Analysis of Wall Glucans from Yeast, Hyphal and Germ-tube Forming Cells of *Candida albicans*

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Acid-soluble and alkali-insoluble glucan fractions were prepared from yeast, hyphal and germ-tube forming cells of *Candida albicans*. Alkali-insoluble glucan was also extracted from purified yeast cell walls. Paper chromatography of partial acid hydrolysates confirmed that the glucan preparations contained $\beta(1\rightarrow3)$ - and $\beta(1\rightarrow6)$ -chains but no mixed intra-chain $\beta(1\rightarrow3)/(1\rightarrow6)$ linkages. Methylation and ¹³C-NMR analyses showed that the acid-soluble glucan consisted of a highly branched polymer composed mainly (67.0% to 76.6%) of $\beta(1\rightarrow6)$ -linked glucose residues. The alkali-insoluble glucan from yeast and hyphal cells contained from 29.6% to 38.9% $\beta(1\rightarrow3)$ and 43.3% to 53.2% $\beta(1\rightarrow6)$ linkages. Alkali-insoluble glucan from germ-tube forming cells consisted of 67.0% $\beta(1\rightarrow3)$ and 14% $\beta(1\rightarrow6)$ linkages. Branch points accounted for 6.7%, 12.3% and 17.4% of the residues in the alkali-insoluble glucan of yeast, germ-tube forming and hyphal cells, respectively.

INTRODUCTION

The cell wall of the dimorphic yeast *Candida albicans* is qualitatively similar in composition to that of *Saccharomyces cerevisiae* (for reviews see Cabib *et al.*, 1982; Ballou, 1982; Duffus *et al.*, 1982). Glucans, which include the main structural component, and mannoproteins constitute at least 80% of the *C. albicans* wall (Sullivan *et al.*, 1983). Chitin, 0.6% of *C. albicans* yeast walls (Sullivan *et al.*, 1983), is located mainly (90%) in the bud scars but is also dispersed around the wall (Cabib *et al.*, 1982; Tronchin *et al.*, 1981).

Germ-tube forming cells, which constitute the initial stage in the yeast-mycelium transition, and hyphal cells contain three to five times more chitin than yeast cells (Braun & Calderone, 1978; Sullivan *et al.*, 1983; Chattaway *et al.*, 1968). Cassone *et al.* (1973) and Scherwitz *et al.* (1978) have shown that the layered organization of the wall, visualized by electron microscopy, changes significantly with the onset of germ-tube formation; in particular an inner electron-translucent layer becomes dominant in the germ-tube.

Previous studies suggest that the fine structure of the cell wall glucan changes with the age of the cells and the morphology. Gale *et al.* (1980) showed that the increased resistance to amphotericin exhibited by stationary-phase yeast cells was due to changes in the cell wall and that treatment with glucanases decreased the resistance. Chattaway *et al.* (1968) reported that although the alkali-insoluble glucan fractions from hyphal and yeast cells had similar glucose contents, treatment with a β -glucanase released 19% and 39% of the glucose, respectively.

Reports of detailed structural analysis of the *C. albicans* glucans are limited. Bishop *et al.* (1960) and Yu *et al.* (1967) extracted a glucan fraction from *C. albicans* serotypes A and B with boiling 3% NaOH. Non-reducing terminal and $\beta(1\rightarrow6)$ -linked residues accounted for more than 70% of the glucose. These preparations contained only a trace (serotype A) and 17% (serotype B) of $\beta(1\rightarrow3)$ -linked residues. However, the alkali-soluble fraction represents less than 10% of the

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total glucan (Sullivan *et al.*, 1983) and boiling NaOH may have caused some degradation of $\beta(1\rightarrow3)$ linkages by β -elimination. Gopal *et al.* (1984) analysed glucan nets from regenerating spheroplasts of *C. albicans*: the nets consisted of 43% $\beta(1\rightarrow3)$ -linked, 30% $\beta(1\rightarrow6)$ -linked and 9.5% branch point residues. This work gives some indication of the glucan structure in the intact walls, but glucan synthase activity may have been modified during the preparation of the spheroplasts (Duffus *et al.*, 1982). In the present paper we describe the structural analysis of glucan fractions from yeast, hyphal and germ-tube forming cells of *C. albicans*.

