

## Chitin Synthesis in *Candida albicans*: Comparison of Yeast and Hyphal Forms

PHYLLIS CELLINI BRAUN\* AND R. A. CALDERONE

Department of Microbiology, Georgetown University, Schools of Medicine and Dentistry, Washington, D.C. 20007

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Chitin synthesis was studied in both yeast and hyphae of the dimorphic fungus *Candida albicans*. Incorporation of *N*-acetyl-D-[1-<sup>3</sup>H]glucosamine ([<sup>3</sup>H]GluNAc) into an acid-alkali-insoluble fraction was 10 times greater in hyphal-phase cells. A crude preparation of chitin synthetase was obtained from sonically treated protoplasts of both forms of *Candida*. Enzyme activity, which was determined by using [<sup>14</sup>C]UDP-GluNAc as a substrate, was exclusively associated with the 80,000 × *g* pellet from sonically treated protoplasts of both forms. It was determined that enzyme activity (nanomoles of [<sup>14</sup>C]UDP-GluNAc incorporated per milligram of protein) was approximately 2 times greater in hyphae versus yeast cells. Enzyme activity in both yeast and hyphae increased six- to sevenfold when the enzyme preparations were preincubated with trypsin. A vacuolar fraction, obtained from yeast cells but not from hyphae, stimulated enzyme activity when incubated with either yeast or hyphal enzyme preparations. Membrane fractions from protoplasts coated with [<sup>3</sup>H]concanavalin A before disruption were isolated by Renografin density gradient centrifugation. Chitin synthetase activity was preferentially associated with the concanavalin A-labeled fraction, suggesting that the enzyme was located on the plasma membrane. In addition, enzyme activity in protoplasts treated with cold glutaraldehyde before disruption was significantly greater than in protoplasts that were sonically disrupted and then treated with cold glutaraldehyde, indicating that the enzyme resides on the inner side of the plasma membrane.

Differentiation from yeast to hyphal growth by dimorphic fungi is accompanied by significant changes in the chemical composition of the newly formed cell wall. For instance, with *Histoplasma capsulatum* Darling and *Blastomyces dermatitidis* Gilchrist and Stokes, mycelial-phase cell walls have less chitin, higher proportions of mannose, and a lower content of ethylenediamine-soluble material than yeast-phase cells (8). In *Candida albicans* (Robin) Berkhout, Chattaway et al. (7) have shown that an alkali-insoluble fraction from mycelial-phase cells contained 3 times as much chitin as that from yeast-phase cells. Other quantitative differences in protein and carbohydrate components in alkali-soluble fractions were observed.

The regulation of these events, especially chitin synthesis, in dimorphic fungi has not been characterized. In the yeast *Saccharomyces cerevisiae* (Hansen) Meyen, regulation of chitin synthesis has been described in an elegant series of experiments by Cabib et al. (3-6) and by Duran et al. (9). In that system, chitin synthetase zymogen is attached to the yeast plasma

membrane and is activated by a protease contained in a vacuolar fraction from *S. cerevisiae*. This compartmentalization of enzyme and activating factor (protease) may account for the localized activation of the zymogen at the site of primary septum formation in budding cells. A third component of the system, a heat-stable protein found in the cytosol, acts as an inhibitor of the activating factor and may function as a regulatory factor by inactivating the vacuolar protease that spills into the cytoplasm.

The purpose of this study was to characterize chitin synthetase from both yeast and hyphal forms of *C. albicans*. We have found similarities to the *S. cerevisiae* system described by Cabib (3-6) although chitin formation and enzyme activity are much greater in hyphae.

### MATERIALS AND METHODS

**Cultivation of organism.** The isolate of *C. albicans* used in this study has been described previously (13). Cloned stock cultures, which were transferred monthly, were grown at 37°C and maintained at 4°C on brain heart infusion slants. The inoculum for all

experiments was obtained in the following manner: *C. albicans* was grown at 37°C for 18 h on brain heart infusion agar plates. Yeast cells were collected in 0.9% saline (wt/vol), centrifuged (3,000 × *g*, 4°C), washed twice, and then harvested and suspended in 0.9% saline. Approximately 0.5 g (wet weight) of yeast or hyphae (mycelium) was obtained by culturing the organism in either Sabouraud-dextrose broth (yeast) or a synthetic medium (hyphae) consisting of 0.01 M glucose, 0.02 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 9.0 mM KH<sub>2</sub>PO<sub>4</sub>, 0.2 mM MgSO<sub>4</sub>, 0.7 mM MgCl<sub>2</sub>, 250 μg of biotin per liter, 10% rabbit serum (vol/vol), and deionized water. Cultures were grown in 70 ml of medium at 37°C in shake culture (150 rpm) for approximately 4 h to obtain the desired wet weight. After incubation, all cultures were centrifuged, washed twice with saline, and suspended in saline.

