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Exo-1,3-β-glucanase Activity in *Candida albicans*: Effect of the Yeast-to-mycelium Transition

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Yeast cells of *Candida albicans* 1001 produced glucan-hydrolysing activity, most of which was due to an exo-1,3-β-glucanase. The enzyme was periplasmically located; it could be found in culture medium samples, and was secreted by protoplasts when cultured under regeneration conditions. In contrast to most yeast exoglucanases, this enzyme was practically inactive against *p*-nitrophenyl-β-D-glucoside, hydrolysis of this substrate being carried out by a β-glucosidase located inside the cytoplasmic membrane and not secreted to the external medium. Supernatant fluids from cell-free extracts reached their maximum glucanase level after several days at 0 °C, suggesting that the active enzyme was formed from an inactive precursor. Glucanase activity substantially decreased and sometimes disappeared from the cells when the yeast-to-mycelium transition was induced, but a significant (though lesser) reduction was also observed in yeast cells incubated in the same medium under conditions (temperature, cell concentration) that did not lead to formation of hyphae. It is suggested that *C. albicans* exo-1,3-β-glucanase may not be necessary for mycelial growth.

INTRODUCTION

Candida albicans can grow as an oval-shaped, unicellular yeast or as a filamentous fungus, when appropriate changes in the environment determine that yeast cells (blastospores) give rise to hyphae. Whereas much information exists regarding environmental factors that control the morphological transition in this yeast (Lee *et al.*, 1975; Manning & Mitchell, 1980; Shepherd *et al.*, 1980; Odds, 1985), data concerning biochemical changes involved in the differentiation process are scarce (Chiew *et al.*, 1980; Niimi *et al.*, 1980; Sullivan *et al.*, 1983) and approaches towards an understanding of the genetic basis of dimorphism in *C. albicans* have only begun (Pomés *et al.*, 1985; Hubbard *et al.*, 1986).

As in the case of many other fungi, glucan, with either 1,3-β- or 1,6-β-linkages, represents the main structural component of the cell wall of *C. albicans* (Sullivan *et al.*, 1983; Gopal *et al.*, 1984), and this species produces glucanases. *C. albicans* thus provides a model system for studying the properties of these cell-wall-degrading enzymes and their role in growth and morphogenesis. There are few reports on glucanases produced by *C. albicans* and the results are conflicting in some respects. Barrett-Bee *et al.* (1982) studied variations in glucanase levels during the growth cycle of *C. albicans*. Notario (1982) detected an endo-β-glucanase and an exo-β-glucanase (which also had β-glucosidase activity as it hydrolysed the synthetic derivative *p*-nitrophenyl-β-D-glucoside) in cell-free extracts, culture fluids and cell wall autolysates. On the other hand, Ram *et al.* (1984), without making a very thorough fractionation of cell-free extracts, presented evidence suggesting that *C. albicans* exo-β-glucanase and β-glucosidase are different enzymes.

In this report, we examine some aspects of the production, localization and activation of glucanase in *C. albicans* and changes in enzyme levels that occur as a result of the morphological transition.

Abbreviation: pNPG, *p*-nitrophenyl β-D-glucoside.

METHODS

Chemicals. Laminarin and *p*-nitrophenyl β-D-glucopyranoside (pNPG) were purchased from Sigma. Pustulan was obtained from Calbiochem-Behring and salicin from Merck. Sephadex G-200 was purchased from Pharmacia, DEAE-Biogel from Bio-Rad and glusulase from Dupont Pharmaceuticals. All other reagents were of analytical grade from commercial sources.

Organism, media and growth conditions. Candida albicans 1001, from the Spanish Type Culture Collection (CECT), was used throughout. Yeast cells were grown in YED medium (containing 15 g Difco yeast extract and 30 g glucose per litre), by shaking at 28 °C in an orbital incubator (Gallenkamp) and collected in the late-exponential phase, which was usually reached after approximately 15 h in this medium. Yeast cells were efficiently converted into mycelial forms (hyphae) by suspending them in YE + Pro medium (containing 1 g Difco yeast extract and 0·1 g proline per litre), at a concentration not higher than 5×10^6 cells ml^{-1}, and shaking at 37 °C. Under these conditions, a very high percentage of yeast cells gave rise to hyphae after 3–5 h, as monitored by microscopic counts using a Neubauer haemocytometer. Cell concentration or incubation temperature were modified, where indicated, to test for their effect on mycelium formation and other phenomena under study.

