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Monitoring polysaccharide synthesis in *Candida albicans**

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ABSTRACT

A method is described that measures the rate of [^{14}C]-labelling of (1→6)-β-D-glucan, mannoprotein, and (1→3)/(1→6)-β-D-glucan during approximately one-tenth of the doubling time of *Candida albicans*. The labelling pattern can be used as a measure of cell-wall synthesis and to detect changes in the composition of newly constructed cell walls that might otherwise be masked by previously deposited envelope.

INTRODUCTION

The structure of the yeast cell wall alters in the transition between the phases of growth and the morphological form. *Candida albicans* grows both as a single-celled yeast and in filamentous form[1], the morphology adopted being dictated by the cellular environment. Electron microscopy has revealed changes in both laminar structure and proportion of cell-wall components[2]. Gale *et al.*[3,4] reported changes in resistance to the lytic action of amphotericin B, which are attributed to alterations in cell-wall structure occurring in the transition from the exponential to the stationary growth phase. Differences in layer structure[5] and proportion of β-D-glucans have also been noted[6]. Gopal *et al.*[7] described differences in the structure of β-D-glucans isolated from the germ-tube and filamentous forms of yeast. These compositions, however, provide no clue as to the mechanism or rapidity of change of the cell-wall architecture.

We now describe a radio-labelling protocol of the intact cell that indicates the rate of synthesis of (1→6)-β-D-glucan, mannoprotein, and (1→3)/(1→6)-β-D-glucans during a 10-min period of yeast cell growth. These polysaccharides could be the major cell-wall components and the method can be used to detect changes in the pattern of biosynthesis of newly constructed cell wall and the subsequent changes not possible by established procedures of cell-wall analysis.

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EXPERIMENTAL

Organism and conditions of culture. — *Candida albicans* (ATCC 10261) was maintained on malt-extract agar slopes. Transfer was by loop to a medium comprising (per L) yeast extract (5 g), peptone (10 g), and D-glucose (20 g) which was then shaken at 27° and 200 r.p.m. until the stationary phase of growth was reached. An inoculum of this culture (50 μL per 500 mL) was introduced into a medium comprising (per L), $(NH_4)_2SO_4$ (1 g), KH_2PO_4 (1 g), $MgSO_4$ (0.05 g), $CaCl_2$ (0.05 g), D-glucose (15 g), bactopeptone (1 g), and biotin (25 μg), and shaken at 27° and 200 r.p.m. until the early exponential phase of growth was reached.

[^{14}C]-Labelling of cells. — Cells (100 mL) in the early exponential phase of growth were harvested at room temperature, the supernatant solution was discarded, and the pellet was resuspended in fresh D-glucose–salts–biotin medium (20 mL) but with D-glucose reduced from 0.083 to 0.01M. Shaking at 27° was recommenced and, after 5 min, D-[^{14}C]glucose (25 μCi/0.92 MBq) was added and shaking was continued for a further 10 min. The assimilation of D-[^{14}C]glucose was halted by mixing suspensions of cells with ethanol (2 vol.) at 0°. After storage at 0° for < 90 min, these cells were heated at 80° for 5 min, cooled, washed three times by centrifugation with aqueous 66% ethanol, suspended in water, stored at −10°, and termed extracted cells.

The effect of the environment was examined by washing and suspending cells dispensed from common stock in (*a*) the above glucose–salts–biotin (GSB) medium, (*b*) GSB adjusted to pH 7 with Tris–HCl at a final concentration of 50mM (GSB/Tris), and (*c*) GSB with peptone and NH_4^+ omitted (GSB-N). These media were maintained during labelling and chasing.

Digestion of labelled cells with Zymolyase 20T. — Suspensions of extracted cells were shaken for 1 h in 0.05M Tris–HCl (pH 7.2) at 30°. Zymolyase (0.1 mg/mL) was added and the digestion continued for 5 h. The liberation of soluble [^{14}C]-labelled material was measured by centrifuging samples at 5000*g* for 5 min and removing aliquots of the supernatant solution for scintillation counting. Comparisons of these measurements with those for uncentrifuged suspensions provided a measure of the solubilisation of [^{14}C]-labelled material. When the digestion was complete, cellular debris was removed by centrifugation, and the supernatant solution was heated at 100° for 5 min before storage at −10°. The Zymolyase-resistant insoluble debris was also stored at −10°.