**Uptake and incorporation of [<sup>3</sup>H]GluNAc.** Hyphal and yeast suspensions (20 mg [dry weight]/10 ml of saline) were incubated with 55 μCi of *N*-acetyl-D-[<sup>3</sup>H]glucosamine ([<sup>3</sup>H]GluNAc; specific activity, 5 Ci/mmol, New England Nuclear Corp.) at 37°C in shake culture (0 to 30 min). To measure uptake, duplicate cell suspensions (0.5 ml) were removed at designated time intervals and collected on glass fiber filters (Whatman GF/A) presoaked with cold GluNAc. For incorporation studies, duplicate cell suspensions (0.5 ml) were removed at designated times and precipitated on ice with cold 15% trichloroacetic acid. Subsequently, all trichloroacetic acid-treated cell suspensions were filtered onto glass fiber filters and washed with 5% trichloroacetic acid and 95% ethanol. Nonspecific trapping of radiolabel on filters was measured as described previously (13).

All radioactive measurements were made in an Intertechnique liquid scintillation counter in a scintillation liquid of the following composition: 0.1 g of *p*-bis[2-(5-phenyloxazolyl)]-benzene and 5 g of 2,5-diphenyloxazole dissolved in 1.0 liter of scintillation-grade toluene.

**Autoradiography.** Cells were incubated with 10 μCi of [<sup>3</sup>H]GluNAc, and, at designated time intervals, portions were removed and the reaction was stopped with 2 M HCl. Chitin-specific incorporation was determined by the following procedure: cells (2 × 10<sup>7</sup>/ml) were digested for 90 min with 1 N HCl at 100°C. The suspensions were washed twice with 0.9% saline, suspended in saline, and then digested with 1 N KOH for 90 min at 100°C (1, 2). After a final washing, the cells were fixed with 95% ethanol on glass cover slips and covered with melted Ilford K-2 emulsion. The cover slips were exposed for 10 days (4°C), developed, and then stained with 1% Congo red. This digestion procedure was also used to quantitate incorporation of [<sup>3</sup>H]GluNAc into acid-alkali-insoluble fractions by yeast or hyphal cells as described in the previous section. Other labeled cell suspensions were prepared for autoradiography without prior digestion.

**Protoplast formation.** The protoplast formation method used was that of Partridge and Drewe (12) for *C. albicans* and *C. tropicalis*. A 0.5-g (wet weight) portion of hyphal and yeast cells was treated for 30 min with 35 ml of 0.5 M sodium thioglycolate in 0.1 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.7), washed with a 0.2 M phosphate-0.1 M citrate buffer

containing 1.0 M mannitol (PCM buffer, final pH 6.5), and suspended in the PCM buffer containing snail gut enzyme (final concentration, ca. 1,000 U of β-glucuronidase per ml; Sigma Chemical Co.) for 60 min at 37°C. Protoplasts were washed and suspended in the PCM buffer described above.

**Chitin synthetase assay.** Chitin synthetase was assayed according to the method of Cabib (3). Protoplasts of *C. albicans* from hyphal and yeast forms were sonically treated in buffer (0.05 M imidazole, 2.0 mM MgSO<sub>4</sub>, and 7.0% mannitol [pH 6.5]). The sonic extract was then centrifuged at 80,000 × *g* for 30 min, and the pellet was washed and suspended in 0.5 ml of imidazole buffer. The supernatant obtained after sonic oscillation was also assayed for activity.

The enzyme assay mixture contained 40 mM MgSO<sub>4</sub>, 25 mM imidazole-chloride (pH 6.5), 45 mM GluNAc, 0.049 μM UDP-GluNAc, 0.001 μM [<sup>14</sup>C]UDP-GluNAc (specific activity, 53 mCi/mmol), and variable amounts of enzyme in a total volume of 50 to 53 μl.