METHODS

Organisms and culture conditions. Conditions for the maintenance of cultures, growth of yeast cells and germ-tube formation have been described previously (Shepherd & Sullivan, 1976; Shepherd *et al.*, 1980). *C. albicans* ATCC 10261 was used to produce yeast cells and germ-tube forming cells. Strain hOG301 was kindly provided by Dr R. T. M. Poulter, University of Otago, New Zealand. This mutant, isogenic with ATCC 10261, grows in a hyphal form at 28 °C in yeast growth medium.

Isolation of cell wall glucans. Two methods were used to obtain wall glucans. In method I cell walls were prepared from a batch (298 g wet wt) of exponential-phase yeast cells (Sullivan *et al.*, 1983). After lipid extraction the final yield of walls was 11.9 g dry wt. The cell wall preparation was extracted with 0.25 M- Na_2CO_3 (500 ml per g dry wt) at 90 °C for 45 min. The soluble extract of alkali-soluble glucan and mannoprotein (Stewart, 1975) was discarded and the insoluble fraction was washed extensively with distilled water. The insoluble residue was treated with pancreatin as described by Ram *et al.* (1981). Each incubation contained 20 ml 0.05 M-Tris/HCl buffer (pH 8.1), 40 μmol CaCl_2 , 20 mg pancreatin and 400 mg of the insoluble wall fraction. After incubation for 24 h at 37 °C, the insoluble residue was recovered by centrifuging, washed twice with distilled water and stored as a frozen pellet; the yield was 2.85 g. In method II intact cells (yeast, hyphal or germ-tube forming cells) were sequentially extracted with alkali and acid as described by Manners *et al.* (1973*a*). In this procedure a known amount (10–15 g wet wt) of cells was extracted with 6 vols 3% (w/v) NaOH at 70 °C for 6 h. The alkali-soluble extract was not analysed in this work. The cell residue was washed three times with water and the pooled extracts and washings were dialysed for 12 h against running tap water. The cell residue was extracted 15 times with 100 ml volumes of 0.5 M-acetic acid at 100 °C, each for 4–6 h. The combined extracts were dialysed, lyophilized and treated with α -amylase. Each preparation, 150–200 mg, was suspended in 2.0 ml 0.1 M-sodium phosphate buffer (pH 6.9) containing 0.6 M-NaCl, 0.02% (w/v) NaN_3 , chloramphenicol (50 $\mu\text{g ml}^{-1}$) and 4 mg α -amylase. After 12 h incubation at 30 °C, each acid-soluble preparation was dialysed against tap water for 12 h and freeze-dried. The insoluble residue remaining after the sequential acid extractions contained insoluble glucan. Each residue was washed with distilled water, treated with α -amylase as described above, washed with water and stored as a pellet at –20 °C. Neither the pancreatin used in method I (above) nor the α -amylase used in method II exhibited β -glucanase activity towards laminarin and pustulan.

Analysis of glucan fractions. The methods used for methylation of glucan fractions, GLC of alditol acetate derivatives, partial acid hydrolysis of glucans and paper chromatography of the hydrolysates were as described previously (Gopal *et al.*, 1984). Alditol acetate standards (Ram *et al.*, 1981) were used to identify peaks in the GLC analyses. Partial acid hydrolysates of laminarin and pustulan were used as standards for paper chromatography (Gopal *et al.*, 1984). Total carbohydrate was determined by the method of Dubois *et al.* (1956) using glucose as a standard.

^{13}C -NMR spectroscopy. ^{13}C -NMR spectra were recorded by Dr H. Wong, Chemistry Division, DSIR, Gracefield, New Zealand, with a Varian FT80A spectrometer operating at 20 MHz in the pulsed mode [20 μs (90%) pulse width, 0.82 s repetition time]. The solvent deuterium oxide signal was used as a field frequency lock signal and chemical shifts were expressed in p.p.m. downfield from external trimethyl silane. All spectra were recorded at 90 °C and a spectral width of 5 kHz.

