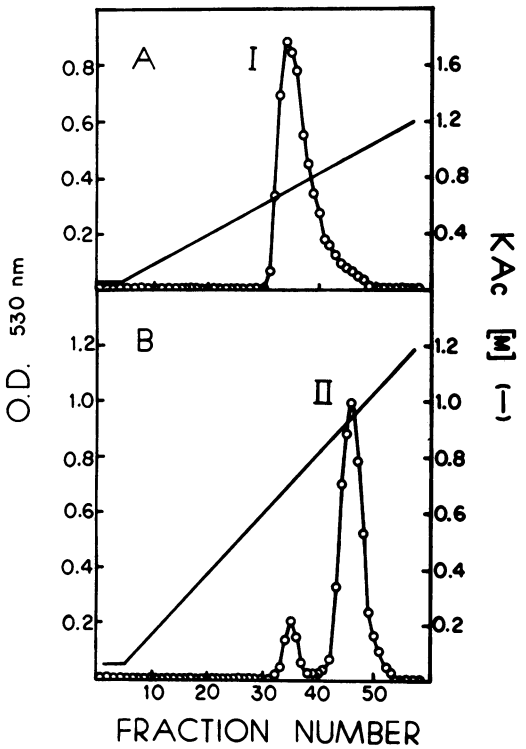

FIG. 2. Ion-exchange chromatography on DEAE-Sephadex of polyuronides extracted from yeast walls by sequential treatments with lithium chloride (A) and potassium hydroxide (B). Chromatograms were developed with an acetate gradient.

Fractionation of the KOH-extractable material from both sources showed a small peak of carbazole-positive material at 0.72 M potassium acetate and a much larger peak with maximum concentration of 0.96 M potassium acetate (polymer II, Fig. 1B and 2B). These results suggest the presence of at least two different polyuronides in both wall types. Fractions 40 through 52 were pooled, dialyzed, and lyophilized.

Extractability of polymers. Although most of the uronic acid could be extracted from yeast walls by sequential treatments with LiCl and KOH, only about 50% of the uronide of mycelial walls was extracted by these two treatments. The remaining uronide could be extracted with alkali after freezing and thawing or by depolymerization of the wall chitosan by nitrous acid. The elution profile of these residual polymers on ion-exchange chromatography showed two peaks with the same elution positions as polymers I and II. In all subsequently reported experiments, the properties of the less acidic and more acidic polymers were indistinguishable from those of mycelial polymers I and II, respectively.

General properties of polymers I and II. All preparations were free of protein and phosphate. Polymer I from both sources was readily soluble in water and was not precipitated by acetic acid or HCl at a final concentration of 1 M or by $CaCl_2$. In contrast, polymer II from both sources was not readily soluble in water but was soluble at pHs above 5. The polymer could be precipitated from neutral aqueous solution by the addition of $CaCl_2$ and was insoluble in 5.6 M LiCl. The polymer was quantitatively precipitated by 1 M acetic acid or 10 mM HCl. Both polymers had a pK_a of ~4.0 as determined by titration.

Amounts of polymers I and II in walls. The differential response of polymers I and II to precipitation by acid was used to estimate relative amounts of uronic acid in wall extracts and hence in the whole wall since all the uronide could be extracted by the sequential procedure. Our analyses indicated 57.1 mg of uronic acid as polymer I and 66.1 mg as polymer II per g of yeast cell wall, whereas mycelial wall yielded 56.1 mg of uronic acid as polymer I and 62.3 mg as polymer II. Thus, little difference is seen between the yeast and mycelial walls in total content of uronic acid. Similar results were reported by Bartnicki-Garcia and Reyes (8).

Molecular weights of polymers. Plots of c versus r^2 (where c is the concentration in grams per liter and r is the radial distance in centimeters) yielded straight lines with linear correlation coefficients of 0.97 or greater for all polymers (data not shown). Data from these plots yielded weight average molecular weights as follows: polymer I (yeast), 34,800; polymer I (mycelial), 19,200; polymer II (yeast), 33,000; polymer II (mycelial), 32,000. Aune (3) has pointed out that such linear plots do not necessarily indicate the presence of a single homogeneous species. The closeness of the molecular weights of polymer II and the differences in those of polymer I were confirmed by gel chromatography on Sepharose 6B (Fig. 3). The broadness of the elution profiles, particularly of polymer II, suggest a range of molecular weights.