Protoplast preparation. Yeast cells were collected, washed twice with sterile distilled water and pretreated for 45 min at 30 °C by suspending them in 10 mM-Tris/HCl, pH 8·5, containing 5 mM-Na_2EDTA and 1% 2-mercaptoethanol. Pretreated cells were washed twice with 1 M-sorbitol and suspended in the same solution supplemented with a snail enzyme preparation (glusulase) (30 μl ml^{-1}). Approximately 2×10^8 cells were suspended per ml of lytic solution and the suspension was incubated for 60 min at 30 °C, with gentle shaking in a water bath, to achieve a protoplast yield of more than 95%. Protoplasts were finally washed in 50 mM-acetate, pH 5·3, containing 0·9 M-sorbitol and 10 mM-$MgSO_4$.

Enzyme preparations. The enzymes under study were assayed in supernatant fluids from cell-free extracts, obtained by mechanical breakage of the cells, in preparations obtained from protoplasts and in samples of culture fluid. These enzyme preparations were also analysed by various types of column chromatography.

For the preparation of extracts, yeast cells were harvested by low-speed centrifugation and hyphae were collected by filtration through Millipore filters (3 μm pore diameter); they were washed twice with 0·01 M-sodium succinate buffer, pH 5·3, and resuspended in the same buffer at a concentration of $2–5 \times 10^9$ cells ml^{-1}. Cell suspensions were homogenized with ballotini glass beads in a Braun homogenizer as described by Santos *et al.* (1979). Crude extracts were usually centrifuged at 1500 *g* to remove cell walls and debris, centrifuged again for 30 min at 48000 *g* to sediment membrane material, and the resulting supernatant fluid dialysed against the suspension buffer. Any precipitate that formed during dialysis was removed by another 30 min centrifugation at 48000 *g*. Where indicated, several variations were introduced in the procedures to clarify and dialyse the extracts, but in all cases a final 48000 *g* centrifugation for 30 min was carried out after dialysis before assay or chromatography.

In some cases, isolated cell walls were washed with buffer at the same concentration as that used for cell suspensions; they were centrifuged at 1500 *g* and then at 48000 *g*, the supernatant fluids being dialysed and used as an enzyme preparation.

To study the location of enzymes, cell-free extracts were obtained from cells treated with 0·1 M-HCl (at a concentration of $2–3 \times 10^8$ cells ml^{-1}) at 4 °C for 15 min. After the acid treatment, used to destroy periplasmic enzymes, the suspension was neutralized by addition of NaOH, the cells were washed twice with 0·01 M-sodium succinate buffer, pH 5·3, and the cell-free extracts were prepared as described above. Untreated cells were used as a control.

Protoplast lysates were obtained by suspending protoplasts in 0·01 M-sodium succinate buffer, pH 5·3, at a concentration of $1–2 \times 10^9$ protoplasts ml^{-1} and by homogenizing the lysed suspension in a Potter homogenizer. The preparation was centrifuged at 48000 *g* for 30 min, dialysed against the suspension buffer and recentrifuged at the same speed, if necessary for clarification and removal of precipitates.

Protoplast secretion products were obtained by incubating protoplasts, with gentle shaking, for 5 h at 28 °C in regeneration medium (10 g Difco yeast extract and 20 g glucose per litre, supplemented with 0·9 M-sorbitol and 10 mM-$MgSO_4$), at a concentration of 10^8 protoplasts per ml of medium. The protoplasts were then removed by centrifugation at 2000 *g* for 10 min, and the supernatant fluid was centrifuged at 48000 *g* for 30 min and dialysed against 0·01 M-sodium succinate buffer, pH 5·3.

Culture fluid samples, for enzyme studies, were prepared by centrifugation of cultures at a low speed and at 48000 *g* for 30 min, followed by dialysis of the supernatant fluids against 0·01 M-sodium succinate buffer, pH 5·3. When needed, preparations of protoplast secretion products or culture fluids were concentrated by dialysis against polyethylene glycol.

