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Protoplasts from Yeast and Mycelial Forms of *Candida albicans*

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Protoplasts have been obtained in high yields from the yeast and mycelial forms of a variety of strains of *Candida albicans* by enzymic digestion of cells with commercially available lytic enzymes. The protoplast formation procedure was equally effective for exponential and stationary phase cells. Pretreatment with dithiothreitol and Pronase in the presence of EDTA and Tris was necessary. Other thiol reagents and conditions did not release protoplasts from all the strains of *C. albicans* tested. Treatment with digestive juice of the snail *Helix pomatia* required the addition of chitinase for the release of protoplasts from most strains tested. Conditions for maximizing the yield of protoplasts and the activities of β-glucuronidase and chitinase were determined. Electron microscopy of *C. albicans* showed that the pretreatment conditions removed the outer layers and the treatment itself completely removed the inner layers of the cell wall. More than 90 % of the protoplasts produced by this method were viable as assessed by vital staining with Janus Green B.

INTRODUCTION

The opportunistic pathogen *Candida albicans* has a temperature- and/or nutrition-dependent dimorphism (Romano, 1966; Dabrowa *et al.*, 1970). Recently, simple methods have been developed for obtaining a synchronous pure yeast culture and for the semi-synchronous induction of germ tubes in an entire cell population (Chaffin & Sogin, 1976; Soll & Bedell, 1978). In order to carry out biochemical and transcriptional studies on the mechanism of the conversion, it was necessary to develop a procedure for producing protoplasts that was equally effective for the yeast and mycelial forms as well as for exponential and stationary phase cells.

The cell wall of the yeast form of *C. albicans* is a complex structure composed of a highly-branched glucan (Yu *et al.*, 1967*a*), part of which is associated with chitin (Domanski & Miller, 1968; Chattaway *et al.*, 1976) and mannans which are firmly bound to protein (Yu *et al.*, 1967*b*). The wall of the mycelial form has a similar structure, but the wall thickness and the polysaccharide and protein composition are qualitatively and/or quantitatively different (Chattaway *et al.*, 1968, 1976; Cassone *et al.*, 1973). Protoplasts or spheroplasts from the yeast form of *C. albicans* have been obtained by degrading the wall with enzyme mixtures from either the gut of *Helix pomatia* (Kobayashi *et al.*, 1964) or micro-organisms (Domanski & Miller, 1968). Spheroplasts from the mycelial form have also been obtained (Kerridge *et al.*, 1976).

The sensitivity of yeast cells to protoplast formation varies from strain to strain (Partridge & Drewe, 1974; Schwencke *et al.*, 1977) and is highly dependent on the physiological state of the yeast (Brown, 1971; Deutch & Parry, 1974). Pretreatment of cells with certain thiol compounds and proteases has been reported to improve the efficiency of protoplast forma-

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tion from stationary phase yeast cells (Russell *et al.*, 1973; Schwencke *et al.*, 1977), but rapid and complete conversion of stationary phase yeast and mycelial forms from different strains of *C. albicans* has not been achieved. We describe here a simple and rapid method by which cells from a variety of strains, and at a different stage of the *C. albicans* cell cycle, can be completely and efficiently converted into protoplasts with commercial lytic enzymes.

METHODS

Nomenclature. The term protoplast is used to denote an osmotically sensitive cell only when it has been established that the entire cell wall has been removed. However, a cell may be osmotically fragile even when some degraded wall material surrounds the cytoplasm; in this case the term spheroplast is used (Partridge & Drewe, 1974). The germ tube outgrowth from a yeast cell is considered a mycelium after the formation of the first true septum.

Organisms. The five strains of *Candida albicans* used in these studies were obtained from Dr Jeffrey Becker, University of Tennessee, Knoxville, Tenn., U.S.A., and are listed in Tables 1 and 2. The strains were confirmed as *C. albicans* by standard tests (Silva-Hunter & Cooper, 1974). Stock cultures were maintained on Sabouraud agar Modified (Difco) at 4 °C after 48 h growth at 25 °C. Working cultures were prepared from stocks weekly on Sabouraud agar. The stocks were transferred monthly and cloned every 4 months.