In some experiments, the enzyme preparation was preincubated with 0.6 μg of trypsin for 15 min to determine whether activation could occur. The reaction was terminated by the addition of 0.9 μg of soybean trypsin inhibitor (Sigma Chemical Co.). The chitin synthetase reaction was initiated by the addition of substrate ([<sup>14</sup>C]UDP-GluNAc). After incubation for various intervals up to 1 h at 30°C, the reaction was stopped by the addition of 1 ml of 66% ethanol. The tubes were centrifuged at 2,000 × *g* for 5 min. The pellets were washed twice with 1 ml of 66% ethanol containing 0.1 M ammonium acetate. The pellets were then suspended in 0.4 ml of 95% ethanol, placed into scintillation vials, and counted as described previously except that the scintillation fluid contained 5 g of 2,5-diphenyloxazole, 3.2 g of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene, and 43 g of (Cabsil) thiotropic gel (Packard Co.) dissolved in 1 liter of scintillation-grade toluene.

**Preparation of activating factor.** Methods used are similar to those described by Cabib et al. (6). Protoplasts (0.5 g [wet wt]) were suspended in 0.5 ml of phosphate-citrate buffer containing 10% mannitol. The protoplasts were then placed in 35 ml of a lysis medium (pH 6.5) containing 8% mannitol, 0.01 M imidazole, 0.5% glucose, and 6 mM citric acid (Tris-chloride) and incubated at 30°C in shake culture. After 30 min, 99% lysis had occurred as established by counting portions of the protoplast preparation microscopically with a hemacytometer. The lysate was centrifuged for 5 min at 4,000 × *g*. After centrifugation, the pellet was suspended in 0.63 ml of a solution containing 8% mannitol, 0.05 M imidazole (pH 6.5), and 6.0 mM citrate and homogenized with a Teflon hand homogenizer. The homogenate was diluted with 2.5 ml of a solution containing 8% mannitol, 0.05 M imidazole (pH 6.5), and 2.0 mM MgSO<sub>4</sub> and centrifuged for 10 min at 12,000 × *g*. The pellet, which contained vacuoles of various sizes as observed by phase-contrast microscopy, was suspended in 0.35 ml of a solution containing 8% mannitol and 0.05 M imidazole (pH 6.5), and 2 mM MgSO<sub>4</sub> was added. The vacuolar suspension was then sonically treated for 20 s at a setting of 2 with a Sonifier cell disruptor (Heat Systems, Plain View, N.Y.). After centrifugation for 30 min at 80,000 × *g*,

the supernatant fluid was assayed for activity as described previously for trypsin activation, but without the addition of the inhibitor.

Protein was measured according to the procedure of Lowry et al. (10). Enzyme activity is expressed as the number of nanomoles of [ $^{14}\text{C}$ ]UDP-GluNAc incorporated per milligram of protein. All assays were run in duplicate. The enzyme preparations from other extractions showed similar activity.

**Utilization of Renografin gradient.** The Renografin gradient method used was that described by Duran et al. (9). Protoplasts were obtained as described previously and suspended in PCM buffer at a final concentration of 1 g/ml (wet weight). To 0.5 ml of this suspension was added 3 ml of 1.0 M mannitol containing 0.05 M Tris-chloride (pH 7.5) and 10 mM  $\text{MgSO}_4$ , followed by 3.5 ml of the same solution, supplemented with 0.5 mg/ml of concanavalin A (ConA). After 10 min at room temperature, the protoplasts were centrifuged for 1 min at  $1,000 \times g$ . The pellet was carefully suspended in 6.5 ml of the solution containing mannitol-Tris-chloride- $\text{MgSO}_4$  and recentrifuged for 3 min at the same speed. The final pellet was suspended in 8.5 ml of 10 mM Tris-chloride (pH 7.5) containing 5 mM  $\text{MgSO}_4$ , 1 mg of deoxyribonuclease per ml, and 0.6 mM phenylmethylsulfonylfluoride. The mixture was homogenized for 3 min at  $0^\circ\text{C}$  in a Sorvall Omni-mixer, and the homogenate was incubated for 15 min at  $30^\circ\text{C}$ . A 3-ml portion of this solution was layered on 9-ml linear gradients of Renografin (5.8 to 50%) containing 20 mM Tris-chloride (pH 7.5). The gradients were centrifuged for 1 h in an SW41 rotor at  $93,000 \times g$ . An Isco model 640 density gradient fractionator was used to collect 0.6-ml fractions from the top. Each fraction was diluted 10-fold with 5 mM Tris-chloride (pH 7.5) containing 2 mM  $\text{MgSO}_4$  and centrifuged for 30 min at  $80,000 \times g$ . The pellet was suspended in the same buffer and used for the enzyme assay. All fractions were preincubated with  $0.6 \mu\text{g}$  of trypsin and assayed as described previously.