Fractionation of soluble [^{14}C]-labelled products liberated by Zymolyase. — Samples (1–2 mL) were applied to a column (1 × 34 cm) of Bio-Gel P-30 and eluted with 5mM sodium azide at 2 mL/h at room temperature. The eluate was monitored for [^{14}C]. Combined fractions (Fig. 1) were concentrated, the residue was dissolved in a small volume of water, and the solution was stored at −10°.

Digestion with amyloglucosidase and alpha-amylase: removal of glycogen. — Samples of fractions 1 and 2 (Fig. 1) were dissolved in 0.05M Tris–HCl (pH 7.2) at 30°, and glycogen (5 mg) was added as an internal control, followed by amyloglucosidase or alpha-amylase (20 μkat/mL). The release of glucose[8] by amyloglucosidase or reducing

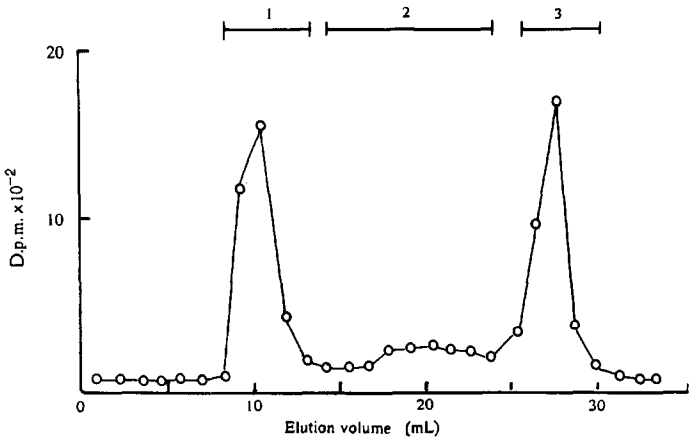


Fig. 1. Gel-filtration chromatography through Bio-Gel P-30 of the solubilised polysaccharides released from Zymolyase-digested extracted cells.

equivalents[9] by alpha-amylase was monitored and the reaction was terminated when no more products were released during a period of 1 h. The enzymes were inactivated by heating the digests at 100° for 5 min. The products of hydrolysis were fractionated on a column of Bio-Gel P-30 as described above, the fraction of low M_r was concentrated, and the residue was analysed by p.c. For the routine removal of glycogen, samples were digested with amyloglucosidase and the products were fractionated by gel-filtration chromatography. The amyloglucosidase-resistant fraction of high M_r was concentrated prior to further fractionation.

Separation of the glucan and mannan[10]. — A sample (1 mL) of fraction 1 containing 1–2 mg of carbohydrate, from which glycogen had been removed, was applied in 0.02M Tris–HCl–0.5M NaCl (pH 7.4) to a column (0.8 × 3 cm) of Sepharose–concanavalin A, and eluted with 15 mL of the same buffer followed by eluant containing 0.2M methyl α-D-mannopyranoside (10 mL). There was a clear separation of bound and non-bound material, which were subsequently shown to be mannan and glucan, respectively.

Analytical methods. — Radioactivity was measured using scintillation fluid comprising (per L) toluene (727 mL), Triton X-100 (273 mL), and 2,5-diphenyloxazole (3 g). Aqueous samples (0.5 mL) were mixed with 4.5 mL of fluid; glass-fibre filters and p.c. strips were immersed in 5 mL of fluid. The [^{14}C] content of cells was measured by filtering aliquots of cell suspensions through GF/C filter pads (25 mm, Whatman) and washing with water cooled to 0° or with aqueous 66% ethanol at 0° if the cells had been extracted. Filters were dried at 60° for 12 h before counting. Sugar analysis of labelled cells was performed by heating samples in aqueous 90% formic acid (1 mL) for 1 h at 100°, concentration, and heating the residue in 2M H_2SO_4 (3 mL) for 4 h at 100°. The hydrolysate was cooled to 0°, then neutralised with $BaCO_3$, and the insoluble material was collected and washed with water by centrifugation. The supernatant solution and washings were combined and concentrated, and a solution of the residue in the

minimum volume of water was fractionated by p.c. For the analysis of soluble polysaccharides, the hydrolysis with formic acid was omitted. P.c. was performed on Whatman 3MM paper, using 10:4:3 ethyl acetate–pyridine–water for mono-, di-, and oligo-saccharides, and 8:2:1 for the products of periodate oxidation. The dried paper was cut into 0.5-cm strips and assayed for radioactivity. Marker strips were stained with silver nitrate[11].