Cultivation of yeast form. Yeast cells from Sabouraud agar cultures were transferred to the amino acid synthetic medium described by Lee *et al.* (1975) and allowed to grow for 12 h at 25 °C with rotary agitation at 200 rev. min^{-1}. These cultures were diluted with fresh Lee's medium to $0{\cdot}5 \times 10^7$ to $1{\cdot}0 \times 10^7$ cells ml^{-1} and incubated at 25 °C as above. Starved cells were taken from the mid-stationary phase (see Results) and resuspended in distilled water for 24 h at 25 °C with agitation.

For some experiments, yeasts were grown in rich medium [1 % (w/v) yeast extract, 2 % (w/v) Difco Bacto-peptone, 2 % (w/v) glucose] at 25 °C with agitation for 12 h in the case of exponentially grown cells and 34 h for stationary phase cells.

Induction of germ tubes and mycelial growth. Germ tubes were induced by suspending stationary phase cells (Chaffin & Sogin, 1976; L. J. Torres, unpublished results) at $0{\cdot}5 \times 10^7$ to $1{\cdot}0 \times 10^7$ cells ml^{-1} at 37 °C in prewarmed Lee's medium with rotary agitation at 200 to 250 rev. min^{-1}. After 4 h, more than 90 % of the yeasts had germ tubes (10 to 35 μm). Cultures at 4 h, 9 to 12 h, and 22 to 24 h after germ tube induction were used for protoplast formation. Blastospores were separated from mycelia by extensive filtration (Chattaway *et al.*, 1973). The percentage of mycelia after induction of yeast cultures was determined as described by Land *et al.* (1975).

Preparation of cells for protoplast formation. Cells were harvested by centrifuging, washed twice with distilled water, then resuspended in one of the four pretreatment media under the conditions described in Table 1 (Partridge & Drewe, 1974; Schwencke *et al.*, 1977). The cells were incubated at 32 °C with gentle agitation (100 rev. min^{-1}), usually for 60 to 70 min (see Table 1), then harvested by centrifugation and washed once with distilled water.

Treatment. Cells recovered from 0·5 or 0·75 M-sodium thioglycollate medium were washed with 1 M-$MgSO_4$/0·05 M-K_2HPO_4 titrated to pH 4·1 with solid citric acid (Partridge & Drewe, 1974). The washed cells were resuspended at 200 mg (wet wt) ml^{-1} in fresh solution containing 10000 Fishman units of β-glucuronidase ml^{-1} (Fishman & Bernfeld, 1955). Cells recovered from 50 mM-dithiothreitol medium or 0·2 M-mercaptoethanol medium were washed once with 0·6 M-KCl (or 1 M-sorbitol for some experiments) and then resuspended in fresh solution under the same conditions as above.

For experiments in which chitinase was added, the 0·6 M-KCl was buffered with 0·04 M-K_2HPO_4 titrated to pH 6·0 with concentrated HCl, and the washed cells were resuspended under the same conditions as above with the addition of 0·25 mg chitinase ml^{-1} [Calbiochem, sp.act. 0·4 mg glucose equivalent release (mg enzyme)$^{-1}$] and made to 7 mM-mercaptoethanol. Snail enzymes and chitinase were dissolved in the same medium before addition.

Measure of protoplast formation. Samples of the digested suspension were examined at 15 to 30 min intervals for 3 h. The samples were halved and each half was diluted sixfold with either distilled water or stabilizing buffer. After 10 min, the number of osmotically sensitive cells was determined by differential cell counts using a haemocytometer. The numbers of osmotically sensitive cells and of true protoplasts (as judged by phase contrast microscopy) were expressed as percentages of the total cell count. Agreement between the two methods and replicate trials was usually within 10 % and was expressed as a range (e.g. 82 %, 88 % were scored as 80 to 90 %).

In the case of mycelium treated with snail enzymes and chitinase, complete degradation was scored as 100 % protoplast formation. Electron microscopy (see below) was used to confirm the absence of cell wall from the protoplasts.

Purification and stability of protoplasts. Protoplasts were purified by washing with stabilizing buffer and then with 1 M-sorbitol, or as described by Schwencke *et al.* (1977). The purified protoplasts could be stored in the medium described by Schwencke *et al.* (1977), modified to raise the KCl concentration to 0·4 M. Viability of intact cells was assessed by vital staining with 0·1 % (w/v) Janus Green B in 1 M-sorbitol (Partridge & Drewe, 1974).