Partial acid hydrolysis of polymers. On hydrolysis of polymer I from either source in 1 M HCl for 1 h at 100°C or in 1 M TFA for 1 h at 120°C, a precipitate formed which contained 34% of the total uronic acid in the hydrolysate. Paper chromatography of the supernatant fraction showed the presence of the neutral sugars galactose, fucose, and mannose. Glucose was not detected. The anhydroglucuronic acid content of the precipitate was 95% as determined by the carbazole assay. Reduction and further hydrolysis of this material with TFA gave glucose alone as deter-

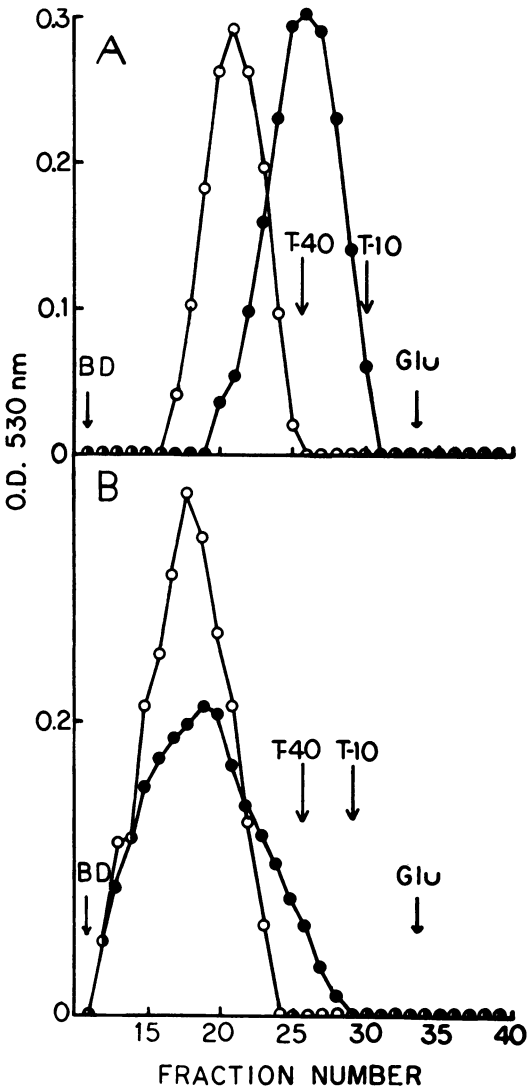

FIG. 3. Sepharose 6B chromatography of polyuronides from yeast (○) and mycelial (●) cell walls. (A) Polymer I; (B) polymer II. The elution positions of blue dextran (BD), dextran T-40 (T-40), Dextran T-10 (T-10), and glucose (Glu) are shown.

mined by paper chromatography. Thus, the insoluble material was probably a homopolymer of glucuronic acid. Another sample of the precipitate was dissolved in a minimum volume of 0.01 M KOH, and the solution was adjusted to pH 8. Gel chromatography of this material on Bio-Gel P-10 gave a single peak for both yeast and mycelial polymers (Fig. 4A). Ion-exchange chromatography of this material on DEAE-Sephadex showed a broad peak with maximum concentration corresponding to 0.56 M potassium acetate.