Materials. α -Amylase (from human saliva) and pancreatin were from Sigma. Laminarin was purchased from the US Biochemical Corp., Cleveland, Oh., USA, and pustulan was obtained from Calbiochem.

RESULTS

Isolation of cell wall glucan

Method I, used to obtain glucan from purified yeast cell walls, involved mild alkaline treatment followed by incubation with pancreatin to remove residual glycogen. The final yield of 2.58 g was 24% of the original wall preparation and was comparable with the amount of insoluble

glucan obtained in a previous study of the *C. albicans* wall (Sullivan *et al.*, 1984). This glucan preparation was obtained by mild procedures so that the subsequent structural analyses would reflect the relative abundance of $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linkages in the cell wall. Method II used intact cells as the starting material and yielded three glucan fractions: an alkali-soluble fraction which was not analysed in this work, an acid-soluble fraction and insoluble glucan. These fractions were prepared from the three morphological forms of *C. albicans*. The crude fractions were contaminated with glycogen (12–14% of the insoluble fractions and 35–44% of the crude acid-soluble fractions). After treatment with amylase the carbohydrate present in the insoluble and acid-soluble fractions, respectively was (mg): yeast, 375 and 136; hyphal, 150 and 60; and germ-tube cells, 255 and 94. These data should not be interpreted as quantitative yields since the objective in this extraction procedure was purity rather than recovery. It should be noted, however, that the relative yields of insoluble glucan and acid-soluble glucan were approximately 2.7:1 for each type of cell.

Partial acid hydrolysis

Paper chromatography of partial acid hydrolysates of insoluble glucan and acid-soluble glucan prepared by method II revealed glucose, laminaribiose and oligomers of this series up to laminaritetrose, gentiobiose and oligomers up to gentiotetrose. The hydrolysates did not contain any unidentified sugars. These results suggested that the glucan fractions were substantially free of other glucans and that the glucan chains did not contain mixed intrachain $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linkages.

Methylation analysis

The acid-soluble glucan fraction from germ-tube forming cells was not analysed further. The other glucan fractions were subjected to three cycles of methylation. The absence of infra-red absorption in the region 3200–3600 cm^{-1} indicated that essentially all the available hydroxyl groups had been methylated. Hydrolysates of these preparations contained the four *O*-methyl glucitol acetate derivatives expected from branched $(1\rightarrow3)$ – $(1\rightarrow6)$ - β -glucans. A small amount (2–3%) of 4,6-di-*O*-methyl glucitol acetate was also detected in some hydrolysates. This arises because of undermethylation or demethylation and the peak was not included in the calculations of percentage composition.

The insoluble glucan fraction from purified yeast cell walls contained near equimolar amounts of $\beta(1\rightarrow3)$ - and $\beta(1\rightarrow6)$ -linked glucose (Table 1). This glucan fraction is highly branched since 10% and 7.6% of the glucose residues were non-reducing end groups and branch points, respectively.

Table 1. *Methylation analysis of the insoluble glucan fraction from yeast cell walls*

The insoluble glucan fraction was extracted from purified yeast cell walls by method I, methylated, hydrolysed and acetylated (see Methods). The peaks were identified from GLC retention times and also by adding authentic samples as internal standards. The percentage composition was determined by making three copies of the GLC traces, cutting and weighing each peak. Individual percentages were calculated from the individual and total weights; molar ratios were calculated from the percentage composition data.

<i>O</i> -Methyl D-glucitol acetate derivative	Type of linkage	Percentage composition	Molar ratio
2,3,4,6-Tetra- <i>O</i> -	Non-reducing end group	10.0	1.0
2,4,6-Tri- <i>O</i> -	(1→3)	38.9	4.7
2,3,4-Tri- <i>O</i> -	(1→6)	43.3	5.3
2,4-Di- <i>O</i> -	Branch point C-1, C-3, C-6	7.6	0.86

Table 2. *Methylation analysis of glucan fractions from yeast, hyphal and germ-tube forming cells*

The glucan fractions were extracted from intact cells by method II, methylated, hydrolysed and acetylated (see Methods). The analyses were done as summarized in Table 1.