Enzyme assays. β-Glucuronidase activity of snail enzymes was estimated from the hydrolysis of phenolphthalein glucuronide according to the directions of the manufacturer, except as noted. One unit of β-glucuronidase is defined as the quantity of enzyme required to hydrolyse 1·0 μg phenolphthalein from phenolphthalein glucuronide h^{-1} at 32 °C, under the above conditions.

Chitinase activity on colloidal chitin (Berger & Reynolds, 1958) was determined by the release of *N*-acetylglucosamine (Reissig *et al.*, 1955). Reaction mixtures (1 ml) contained 0·6 M-KCl, 0·04 M-K_2HPO_4/HCl buffer, pH 6·0, and 10000 units β-glucuronidase, with or without 7 mM-mercaptoethanol, as well as 5 mg colloidal chitin and 250 μg chitinase [Calbiochem, sp.act. 0·4 mg glucose equivalent release (mg enzyme)$^{-1}$]. The reaction was stopped after 1 h by heating at 100 °C for 10 min. A reaction mixture in 0·04 M-K_2HPO_4/HCl buffer, pH 6·0, was the positive control and the negative control contained 0·04 M-K_2HPO_4/HCl buffer, pH 6·0, 10000 units of β-glucuronidase and no chitinase. One unit of chitinase was defined as that which produced 1 μg *N*-acetylglucosamine h^{-1} under the above conditions.

Protease activity was determined from the hydrolysis of 2·5 % (w/v) casein (in 2 ml), essentially as described by Chattaway *et al.* (1976). A reaction mixture containing casein in 0·1 M-Tris (pH 7·5) was the positive control. One unit of protease was defined as that which produced a unitary increase in A_{280} h^{-1}.

Electron microscopy. Candida albicans cells were fixed in 100 mM-Na_2HPO_4/NaH_2PO_4 buffer, pH 7·4, containing 3 % (w/v) glutaraldehyde and 0·25 M-sucrose (1 h, room temperature). They were then rinsed in the same sucrose buffer and post-fixed in sucrose buffer solution containing 2 % (w/v) osmium tetroxide (1 h, room temperature). The cells were dehydrated through a series of ethyl alcohol washes from 30 to 100 % (v/v), embedded in Epon 812 and sectioned on a Sorvall Ultramicrotome MT-1. The silver to pale-gold sections were picked up on copper grids and examined with a RCA EMU-4 electron microscope.

Chemicals and enzymes. Snail enzymes (glusulase) were obtained from Endo Laboratories, Garden City, N.Y., U.S.A. Pronase, chitinase and mercaptoethanol were from Calbiochem, while dithiothreitol, chitin (practical grade), phenolphthalein, phenolphthalein glucuronide and *N*-acetylglucosamine were from Sigma. All other chemicals were standard reagent grade.

RESULTS AND DISCUSSION

Growth kinetics of yeast form

At 25 °C, the major experimental strain, *C. albicans* H317, grew exclusively in the budding yeast form in both Lee's medium and in rich medium. After 6 to 14 h in Lee's medium, cultures contained cells in the exponential phase; at 18 to 24 h, cells were in the early-stationary phase; at 26 to 36 h, cells were in the mid-stationary phase; and at 38 to 72 h, cells were in late-stationary phase (less than 10 % budding cells after 4·2 generations). Similar growth kinetics in Lee's medium were obtained for strains H350 and 4W. All strains grown in rich medium were in the exponential phase of growth for at least 12 h and reached stationary phase before 34 h.

Induction of germ tube and mycelial growth

Chaffin & Sogin (1976) and Soll & Bedell (1978) demonstrated that cells of a stationary phase culture of *C. albicans* grown in Lee's medium could be induced to form germ tubes and develop into mycelia by dilution in prewarmed growth medium followed by incubation at 37 °C. After 3 h of induction of stationary phase yeast cultures, the germ tube formed a true septum (Figs 1*a* and 2*c*) and mycelial growth developed in cell cultures. However, depending on the strain and on pre-induction conditions, hyphal growth could be characterized either as lengthening accompanied by the production of secondary blastospores, or as lengthening essentially free of blastospore production for at least 24 h (L. J. Torres, unpublished results). The range of number of septa for the 4 h mycelium was 1 to 2, for the 8 h mycelium from 2 to 7, and for the 24 h mycelium from 4 to 10. Although the percentage of mycelia produced after 4 h varied from strain to strain, the kinetics of mycelial induction were similar for all strains.