Enzyme assays. Assays of 1,3-β-glucanase and 1,6-β-glucanase were based on the release of reducing sugar from laminarin and pustulan respectively. Reducing sugar was determined by the method of Somogyi (1952) and Ashwell (1957). Determination of exoglucanase activity was based on the release of glucose residues, from laminarin, as measured with glucose oxidase coupled to peroxidase (Keston, 1956). Assay of β-glucosidase was

based on the release of *p*-nitrophenol from pNPG, measured as described previously (Santos *et al.*, 1979). Where indicated, β-glucosidase activity was also determined by quantifying the glucose (as reducing sugar) released from salicin.

In all cases reaction mixtures contained the enzyme preparation in a total of 250 µl 0·01 M-sodium succinate buffer, pH 5·3. The corresponding substrate was added to a final concentration of 0·25% (w/v) and incubations were performed at 37 °C for 30 min, unless indicated otherwise. The reaction was stopped by adding the reagents used for determination of the product released by the action of the enzyme (see above) and, when needed, removing the precipitated protein by low-speed centrifugation, the concentration of the reaction products being measured in the supernatant fluids. One unit of activity (U) was defined as the amount of enzyme which released 1 nmol of the measured reaction product min^{-1} under the conditions of the reaction. Protein was determined by the Lowry method, with bovine serum albumin as standard, or estimated spectrophotometrically by monitoring absorbance at 280 nm in column eluates. Specific activity was defined as units of enzyme activity $(mg\ protein)^{-1}$.

Column chromatography. Sephadex G-200 gel filtration was performed on a column (58 × 0·95 cm) previously equilibrated with 0·025 M-sodium succinate buffer, pH 5·3. Samples were eluted from the column with the same buffer at a flow rate of 2 ml h^{-1}, 1 ml fractions being collected. DEAE-Biogel A chromatography was carried out on a column (13 × 1·5 cm) previously equilibrated with 0·01 M-sodium succinate buffer, pH 5·3. The column was eluted at a flow rate of 12 ml h^{-1}, first with the initial buffer and then by applying a linear concentration gradient of NaCl (0–0·75 M) in the same buffer. Fractions of 2·5 ml were collected.

RESULTS

Production and activation of C. albicans β-glucanase

As shown by other workers (Notario, 1982; Ram *et al.*, 1984), *C. albicans* 1001 produced a measurable amount of laminarinase (1,3-β-glucanase) that was clearly detectable in cell extracts and culture fluids (Table 1). However, no activity against pustulan (1,6-β-glucan) could be detected in these preparations. 1,3-β-Glucanase was completely inactivated by treatment of the cells with 0·1 M-HCl, which is known to destroy periplasmic enzymes, and, consistently, it was not detected in protoplast lysates. However, protoplasts incubated under regeneration conditions were capable of secreting substantial amounts of glucanase (Table 1). Activation of the glucanase was observed when extracts were maintained at 0 °C (Table 1). The degree of activation varied in different experiments, as will be illustrated below, and it occurred only in cell extracts and not in culture supernatants or preparations of protoplast secretion products. The results suggested a periplasmic location for 1,3-β-glucanase in *C. albicans* and contrasted with those obtained for β-glucosidase (measured as pNPG-hydrolysing activity). The bulk of this activity was detected in protoplast lysates and was neither destroyed by acid treatment of the cells nor secreted by protoplasts (Table 1). Acid treatment destroyed a small proportion of pNPG-hydrolysing activity that was also secreted by protoplasts; this corresponded to the very reduced activity against this substrate of the major glucanase (see below), located in the periplasmic space.

Table 1. *Activity of 1,3-β-glucanase and β-glucosidase in different preparations from C. albicans*

The results correspond to preparations obtained from the same amount of cells (approximately 5·6 × 10^{10}) or their culture fluid at the time of collection. The enzymes were assayed after dialysing (fresh prep.) or after maintaining the preparations for 5 d at 0 °C (5 d prep.).