On hydrolysis of polymer II from both sources in 1 M HCl for 1 h at 100°C, only 7% of the sugar residues were solubilized as determined radiometrically. Treatment with 1 M HCl for 6 h at 100°C or in 1 M TFA for 1 h at 120°C did not change these results. Paper chromatography of the supernatant fraction showed the presence of glucose and mannose with trace amounts of fucose and galactose. More than 98% of the carbazole-positive material in the hydrolysate was present in the precipitate. The precipitated material had an anhydroglucuronic acid content of 85% as determined by the carbazole assay. Reduction and further hydrolysis of this material with TFA gave glucose and traces of fucose as determined by paper chromatography. This suggests that the material was largely a homopolymer of glucuronic acid. Another sample from both sources was solubilized. Gel chromatography of this material on Bio-Gel P-10 gave a single peak, although a broad one (Fig. 4B). As the original polymers were eluted in the blue dextran void volume, considerable breakdown must have occurred. The elution profiles suggest a higher average molecular weight for the glucuronan derived from polymer II than for that from polymer I. On DEAE-Sephadex, the material from both sources was eluted as a single peak with maximum concentration corresponding to 0.84 M potassium acetate. This is different from the elution position of the homopolymeric glucuronan derived from polymer I.

Monosaccharide composition of polymers. It is well known that hydrolysis of polymers rich in uronic acids presents difficulties. Preliminary reduction with lithium aluminium hydride failed to render the polyuronides from *M. mucedo* fully hydrolyzable (10). In that case, the composition of the polymer was estimated by using gas chromatography of hydrolysates and colorimetric determination of glucuronic acid in a hydrolysis-resistant part of the molecule. Reduction of polymer I by the method of Taylor and Conrad (17) was successful as judged by the absence of a precipitate upon hydrolysis of the reduced polymer, by the absence of glucuronic acid in the soluble fraction, and by the appearance of glucose as the major sugar (glucose was not detected in the original polymer). Neutral sugar compositions are given in Table 1.

Reduction of polymer II was less successful. Precipitation occurred during the activation step with EDC, and this presumably contributed to less than complete reduction upon subsequent treatment with sodium borohydride. After hydrolysis, the reduced polymer gave a precipitate which contained carbazole-positive material (about 20% of the original). Gas chromatography of the alditol acetates revealed a large peak of glucitol, a much smaller peak of mannitol, and traces of fucitol and galacitol. This pattern was

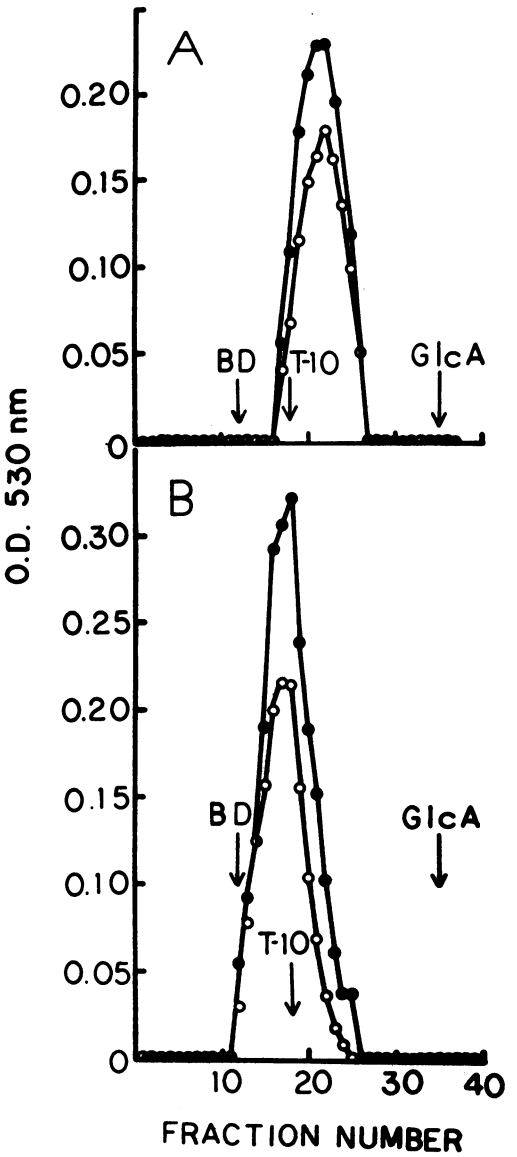

FIG. 4. Bio-Gel P-10 chromatography of homopolymeric glucuronans derived from yeast (○) and mycelial (●) type polymer I (A) and II (B) by partial acid hydrolysis. The elution positions of blue dextran (BD), dextran T-10 (T-10), and glucuronic acid (GlcA) are shown.