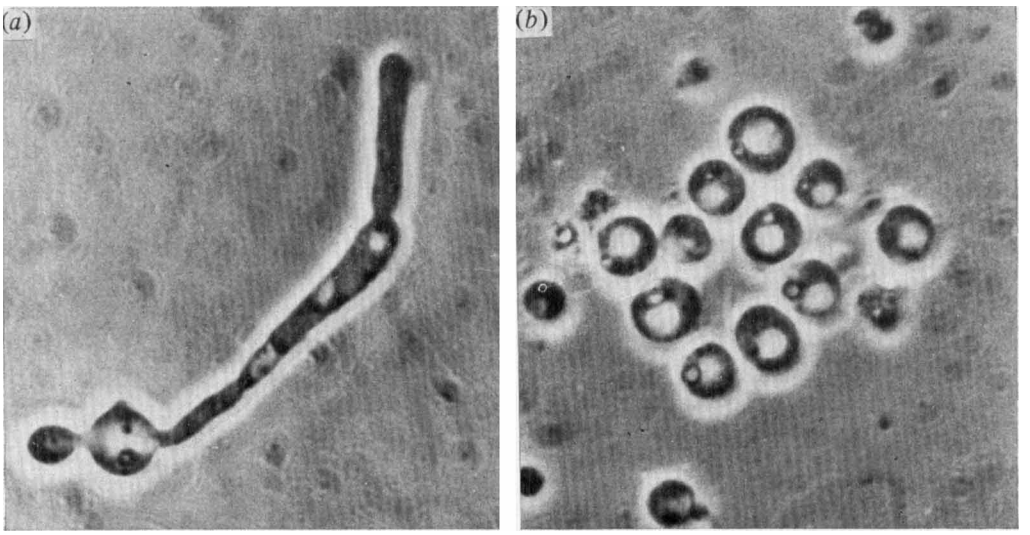

Fig. 1. Phase contrast micrographs of *C. albicans* H317: (*a*) mycelial form from a 5 h culture – a secondary blastospore can be seen on the mother cell; (*b*) yeast protoplasts from a stationary phase culture.

Effects of pretreatment media on protoplast formation

The effect of three thiol reagents in alkaline conditions on protoplast formation was tested (Table 1). Dithiothreitol in the presence of EDTA, Tris and 1 mg Pronase ml^{-1} (Schwencke *et al.*, 1977) gave the best results at high cell concentration and short incubation times during pretreatment. Incubation times for pretreatment and treatment longer than those shown in Table 1 did not change the results significantly. Higher concentrations of sodium thioglycollate (0·5 and 0·75 M) and mercaptoethanol (0·2 M) were less effective than lower concentrations (50 mM) of dithiothreitol. In both mercaptoethanol and sodium thioglycollate media (results not shown) there was a decrease in the percentage of protoplast formation with increased cell concentration in the range 20 to 200 mg (wet wt) ml^{-1}, while the percentage of protoplast formation in dithiothreitol medium was not affected in this range of cell concentration. When EDTA was omitted from dithiothreitol medium, there was a sharp drop in protoplast formation with strain H317. Moreover, cells grown in rich medium required an additional wash with 5 mM-EDTA (pH 7·5) before pretreatment to obtain results similar to those obtained in defined medium. The presence of Pronase in the dithiothreitol medium for stationary phase cells proved to be indispensable for complete protoplast formation and, in general, accelerated the process with all the strains tested (Tables 1 and 2). In summary, all the components of dithiothreitol medium were necessary for protoplast formation in *C. albicans* (see also Schwencke *et al.*, 1969).

Table 1 also shows the effect of growth phase and strain variation on subsequent protoplast formation. Stationary phase cells were more resistant to attack by snail enzymes and stationary phase cultures of strain H317 were resistant to complete conversion under the conditions of Table 1.