Type of preparation: supernatant fluid from	Total U (specific activity in parenthesis) of:			
	β-Glucosidase*		1,3-β-Glucanase†	
	Fresh prep.	5 d prep.	Fresh prep.	5 d prep.
Cell-free extracts	289·8 (2·3)	304·0 (2·4)	372·6 (2·9)	736·0 (5·8)
Extracts of cells treated with 0·1 M-HCl	243·6 (1·7)	260·2 (1·8)	0·0 (0·0)	0·0 (0·0)
Protoplast lysate	223·1 (2·3)	231·7 (2·4)	0·0 (0·0)	0·0 (0·0)
Culture medium	123·0 (0·6)	102·5 (0·5)	3034·0 (14·8)	2911·0 (14·2)
Protoplast secretion products	145·2 (1·1)	132·0 (1·0)	1372·8 (10·4)	1320·0 (10·0)

* Measured with pNPG as substrate. † Measured with laminarin as substrate.

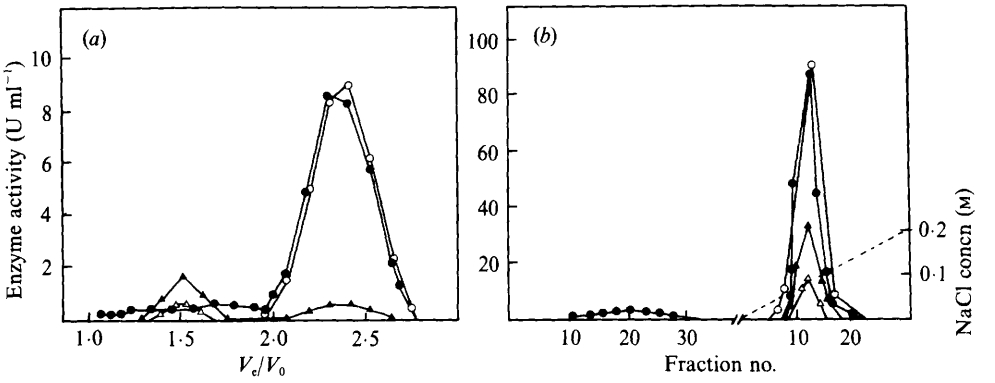

Fig. 1. Column chromatographic analysis of glucan- and pNPG-hydrolysing activities present in supernatant fluids from *C. albicans* cell-free extracts. (*a*) Sephadex G-200 gel filtration. (*b*) DEAE-Biogel ion-exchange chromatography. Activity against laminarin was determined in the fractions by measuring reducing sugars (●) or free glucose (○) released by the action of the enzymes. ▲, Activity against pNPG; △, activity against salicin; - - -, NaCl concentration in the elution gradient. V_e, elution volume. V_0, void volume.

Table 2. *Activity of 1,3-β-glucanase and β-glucosidase in C. albicans extracts prepared by different procedures of differential centrifugation and dialysis*

The cells were homogenized and the corresponding homogenates centrifuged at 1500 *g* (15 min) and then at either 10000 *g* (30 min) or 48000 *g* (30 min). Samples of crude homogenates or supernatant fluids of each of the three indicated centrifugation speeds and cell wall washes were dialysed overnight against the suspension buffer. Dialysed preparations were finally centrifuged at 48000 *g* for 30 min and assayed immediately (fresh prep.) or after maintaining them for 1 d at 0 °C (1 d prep.).

	U ml⁻¹ (specific activity in parenthesis) of:			
	β-Glucosidase*		1,3-β-Glucanase†	
Type of preparation dialysed	Fresh prep.	1 d prep.	Fresh prep.	1 d prep.
Expt 1				
Crude extract	10·1 (2·5)	9·7 (2·4)	18·4 (4·5)	20·3 (4·9)
Supernatant fluid from 1500 *g*	8·9 (2·0)	8·9 (2·0)	3·8 (0·9)	15·8 (3·6)
Supernatant fluid from 10000 *g*	7·8 (1·6)	7·6 (1·5)	0·0 (0·0)	11·8 (2·4)
Supernatant fluid from 48000 *g*	7·1 (1·4)	7·2 (1·4)	0·0 (0·0)	9·9 (1·9)
Expt 2				
Supernatant fluid from 48000 *g*	12·1 (1·2)	13·2 (1·3)	6·6 (0·7)	34·5 (3·4)
Cell wall wash	1·4 (1·5)	1·6 (1·7)	4·6 (4·9)	12·7 (13·6)

* Measured with pNPG as substrate. † Measured with laminarin as substrate.