Periodate oxidation, borohydride reduction, and acid hydrolysis. — Standard procedures were followed. Oxidation of 1–2 mg of material was in 0.024M sodium metaperiodate with glycogen (10 mg) added as the internal standard so that the course of oxidation could be followed. Reactants were stored in the dark at 4° and the oxidation was followed by measuring the absorbance at 230 nm. After 100 h, the excess of periodate was reduced with ethylene glycol, the mixture was dialysed against water and concentrated, and the residue was reduced with 0.1M $NaBH_4$ at room temperature for 18 h. After destruction of the excess of reductant with acetic acid and co-concentration with methanol, the dried residue was hydrolysed (see above).

Chasing labelled cells. — Cells (10 mL) exposed to 0.01M D-[^{14}C]glucose for 10 min were mixed rapidly with fresh medium (90 mL) of the same composition as the labelling medium, but containing 0.1M non-labelled D-glucose, and shaken for a further 60 min. Ethanol (200 mL) at 0° was then added, and the cells were washed with aqeous 66% ethanol, stored at −10°, and termed chased cells.

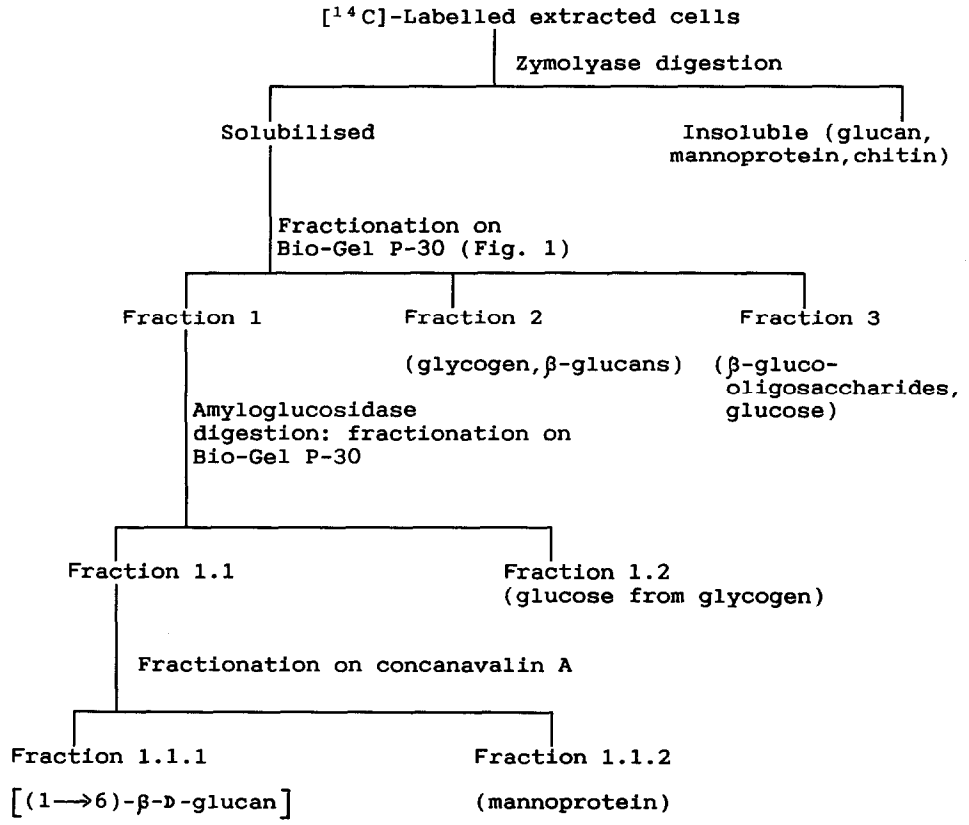
Enzymes. — Zymolyase 20T, a (1→3)-β-D-glucanase from *Arthrobacter luteus*, was purchased from Miles Scientific. No mannanase, chitinase, or protease activity could be detected at the concentration of 0.1 mg/mL used for the digestion of labelled, extracted cells. Porcine alpha-amylase and amyloglucosidase were obtained from Boehringer-Mannheim. Stock alpha-amylase, which was crystalline, contained 1000 U/mg; amyloglucosidase from *Aspergillus niger* was a lyophilised powder with an activity of 14 U/mg.