We recommend dithiothreitol as the thiol of choice in protoplast formation from *C. albicans* because of: (i) its effectiveness at high cell densities (Table 1); (ii) its ability to effect complete removal of the outer layers from strain H317 (Fig. 2*a*, *c*); and (iii) its effectiveness with stationary phase yeasts and mycelium of several strains (Table 2). Observations by other workers using dithiothreitol with a variety of yeasts support these conclusions (Sommer & Lewis, 1971; Schwencke *et al.*, 1977). The enhancing effect of Pronase may

Table 1. *Effect of different pretreatment media on protoplast formation in C. albicans*

Pretreatment medium	Strain	Growth phase*	Pretreatment cell concn [mg (wet wt) ml^{-1}]	Incubation time (min)		Percentage conversion (range)
				Pretreatment	Treatment†	
0·5 M-Sodium thioglycollate in 0·1 M-Tris, pH 9·3	IV	Exponential	30–60	30	100–120	70–80
		Early- and mid-stationary	10–20	30	120	50–70
0·75 M-Sodium thioglycollate in 0·1 M-Tris, pH 9·3	IV	Mid-stationary	10–20	60	120	75–80
50 mM-Dithiothreitol + 5 mM-Na_2EDTA + 0·1 M-Tris, pH 8·9 + 1 mg Pronase ml^{-1}	IV	Exponential	200	30–60	60–100	100
		Early- and mid-stationary	200	60–70	60–120	95–100
	H317	Exponential	200	60–70	120	95–100
		Early- and mid-stationary	200	60–70	120	70–85
0·2 M-Mercaptoethanol + 5 mM-Na_2EDTA + 0·1 M-Tris, pH 8·9 + 1 mg Pronase ml^{-1}	H317	Mid-stationary	200	70	120	45–60

* See Results for a definition of each growth phase in Lee's medium.

† Washed cells were resuspended at 200 mg (dry wt) ml^{-1} (about $0·5 \times 10^{10}$ to $1·0 \times 10^{10}$ cells ml^{-1}) in medium containing 10000 units β-glucuronidase ml^{-1}.

indicate that quantitative and/or qualitative differences occur in the outer mannan–protein complexes of the cell wall with different strains and during stationary phase conditions (Table 2). The results of Deutch & Parry (1974) with inhibitors of RNA and protein synthesis suggest the same conclusion for stationary phase cells.

Enzyme activities under different conditions

The proteolytic activity of Pronase was not significantly affected by the dithiothreitol pretreatment medium. However, the enzyme could not be stored under these conditions for more than 2 d at 4 °C without loss of activity.

β-Glucuronidase activity in phosphate buffer (pH 6·0) with 0·6 M-KCl and with or without mercaptoethanol (7 or 30 mM) was compared with activity in two unbuffered osmotic stabilizers (0·6 M-KCl and 1 M-sorbitol) and with activity in 0·1 M-sodium acetate, pH 5·0, as a positive control. None of the conditions used yielded activity as high as that of the control, but 50 to 70 % of the control activity was obtained with either 0·6 M-KCl or 1 M-sorbitol as the osmotic stabilizer. The presence of 0·04 M-phosphate buffered at pH 6 or of mercaptoethanol had negligible effects.

The snail lytic enzymes in the negative control showed a small amount of chitinase activity on colloidal chitin. However, when the same amount of snail enzymes was added to the treatment conditions (see Methods), the chitinase activity was approximately twice that of the positive control. The presence of 7 mM-mercaptoethanol in the treatment conditions had negligible effect on chitinase activity. The increase of chitinase activity detected with the addition of snail lytic enzymes was probably a chitobiase-like activity of these enzymes. The commercial preparation of chitinase has little chitobiase activity (Jeuniaux,