The results of Table 2 further substantiated the activation of β-glucanase in extracts and the effect of the preparation procedure. The type of preparation dialysed, whether it was a crude homogenate or a supernatant fluid of centrifugation at different speeds, greatly influenced the level of soluble enzyme after dialysis. Dialysis of the whole homogenate, including cells walls and membrane material, determined the highest initial level of 1,3-β-glucanase and a low degree of activation, whereas removal of cell walls or membrane material before dialysis led to preparations with a low, or even undetectable, activity but with the highest activation potential since their activity substantially increased after 1 d at 0 °C. It was also clear that some of the glucanase adhered to cell walls and could be removed by washing the walls to obtain preparations with some activation potential (Table 2). Attempts to activate fresh extracts in a shorter time by incubating them at 25–37 °C were unsuccessful (data not shown). Again β-glucosidase in the same preparation behaved differently as it did not show any activation.

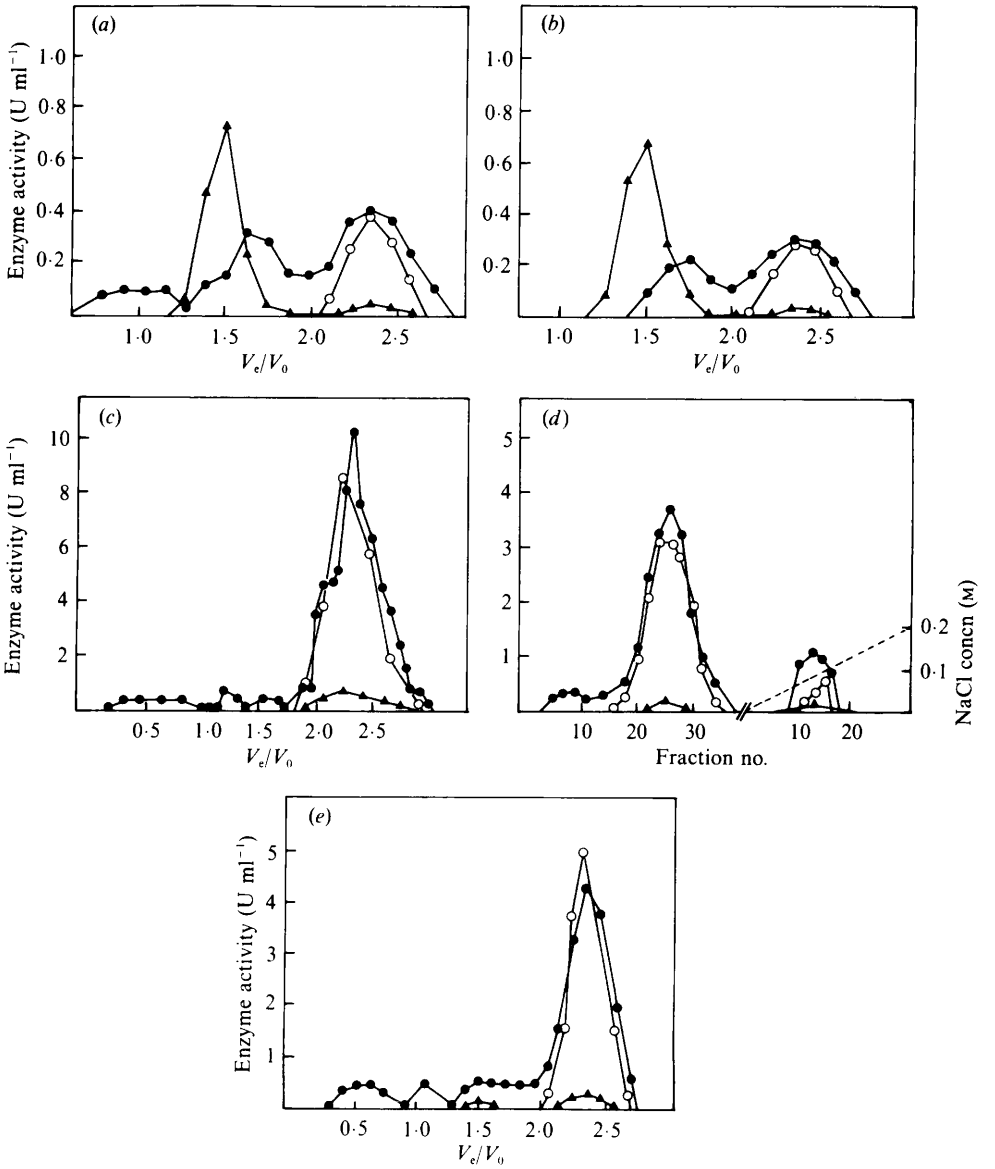

Fig. 2. Column chromatographic analysis of glucan- and pNPG-hydrolysing activities present in different types of sample from *C. albicans*: supernatant fluids from (*a*) extracts of cells treated with 0·1 M-HCl, (*b*) protoplast lysate, (*c*) and (*d*) culture medium, (*e*) protoplast secretion products. (*a*, *b*, *c*, *e*) Sephadex G-200 gel filtration; (*d*) DEAE-Biogel ion-exchange chromatography. Symbols as in Fig. 1. When necessary (*a*, *b*) the time of incubation was increased to 8 h to detect low activities undetectable under standard conditions of enzyme assay, such as those of Table 1.

Characteristics of C. albicans β-glucanase