O-Methyl D-glucitol acetate derivative	Type of linkage	Percentage composition	Molar ratio
Yeast insoluble			
2,3,4,6-Tetra-O-	Non-reducing end group	8.1	1.0
2,4,6-Tri-O-	(1→3)	31.8	3.6
2,3,4-Tri-O-	(1→6)	53.2	6.1
2,4-Di-O-	Branch point C-1, C-3, C-6	6.7	0.7
Yeast acid-soluble			
2,3,4,6-Tetra-O-	Non-reducing end group	6.3	1.0
2,4,6-Tri-O-	(1→3)	11.7	1.4
2,3,4-Tri-O-	(1→6)	76.6	11.8
2,4-Di-O-	Branch point C-1, C-3, C-6	5.3	0.8
Hyphal insoluble			
2,3,4,6-Tetra-O-	Non-reducing end group	5.8	1.0
2,4,6-Tri-O-	(1→3)	29.6	4.9
2,3,4-Tri-O-	(1→6)	47.1	7.2
2,4-Di-O-	Branch point C-1, C-3, C-6	17.4	2.4
Hyphal acid-soluble			
2,3,4,6-Tetra-O-	Non-reducing end group	10.1	1.0
2,4,6-Tri-O-	(1→3)	14.3	1.3
2,3,4-Tri-O-	(1→6)	67.0	7.0
2,4-Di-O-	Branch point C-1, C-3, C-6	8.4	0.8
Germ-tube cells insoluble			
2,3,4,6-Tetra-O-	Non-reducing end group	5.8	1.0
2,4,6-Tri-O-	(1→3)	67.0	10.8
2,3,4-Tri-O-	(1→6)	14.0	2.3
2,4-Di-O-	Branch point C-1, C-3, C-6	12.3	1.9

Table 2 summarizes the methylation analyses of the glucan fractions extracted from intact cells. The acid-soluble glucan fractions consisted predominantly (67–76%) of $\beta(1\rightarrow6)$ -linked glucose residues with smaller amounts (11.7–14.3%) of $\beta(1\rightarrow3)$ linkages. As expected 2,3,4,6-tetra-*O*-methyl glucitol acetate (non-reducing end groups) and 2,4-di-*O*-methyl glucitol acetate (branch points) were present in near equivalent amounts. The relative abundance of branch point residues (5.3–8.4%) indicates that acid-soluble glucans are highly branched.

The insoluble glucan fractions from intact yeast and hyphal cells were quite similar with respect to their percentage $\beta(1\rightarrow3)$ (31.8% and 29.6%) and $\beta(1\rightarrow6)$ linkages (53.2% and 47.1%). Both glucan preparations were highly branched as judged from the percentage of non-reducing end groups but surprisingly there was a threefold excess of di-*O*-methyl glucitol acetate over tetra-*O*-methyl glucitol acetate in the hyphal preparation. A similar result (5.8% tetra-*O*-methyl glucitol acetate and 12.3% di-*O*-methyl glucitol acetate) was obtained with the insoluble fraction from germ-tube forming cells. The latter cells yielded an insoluble glucan with a different composition. In this preparation $\beta(1\rightarrow6)$ -linked glucans only accounted for 14% of the residues while 67% of the glucose units were $\beta(1\rightarrow3)$ -linked.