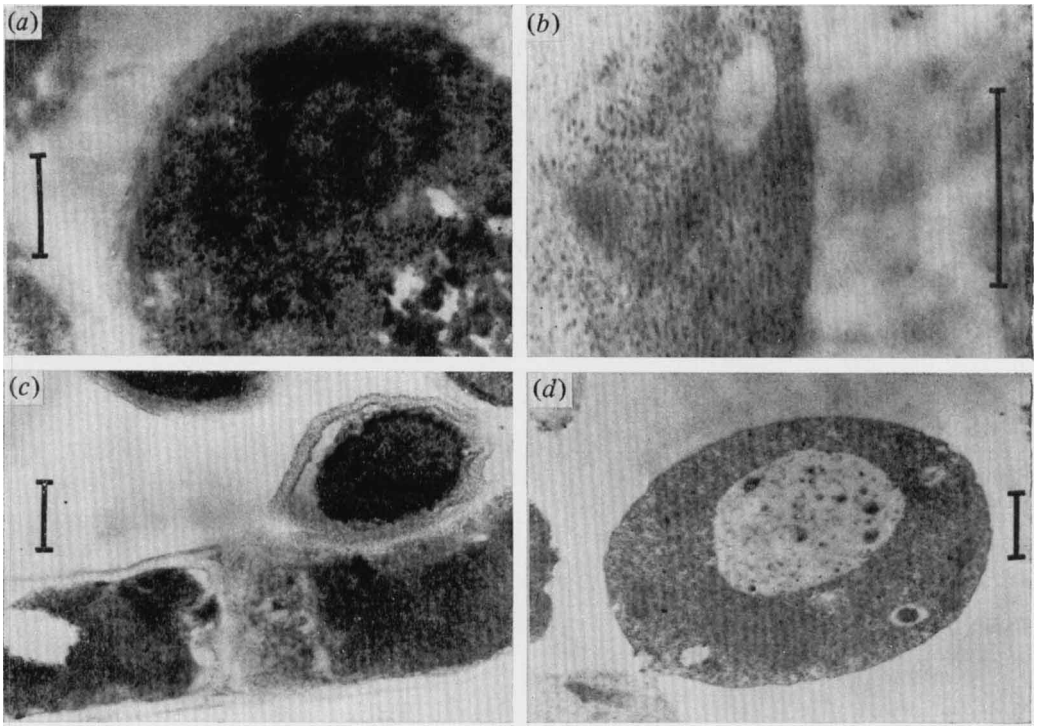

Fig. 2. Electron micrographs showing the effect of pretreatment with dithiothreitol medium and subsequent treatment with snail enzymes and chitinase on yeast and mycelium of *C. albicans* H317: (*a*) yeast cell from a stationary phase culture after pretreatment; (*b*) yeast protoplast from a stationary phase culture after pretreatment and treatment; (*c*) mycelium from a 22 h culture after pretreatment; (*d*) mycelial protoplast from a 22 h culture after pretreatment and treatment. Bar markers represent 0·8 μm.

1966) and the colorimetric method of Reissig *et al.* (1955) measures only the amount of *N*-acetylglucosamine.

Electron microscopy of C. albicans cells

Stationary phase yeast cells pretreated with dithiothreitol medium showed only the inner electron-transparent layers and the innermost layers of the cell wall (Fig. 2*a*). The outer electron-dense layers described by Cassone *et al.* (1973) (layers 1, 2 and 3) and Poulain *et al.* (1978) (layers 1, 2, 3 and 4 of this scheme) were completely removed following pretreatment with dithiothreitol medium. After pretreatment and treatment with snail enzymes and chitinase, the inner layers of the cell wall were completely removed (Fig. 2*b*).

Following pretreatment of 22 h mycelium with dithiothreitol medium, the outer electron-dense layers of the cell wall (Cassone *et al.*, 1973; Scherwitz *et al.*, 1978) were also completely removed (Fig. 2*c*). The same effect was seen on a growing secondary blastospore on the apical zone of the mycelium (Fig. 2*c*). After treatment with chitinase and snail enzymes, the protoplasts were released from the hyphae and the cytoplasmic membrane became the external barrier (Fig. 2*d*).

Protoplast formation has been assayed under mild conditions (dilution in water; Chattaway *et al.*, 1976) and with membrane-disruptive agents (Kobayashi *et al.*, 1964; Bhattacharya & Datta, 1977). We found that the assay using only dilution in distilled water agrees more closely with observations by phase contrast microscopy and with electron microscopy (Figs 1*b* and 2*b*, *d*) than does the assay with membrane disruptive agents.