To investigate the nature of the laminarin- and pNPG-hydrolysing activities, extracts were analysed by column chromatography (Fig. 1). The bulk of the 1,3-β-glucanase eluted in a peak whose activity against laminarin was the same when the products were measured as reducing sugar groups or as free glucose, suggesting that the most abundant 1,3-β-glucanase was an exo-hydrolysing enzyme. Exo-1,3-β-glucanases produced by yeasts are also considered to have β-glucosidase activity since they are usually as active against laminarin as against pNPG (Brock, 1965; Phaff, 1979). However, this was not confirmed in *C. albicans* 1001 since most of the β-glucosidase, measured with pNPG and salicin as substrates, eluted in a different and much less

Table 3. *Effect of media and conditions that determine mycelium formation on the levels of 1,3-β-glucanase and β-glucosidase*

Yeast cells were incubated as indicated, and collected for preparation of extracts and for microscopic examination to verify changes in cell morphology.

Time (h)	Temp. (°C)	Cell concn (cells ml^{-1})	Cell morphology	U ml^{-1} (specific activity in parenthesis) of: β-Glucosidase*	1,3-β-Glucanase†
Expt 1					
0	28	5 × 10^6	yeast	42·3 (25·1)	48·5 (28·8)
3	37	5 × 10^6	hypha	47·1 (38·0)	0·0 (0·0)
Expt 2					
0	28	5 × 10^6	yeast	7·7 (4·2)	27·9 (15·2)
1	37	5 × 10^6	yeast‡	16·0 (8·0)	0·8 (0·4)
3	37	5 × 10^6	hypha	55·0 (32·8)	0·0 (0·0)
Expt 3					
0	28	5 × 10^6	yeast	9·5 (6·4)	66·3 (44·3)
4	25	5 × 10^6	yeast	4·0 (6·5)	11·5 (18·5)
4	37	5 × 10^6	hypha	27·1 (13·6)	2·6 (1·3)
Expt 4					
0	28	5 × 10^6	yeast	3·8 (3·6)	25·8 (24·3)
3	25	5 × 10^6	yeast	2·6 (3·9)	5·5 (8·4)
3	37	5 × 10^6	hypha	9·4 (13·8)	2·4 (3·6)
3	37	5 × 10^7	yeast§	18·0 (9·1)	22·0 (11·1)
3	37	5 × 10^8	yeast	10·6 (5·5)	30·8 (15·9)

* Measured with pNPG as substrate.
† Measured with laminarin as substrate.
‡ Only 10% of cells had a short but distinguishable germ tube.
§ Only 5% of cells had germ tubes.

retarded peak (Fig. 1*a*), the main peak of 1,3-β-glucanase showing almost negligible pNPG hydrolysis. Exo-1,3-β-glucanase and β-glucosidase were retained by DEAE-Biogel and eluted at the same salt concentration (Fig. 1*b*). Both chromatographic procedures revealed the existence of a small amount of laminarinase, detectable only by assaying reducing sugar groups (an indication of an endohydrolytic mode of action) and with some chromatographic heterogeneity.