¹³C-NMR spectroscopy

Laminarin and pustulan used as standard glucans showed six chemical shifts consistent with previously published data (Colson *et al.*, 1974; Saito *et al.*, 1977; Bassieux *et al.*, 1977). The acid-soluble fractions (yeast, germ-tube and hyphal) gave the same well-resolved spectrum and the spectra of laminarin and yeast acid-soluble glucan are shown in Fig. 1. The 105.0 p.p.m. signal is indicative of a β -anomeric carbon. No signal was detected in the 100–103 p.p.m. region thus confirming the absence of α -glycosidic contaminants. Furthermore, while laminarin showed a clear *O*-substituted C-3 signal at 87.0 p.p.m., no such signal was present in the spectra of the acid-soluble glucan or pustulan. The chemical shifts (p.p.m.) of the acid-soluble glucan as shown in Fig. 1 are assigned as follows: C-1 (105.0), C-2 (75.3), C-3 (78.0), C-4 (72.1), C-5 (77.1), and *O*-

Fig. 1. ^{13}C -NMR spectrum of acid-soluble glucan. The spectra of (a) laminarin (100 mg ml^{-1}) and (b) yeast acid-soluble glucan (85 mg ml^{-1}) were recorded in D_2O as described in Methods. The spectra were obtained after 61 000 and 51 000 accumulations, respectively. Values for the chemical shifts are given as p.p.m. The spectrum of pustulan (100 mg ml^{-1}) showed chemical shifts at 104.9, 77.9, 77.0, 75.2, 71.9 and 71.0 p.p.m. (not shown).

substituted C-6 (71.1). These data are essentially the same as those reported for the (1 $\rightarrow$ 6)- β -glucan from *Gyrophora esculenta* (Saito *et al.*, 1977) and pustulan from *Umbilicaria pustulata* (Bassieux *et al.*, 1977). In particular, the acid-soluble glucan and (1 $\rightarrow$ 6)- β -glucans, but not laminarin, have a chemical shift at 70.0 p.p.m. which has been assigned to the O-substituted C-6 (Saito *et al.*, 1977).

DISCUSSION

The present work has established that the alkali-insoluble glucan extracted either from intact cells or from purified cell walls of *C. albicans* contains from 31.8% (yeast cells) to 67.0% (germ-tube forming cells) of $\beta(1\rightarrow3)$ -linked residues. It is generally accepted (Duffus *et al.*, 1982; Cabib *et al.*, 1982) that (1 $\rightarrow$ 3)- β -glucan in the alkali-insoluble fraction of *Saccharomyces cerevisiae* provides the rigid framework of the cell wall. This type of glucan probably fulfils the same role in *C. albicans*. Consistent with this proposal, it has previously been shown that Zymolyase, a cell wall lytic preparation which contains (1 $\rightarrow$ 3)- β - but not (1 $\rightarrow$ 6)- β -glucanase activity readily converts intact yeast cells of *C. albicans* to spheroplasts (Poulter *et al.*, 1981; Gopal *et al.*, 1984). Zymolyase is less effective with the mycelial mutant hOG301 and germ-tube forming cells (R. Surarit, M. J. Hubbard, M. G. Shepherd & P. A. Sullivan, unpublished results) and this may reflect the differing amounts of branching in these cell walls.

From the paper chromatography of partial acid hydrolysates it was concluded that *C. albicans* glucan contains chains of either $\beta(1\rightarrow3)$ - or $\beta(1\rightarrow6)$ -linked glucose but no mixed intra-chain $\beta(1\rightarrow3)/\beta(1\rightarrow6)$ linkages. Further studies would be needed to determine the type of linkages present in the backbone and the side chains. It is clear, however, that insoluble glucan from *C. albicans* and *S. cerevisiae* differs in structural detail. The former is highly branched (6.7%, 12.3% and 17.4% branch point residues in yeast, germ-tube forming and hyphal cells) and contains from 14% to 52.2% of $\beta(1\rightarrow6)$ -linked residues. In contrast, the insoluble glucan from *S. cerevisiae* contains 3% of branch point and $\beta(1\rightarrow6)$ -linked residues (Manners *et al.*, 1973a, b). Bacon (1979)

has suggested that the extent of branching in *S. cerevisiae* insoluble glucan could result in insufficient unsubstituted $\beta(1\rightarrow3)$ sequences for a triple helix conformation (Rees, 1973) in yeast glucan microfibrils. A triple helix conformation would be even less likely on these grounds with *C. albicans* glucan. Ballou (1982) has suggested that $(1\rightarrow3)$ - β -glucan in yeast walls may have a double helix structure involving extensive hydrophobic interactions.