confirmed by paper chromatography of the parent neutral aldoses. Given incomplete reduction, accurate determination of the composition was not possible. However, the unreduced polymer after acid hydrolysis yielded a soluble fraction containing largely mannose and glucose and an insoluble fraction which appeared to be almost entirely uronic acid (see above). Based on the relative contributions of these two fractions, together with the mannose-to-glucose ratio in the supernatant fraction, an estimated composition was obtained (Table 1). Overall, the compositions of polymers I and II were quite different. The major differences between yeast and mycelial walls were in the composition of polymer I.

DISCUSSION

Our results demonstrate that cell walls from the yeast and mycelial phases of *M. rouxii* NRRL 1894 contain two distinct polyuronides. They are probably not part of a larger heteropolymer since they can be resolved by mild extraction with LiCl. This result contrasts with that of Datema et al. (10), who showed that the mycelial walls of *M. mucedo* contained a single heteropolymeric glucuronan of apparently higher molecular weight than that encountered here.

On partial acid hydrolysis, *M. rouxii* polyuronides gave rise to insoluble homopolymeric glucuronans. The ion-exchange data suggested that these derived homopolymers may not be identical. In addition, the mucoric acid fraction isolated from whole walls of *M. rouxii* NRRL 1894 as described by Bartnicki-Garcia and Reyes (8) gave two peaks of uronic acid-containing material on ion-exchange chromatography (data not shown). Therefore, the mucoric acid fraction of this strain of *M. rouxii* may be a mixture of two polymers with different properties. Thirty-four percent of the uronide residues of polymer I, and almost all of the uronide residues of polymer II (from both wall types), are present as homopolymers.

Our results contrast with those of Bartnicki-Garcia and Lindberg (4), who did not report the presence of acid-insoluble polyglucuronic acid as a significant part of the mucoran from the yeast phase of *M. rouxii*. The majority of the uronic acid in that polymer was present as the repeating disaccharide glucuronic acid-(1→3)mannose(4). Partial acid hydrolysis degraded most of the mucoran into dialyzable fragments. Partial acid hydrolysis of the hetero-

TABLE 1. Carbohydrate compositions of polymers I and II from yeast and mycelial cell walls

Sugar	Relative amt (% of total)			
	Mycelial polymers[a]		Yeast polymers[a]	
	I	II	I	II
Glucuronic acid	58.0	93.0	60.9	93.5
Galactose	4.1	Tr	2.1	Tr
Glucose		4.7		5.0
Mannose	9.1	2.3	18.2	1.5
Fucose	28.8	Tr	18.8	Tr

[a] As defined in the text.

polymeric glucuronan from *M. mucedo* walls yielded both insoluble polyglucuronic acid and a soluble fraction containing both glucuronic acid and neutral sugars (10).

The polyuronide of *M. mucedo* is considered to be retained within the cell wall solely by electrostatic interactions with positively charged chitosan since it can be quantitatively extracted with 6 M LiCl (10). In contrast, we found that LiCl did not extract any polymer II from *M. rouxii* walls. The alkali-extracted polymer II was shown to be insoluble in 5.6 M LiCl; therefore, it is still possible that both polyuronides are retained in the wall by purely electrostatic interactions, although the existence of alkali-labile covalent bonds between polymer II and chitin-chitosan cannot be excluded. Sequential treatments with LiCl and KOH extracted almost all of the uronide from yeast walls but only about 50% of the uronide from mycelial walls. The residual uronide could be extracted almost entirely with alkali after freezing and thawing, suggesting that the polymers were probably physically restrained rather than held by alkali-resistant covalent linkages.

Major differences between the polymers of yeast and mycelial walls occurred only within the compositions and molecular weights of polymer I. The significance of these differences for wall properties remains to be tested. A complete understanding of cell wall structure must take into account not only the nature of the polymers but also their fine locations and interactions. Synthesis and turnover also undoubtedly have a crucial role in morphogenesis.

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