The chromatographic properties of β-glucanase and β-glucosidase present in other types of preparation were also examined. Gel filtration of extracts from 0·1 M-HCl-treated cells or of protoplast lysates revealed a peak of β-glucosidase which eluted in a position similar to that of extracts from untreated cells. β-Glucanase was undetectable in these preparations when assayed by the usual procedures (Table 1); however, when column fractions were assayed by incubating for 8 h, a small peak corresponding to the exo-1,3-β-glucanase of cell extracts, together with some other activity, could eventually be observed (Fig. 2*a*, *b*). Samples of culture fluids were devoid of β-glucosidase, but contained a significant level of β-glucanase; this activity eluted as the much retarded peak of exo-1,3-β-glucanase (Fig. 2*c*) observed in cell extracts. However, the charge of the enzyme presumably changes upon its release to the culture medium since most of it was not retained by the ion-exchanger (Fig. 2*d*). Finally, protoplast secretion products were also consistently devoid of β-glucosidase and displayed the major exo-1,3-β-glucanase peak when analysed by gel filtration (Fig. 2*e*).

Effect of mycelium formation of the level of glucanase

A substantial reduction in 1,3-β-glucanase, which became largely undetectable, accompanied the morphological transition from yeast cells to hyphae (Table 3). This was paralleled by an increase in the absolute level and specific activity of β-glucosidase. To ascertain the specificity of these enzyme changes with regard to morphogenesis, cells were incubated in the same medium but at a lower temperature or a higher concentration of yeast cells. Under these conditions hyphae did not develop but similar, although less marked, changes in enzymes were

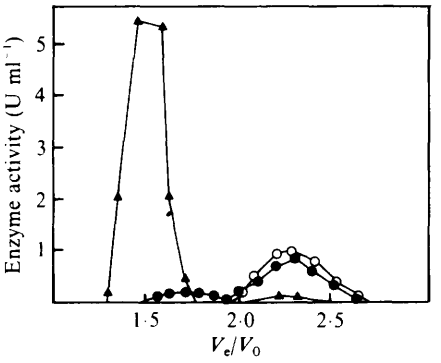

Fig. 3. Analysis by Sephadex G-200 gel filtration of glucan- and pNPG-hydrolysing activities present in supernatant fluids from cell-free extracts of *C. albicans* hyphae. Symbols as in Fig. 1. The time of incubation was 8 h.

observed. The specific activity of 1,3-β-glucanase was reduced and that of β-glucosidase increased substantially (Table 3). This suggested that most of the 1,3-β-glucanase produced by yeast cells was not necessary for mycelium development and that reduction in the glucanase level was not absolutely specific to morphogenesis but determined by the culture conditions themselves. Chromatographic analysis of mycelium extracts revealed the β-glucosidase peak and, when incubation assays were prolonged enough, a slight peak of exo-1,3-β-glucanase, with the same chromatographic pattern as the enzymes in extracts of yeast cells (Fig. 3).

The results regarding location of the enzymes, activation of glucanase in some types of samples, chromatographic properties of the enzymes and decrease or disappearance of glucanase upon the yeast-to-mycelium transition were reproducibly observed throughout several experiments. However, the absolute levels of glucanase varied between experiments, as is reflected in the data that are included; this was to be expected in a system which is activable upon storage.

DISCUSSION

Examination of published data concerning the 1,3-β-glucanase complement of yeasts with glucan in their cell walls does not reveal a unifying picture. Different species produce a multiplicity of enzymes with different properties and modes of action (exo- or endo-hydrolytic), suggesting that the putative role of these enzymes in the controlled modification of cell-wall structural components can be accomplished in various ways. In every species studied the production of more than one glucanase has been observed (Phaff, 1979; Villanueva *et al.*, 1979; Fleet, 1984). Exoglucanases, which hydrolyse the polymer at the chain ends, thus releasing glucose, are the most abundant glucan-hydrolysing enzymes in fungal species. They are usually almost as active on a synthetic glucoside, such as pNPG, as on 1,3-β-glucan itself (laminarin is the substrate used), and workers in the field tend to characterize as exoglucanases those enzymes that are shown to hydrolyse pNPG in addition to laminarin, to differentiate them from endoglucanases that are likewise active against laminarin (Brock, 1965; Phaff, 1979).