The morphology of *C. albicans* is not determined simply by the ratio of $\beta(1\rightarrow3)$ to $\beta(1\rightarrow6)$ linkages, as this value was similar in the insoluble glucan from yeast and hyphal cells. However, insoluble glucan from hyphal and germ-tube forming cells contains more branch point residues (17.4% and 12.3%, respectively) and so this may be a feature of apical wall extension. These values, however, were somewhat surprising. In methylation analyses the amounts of di-*O*-methyl glucitol acetate (branch points) and tetra-*O*-methyl glucitol acetate (non-reducing end groups) are usually equivalent. The molar ratios for branch point residues in insoluble and acid-soluble glucans from yeast varied from 0.70 to 0.86 (Tables 1 and 2). The values for insoluble glucan from germ-tube and hyphal cells were 1.9 and 2.4, respectively. A possible explanation for these data is that the insoluble glucan in germ-tube and hyphal cells is, in part, covalently linked to another wall polymer via non-reducing end groups. Sietsma & Wessels (1981) have reported that the R-glucan in yeasts and fungi is linked in varying amounts to chitin. It is interesting to note that hyphal and germ-tube cells contain three to five times more chitin than do yeast cells (Chattaway *et al.*, 1968; Sullivan *et al.*, 1983).

Perhaps the most striking result from the present work was the increased amount (67%) of $\beta(1\rightarrow3)$ -linked glucose in the insoluble glucan from germ-tube forming cells, compared with 31.8% in yeast cells. This glucan preparation was obtained from intact cells and, taking into account the $(1\rightarrow3)$ -/ $(1\rightarrow6)$ - β -glucan in the mother cell, it seems likely that the glucan in the germ-tube consists mainly, if not entirely, of $\beta(1\rightarrow3)$ -linked glucan. Exclusive synthesis of $(1\rightarrow3)$ - β -glucan may occur during the morphogenic transition and if so this would be somewhat analogous to regenerating spheroplasts, which preferentially synthesize $\beta(1\rightarrow3)$ -linked glucan (Kreger & Kopecká, 1975; Gopal *et al.*, 1984). Changes in the relative amounts of $(1\rightarrow3)$ - and $(1\rightarrow6)$ - β -glucan may contribute to the altered wall layering (Cassone *et al.*, 1973) and increased cell wall porosity (Sullivan *et al.*, 1984) noted during germ-tube formation.

It has been shown during the present study that an acid-soluble $(1\rightarrow6)$ - β -glucan fraction can be extracted from *C. albicans*. Acid-soluble glucan constitutes less than 15% of the *S. cerevisiae* wall, contains 65% $\beta(1\rightarrow6)$, 19% $\beta(1\rightarrow3)$ and 16% branch point residues (Manners *et al.*, 1973*b*). Yeast and hyphal acid-soluble glucans from *C. albicans* contain fewer branch points (5.3% and 8.4%) and $\beta(1\rightarrow3)$ linkages (11.7% and 14.3%), respectively. NMR analysis clearly confirmed the presence of $\beta(1\rightarrow6)$ linkages. The complete absence of a ^{13}C -signal for $\beta(1\rightarrow3)$ linkages in the acid-soluble glucan suggests that these residues were in an immobilized or ordered state. Saito *et al.* (1977) have reported a similar situation for a 16200 molecular weight fraction derived from lentinan. This polymer consisted of a branched $(1\rightarrow3)$ - β -glucan with $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ side chains but it did not exhibit a ^{13}C -signal for $\beta(1\rightarrow3)$ linkages. The acid-soluble glucan from *C. albicans* may therefore consist of an ordered state $\beta(1\rightarrow3)$ backbone with $\beta(1\rightarrow6)$ side chains.

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