Table 2. *Protoplast formation from C. albicans by the action of chitinase and snail enzymes as a function of strain variation, growth form and growth phase*

Cells were pretreated for 70 min in medium containing 5 mM-EDTA, 50 mM-dithiothreitol, 0·1 M-Tris, pH 8·9, and 1 mg Pronase ml^{-1}. They were then washed and resuspended at 200 mg (wet wt) ml^{-1} in medium containing 0·6 M-KCl, 0·04 M-phosphate buffer, pH 6·0, and 7 mM-mercaptoethanol together with 0·25 mg chitinase ml^{-1} and 10000 units β-glucuronidase ml^{-1}.

Strain	Growth form	Growth phase* (yeast form)	Incubation time for 95–100 % conversion (min)
H317	Yeast	Exponential	60–90
	Yeast	Early- to mid-stationary	90–120
	Yeast	Late-stationary	100–130
	Yeast	Starved cells	120
	Mycelium (4 h)		60–90
	Mycelium (12 h)		60–90
	Mycelium (24 h)		70–90
4W	Yeast	Exponential	120
	Yeast	Mid-stationary	120
	Mycelium (4 h)		60
WD-18-4 (Lys^- Met^-)	Yeast	Exponential	90
H350	Yeast	Exponential and early-stationary	90

* See Results for a definition of each growth phase in Lee's medium.

Treatment conditions

Failure to obtain complete conversion of yeast stationary phase cultures to protoplasts (Table 1) after the removal of the outer layers of the cell wall (Fig. 2*a*; Cassone *et al.*, 1978) suggested an inability of snail enzymes to digest an important structural component of the cell wall. Studies by Chattaway *et al.* (1968, 1976) and others (Domanski & Miller, 1968; Cassone *et al.*, 1973) with the cell wall of *C. albicans* have shown that chitin is an important component. As described above, the commercial preparation of snail enzymes had a weak chitinase activity. Using four strains of *C. albicans* at different stages of the life-cycle, we tested the effect of chitinase in concert with snail enzymes on protoplast formation. All cells were effectively converted to protoplasts within 2 h, but variations in the incubation time required were observed (Table 2). This treatment effectively removed the inner layers of *C. albicans* cell wall (Fig. 2*b*, *d*). Our results confirmed the observation of Chattaway *et al.* (1976) that the inner layer alone maintains the rigidity of the cell wall (Fig. 2*a*, *c*) and that chitin is an important component in the structure of the inner layers. In general, the inclusion of a small amount of a thiol reagent (mercaptoethanol) in the treatment medium accelerated the process of protoplast formation in *C. albicans*.

Yield and viability of protoplasts

The yield of protoplasts by cell count varied from 70 to 95 % depending mainly on the phase of growth of the yeast culture. Protoplast production was more efficient in 0·6 M-KCl than in 1 M-sorbitol, as judged by yield. Starved cells and late-stationary phase cells gave the lowest recovery of protoplasts. Approximately 95 % of the protoplasts produced by this procedure were viable and could be stored successfully for several days (see Methods). A high percentage of the protoplasts produced by this procedure could regenerate the cell wall under the conditions described by Ota (1972).

Conditions for C. albicans protoplast formation

Under the experimental conditions employed, all *C. albicans* strains treated with commercial snail enzymes and chitinase produced protoplasts efficiently. The two-step procedure for protoplast/spheroplast formation in yeast (reviewed by Wiley, 1974) proved to be indispensable for rapid production of protoplasts in *C. albicans*. Optimal conditions for the removal of the outer layers of the cell wall required alkaline pH, while the removal of inner layers by chitinase and snail enzymes required acidic pH. The use of a one-step procedure for protoplast/spheroplast formation in *C. albicans* by other workers (Ota, 1972; Chattaway *et al.*, 1976) had required prolonged incubation with similar reagents.

This simple and rapid procedure for the production of protoplasts from *C. albicans* at any stage of its life-cycle will assist the cell biologist studying the role of the stationary phase yeast cell during morphogenesis. Also, protoplasts will facilitate studies on the genome organization and expression of both morphological forms. These topics are now under investigation in our laboratory.

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