The evidence presented above clearly shows that to rely on pNPG hydrolysis for exoglucanase characterization is not of general value. *C. albicans* 1001 produced an exo-1,3-β-glucanase which was the most abundant glucanase in these cells, but which was clearly distinguishable from the enzyme (β-glucosidase) that hydrolysed pNPG and other glucosides such as salicin. Exo-1,3-β-glucanase was located in the periplasmic space, released into the culture medium by growing cells and secreted by protoplasts when cultured in regeneration medium. The activity of this enzyme on pNPG was almost negligible (4% of that on laminarin) but it could be characterized as an exoglucanase because its activity was the same when measured either as reducing sugar groups or as free glucose released by the enzyme. In contrast, the bulk of β-glucosidase (pNPG-hydrolysing activity) corresponded to a protein of higher M_r which was located intracytoplasmi-

cally and was not secreted into the periplasmic space or culture medium. These results are consistent with those of Ram *et al.* (1984) but they conflict with the observations of Notario (1982), who reported that the exo-hydrolysing enzyme in *C. albicans* 6406 (Gale, 1974) was significantly active on pNPG. We can only attribute these discrepancies to strain differences. Given the genetic complexity of *C. albicans* (Whelan & Soll, 1982) and the diversity among cell wall autolysins, these differences among strains are not surprising.

A very minor proportion of the glucanase activity in the cell corresponded to at least one other enzyme distinguishable from the exoglucanase. Although we have not concentrated on this particular enzyme, we obtained indications of an endohydrolytic mode of action. Both exo- and endoglucanases are considered to collaborate in the modification of cell wall structure in fungi (Johnson, 1968; Jones *et al.*, 1974) and these results suggest that in *C. albicans* both types of activity are also operative.

Indications regarding the formation and secretion of active exo-1,3-β-glucanase in *C. albicans* can be obtained from the observed activation of the enzyme in extracts but not in other types of preparation. Protoplast lysates or extracts from 0·1 M-HCl-treated cells were devoid of glucanase activity and only by very prolonged incubation of column fractions from the analysis of these preparations could a very small amount of the exoglucanase be detected. High-speed supernatant fluids from cell-free extracts were usually devoid of or very low in glucanase activity when assayed immediately after their preparation and dialysis. However, the extracts were activated by maintaining them at 0 °C for several days. These observations suggest that the active exo-1,3-β-glucanase is generated from an inactive precursor that is activated as a result of the secretion process, once in contact with whatever activator(s) is (are) needed, outside the cytoplasmic membrane. An activation of this type has been shown to occur with some bacterial autolysins, such as the amidase of *Streptococcus pneumoniae* (Tomasz & Westphal, 1971). We plan to test this hypothesis by isolating whatever precursor of exo-1,3-β-glucanase exists in the cytoplasm with the use of antibodies prepared against the enzyme (M. Molina & C. Nombela, unpublished). No further glucanase activation was observed in preparations, such as culture fluids or protoplast secretion products, that consisted of proteins that had been transported through the membrane. This is consistent with our hypothesis of activation of the glucanase precursor in the course of secretion. Column chromatography revealed a difference in charge, but not in apparent M_r between the active exo-1,3-β-glucanase located periplasmically and the same enzyme released into the culture medium. This difference does not seem to be related to the activation itself, but may indicate a modification of the active protein when it is released through the cell wall to the extracellular medium.

Finally, our results clearly showed a significant decrease in exo-1,3-β-glucanase upon the yeast-to-mycelium transition, indicating that the enzyme may not be necessary for mycelial growth. Under our conditions this was not paralleled by a significant increase of the enzyme in the medium (data not shown), but a similar although less intense diminution of enzyme levels occurred when yeast cells were incubated in filamentation medium but at cell concentrations or temperatures which did not permit hyphal development. The exoglucanase may thus not be necessary for mycelial growth. Whether its disappearance is due to specific inactivation or simply to lack of synthesis during mycelial growth remains to be established. The reduction in enzyme levels under similar conditions not leading to mycelium development illustrates the necessity of proper controls to clearly establish the specificity of biochemical changes that accompany morphogenesis in *C. albicans*.

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