RESULTS

Labelling of cells. — Cells, harvested at a density of 5×10^7/mL, were in the early exponential phase of growth as judged by absorbance. Cells were not limited in growth prior to labelling and, since <20% of the D-glucose was used during labelling, they were not retarded during exposure to D-[^{14}C]glucose. Assimilation of isotope ceased immediately upon immersing cells in aqueous ethanol. Of the label, ~60% was extracted by the solvent and emerged as one peak of labelled material of $M_r < 1000$ when fractionated on Bio-Gel P-30. The isotope remaining in the cell residue was not extracted with 50mM Tris–HCl (pH 7.0) and it was assumed that the solvent had lysed the cytoplasmic membrane. Labelled material remaining in the extracted cells was shown to be only carbohydrate. Thus, in a typical analysis of the label assimilated into polymeric material, 86% was released by Zymolyase. Of this material, all but 9% (8% of total cellular polymeric label) was accounted for as (1→6)-β-D-glucan, mannoprotein, (1→3)-β-D-glucan, and glycogen. The remaining 9% was not identified but comprised

small gluco-oligosaccharides, possibly containing 2-acetamido-2-deoxyglucose and originally bound to a polymer by (1→3)-β-D-glucosidic bonds. Of the remaining insoluble labelled cellular polymer (14%) not solubilised by Zymolyase, 64% was identified as glucan, chitin, and mannan.

Solubilisation of [^{14}C]-labelled polysaccharides from extracted cells. — Digestion of the cells for 4 h by Zymolyase at 0.01 and 0.1 mg/mL solubilised 85–90% of the labelled material. Digestions at 1 mg/mL released 93% of labelled oligo- and polysaccharides. Hydrolyses were carried out at pH 7 with 0.1 mg/mL of Zymolyase in order to avoid hydrolysis of acid- or alkali-sensitive bonds. It is possible that polymers comprising the yeast cell wall are substantially more cross-linked than is currently believed. Even though the nature of these putative bridging structures has not been identifed, it would be prudent to develop conditions of controlled cell-wall dismantling that avoid non-enzymic hydrolysis.



Scheme 1. Fractionation of Zymolyase-solubilised polysaccharides. Fractions 1–3 correspond to those in Fig. 1.

Fractionation and characterisation (Scheme 1). — Solubilised carbohydrates were separated by gel-filtration on Bio-Gel P-30 into three fractions with M_r >40,000 (1), 40,000–1000 (2), and <1000 (3) (Fig. 1). Re-chromatographed fractions were eluted in the same volume, and duplicate digestions of the same or other preparations of extracted cells produced carbohydrate distributions that differed by <5%. Fractions 1 and 2 contained carbohydrate susceptible to digestion by fungal amyloglucosidase. The products (Scheme 1, fractions 1.1 and 1.2) were analysed on Bio-Gel P-30. Fraction 1.1 remained in the void volume and fraction 1.2 was identified as glucose. That the amyloglucosidase-susceptible carbohydrate was glycogen was shown by digestion with porcine alpha-amylase followed by gel-filtration and the identification of the products as glucose (13%) and maltose (87%). The same proportion of undigested material remained in the void volume.

The carbohydrate in fraction 1.1 was resistant to alpha-amylase or amyloglucosidase and contained labelled glucose (55–60%) and mannose (40–45%). Affinity chromatography on concanavalin A gave a non-adsorbed fraction (1.1.1) which contained glucose as the only labelled sugar. Periodate oxidation, borohydride reduction, and acid hydrolysis gave glucose and glycerol in the ratio 1:8.1 together with glycolaldehyde. This result suggested that fraction 1.1.1 was the component of the cell wall that was mainly a (1→6)-β-D-glucan with some branches at positions 3.

The label present in the adsorbed fraction (1.1.2) comprised 96–97% of mannose and 3–4% of glucose. The products of periodate oxidation contained 3% of the label as unchanged glucose, suggesting that the trace glucan was (1→3)-linked. The remaining products were consistent with the structure of the branched mannan found in yeast cell walls.

Glucose was the only labelled sugar in fraction 2 and analysis, as for fraction 1, showed that 23–27% of the polysaccharide was glycogen. Periodate oxidation, borohydride reduction, and acid hydrolysis produced labelled glucose (30%), glycerol (60%), and erythritol (10%), suggesting (1→3)- and (1→6)-linked glucans to be the remaining components of fraction 2.

P.c. of fraction 3 revealed the major products to be D-glucose (20%), laminaribiose (40%), and an oligosaccharide (40%) with a mobility intermediate of those of lamanari-triose and -tetraose, which indicated a branched gluco-oligosaccharide.

The water-insoluble residue of Zymolyase-digested cells contained the remaining 10–12% of labelled polymer originally present in the extracted cells. Acid hydrolysis indicated labelled glucose (50–55%), mannose (35–40%), and 2-amino-2-deoxyglucose (8–10%). The glucose resisted periodate oxidation, indicating (1→3) linkages, and the products remaining after reduction (mannose, glyceraldehyde, glycolaldehyde) were consistent with the presence of yeast mannan.

The procedures for labelling, digestion, and fractionation are summarised in Scheme 1. The distribution of the [^{14}C] label in the extracted cells is shown in Tables I and II. Measurement of isotope incorporation into the glucans and mannan after 2 and 10 min showed a linear incorporation of label except for the (1→6)-linked glucan where there appeared to be a delay of ~ 1 min before incorporation began.

TABLE I

Percentage of isotope distributed among polysaccharides after exposure of the cell to D-[^{14}C]glucose for 10 min (see Experimental)

Polysaccharide	Cell-growth medium		
	GSB (pH 5)	GSB/Tris (pH 7)	GSB-N (pH 5)
(1→6)-β-D-glucan	12	6	10
Mannoprotein	20	14	25
Fraction 2 (glucans)	10	7	6
Fraction 3 (β-gluco-oligosaccharides)	43	49	48
Resistant residue	15	24	11

TABLE II

Change of percentage of isotope distribution among polysaccharides after 60-min cold chase (see Table I and Experimental)

Polysaccharide	Cell-growth medium		
	GSB (pH 5)	GSB/Tris (pH 7)	GSB-N (pH 5)
(1→6)-β-D-Glucan	+3	−1	0
Mannoprotein	+11	0	+1
Fraction 2 (glucans)	−1	+2	−2
Fraction 3 (β-gluco-oligosaccharides)	−23	−26	−26
Resistant residue	+10	+25	+27

DISCUSSION

The principal objective of this investigation was to develop a protocol for the characterisation of the architecture of freshly incorporated cell-wall material. In addition, the procedure had to rapidly halt enzymic activity and avoid the cumbersome and chemically destructive procedures of mechanical disruption and acid–base extraction of the cell[12]. These traditional procedures do not guarantee the immediate inactivation of hydrolases that can modify the structure of the cell wall. At the expense of hydrolysing (1→3)-β-D-glucosidic linkages, the protocol fulfils the requirements and the assumption is made that the polymers examined are those comprising the cell wall.

Exponentially growing cells of *C. albicans* were used. Of the D-[^{14}C]glucose assimilated into cellular polymeric material, 85–90% could be solubilised, of which >90% was accounted for as β-D-glucan, mannan, and glycogen. Of the remaining 10%,

which was insoluble and Zymolyase-resistant, the majority was a mixture of glucan, chitin, and mannan. Treatment of the residue with 0.75M NaOH at 70° for 5 h solubilised 70% of the labelled material and some glucan remained. Apart from glycogen, the structures attributed to the polysaccharides are characteristic of cell-wall polymers. The binding of mannan to concanavalin A and the lack of any other sugar in its composition strongly suggest that it is the mannoprotein of the cell wall.

The assimilation of D-[^{14}C]glucose into polysaccharides during 10 min of exponential growth in GSB medium is shown in Table I. The linearity of uptake of the label during 10 min into the various types of polymer, with the exception of the small delay in entry into the (1→6)-β-D-glucan, indicates the respective rates of synthesis. The structure of the polysaccharides identifies them as components of the cell wall and, indeed, the cell-wall composition reported by Sullivan *et al.*[6] is comparable, namely, mannan (20–23%) and β-D-glucan (48–60%), although the distribution of (1→6)- and (1→3/(1→6)-β-D-glucans within the latter are different.

The distribution of label was used to assess the immediate effect of environmental change on the synthesis of cell-wall components and on their subsequent processing. Labelling patterns were recorded after exposure to D-[^{14}C]glucose after 10 min and again after a 60-min cold chase. The results are shown in Tables I and II. Changes in the rates of biosynthesis that are affected by pH or loss of nitrogen are not great and, taking account of the accuracy of the probe, may not be significant. Alterations in the susceptibility of the cell-wall polymers to digestion after they have been incorporated into the wall are more significant, and they become more resistant to Zymolyase (Table II). It is this post-polymerisation processing that appears to be susceptible to the environment.

Thus, the procedure described can be used to explore the rapidity and extent of changes to yeast and fungal cell-wall assemblies during the transition between growth phases or in morphological changes.

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