[ $^3\text{H}$ ]ConA (20  $\mu\text{Ci}$ ; specific activity 29.0 mCi/mmol) was added to 0.1 ml of protoplast suspension in mannitol-Tris-chloride- $\text{MgSO}_4$  and incubated at room temperature for 10 min. These protoplasts were then washed, diluted with 2.9 ml of ConA-treated protoplasts, and extracted as described above.

## RESULTS

**Whole cell studies.** The uptake and incorporation of [ $^3\text{H}$ ]GluNAc was measured in both yeast and hyphae (mycelium) of *C. albicans*. Over a 30-min incubation period, incorporation by hyphae was over 10-fold that of yeast cells (Fig. 1). In part, this difference is due to the greater uptake of label by hyphae (48% greater than yeast). Also, whereas 84% of the label taken up by hyphae becomes incorporated after 30 min, only 11% becomes incorporated in yeast cells. The data presented in Fig. 1 represent total incorporation per milligram (dry weight) of yeast or hyphae. In other experiments, the amount of label in acid-alkali-insoluble fractions (presumably chitin) was also measured. It was deter-

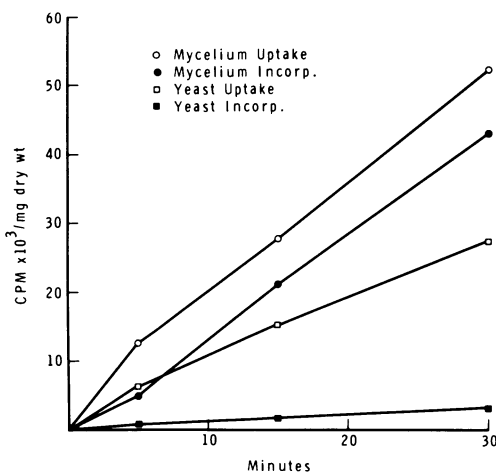


FIG. 1. Uptake and incorporation of [ $^3\text{H}$ ]GluNAc by yeast and hyphae of *C. albicans*.

mined that approximately 52% of trichloroacetic acid-precipitable label was acid-alkali insoluble in both yeast and hyphae (data not presented).

The site of chitin deposition within the cell walls of the yeast and hyphae of *C. albicans* was demonstrated by prelabeling cells with [ $^3\text{H}$ ]GluNAc. Autoradiographs revealed that the radioactivity was preferentially located at the apex of the hyphae (Fig. 2). By contrast, the radioactivity incorporated into yeast cells was predominately deposited at the site of budding in whole cells (Fig. 3).

**Chitin synthetase from yeast and hyphae.** Chitin synthetase activity was measured in sonically treated protoplasts after centrifugation at  $80,000 \times g$ . Enzyme activity was exclusively located in the  $80,000 \times g$  pellet (Table 1). In addition, the activity of the enzyme preparation from both yeast and hyphae was six to seven times greater when preincubated with trypsin ( $0.6 \mu\text{g}$ ) for 15 min (Table 1). Preliminary studies had shown that this concentration of trypsin was optimum for stimulation. It is noteworthy that the specific activity of chitin synthetase from hyphae was approximately twice that observed with yeast cells.

Centrifugation of homogenized protoplasts of both yeast and hyphae by means of a Renografin density gradient revealed four primary bands (Fig. 4 and 5). With both yeast and hyphae, chitin synthetase activity was associated with those bands that possessed [ $^3\text{H}$ ]ConA activity. The membrane fraction that contained enzyme activity in hyphae sedimented at a greater density than the corresponding yeast form. Recovery of the enzyme and specific activity are shown in Table 2. In both yeast and hyphae, the specific activity of chitin synthetase was approximately

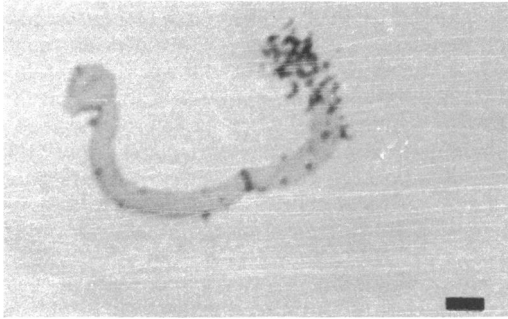


FIG. 2. Autoradiograph of an acid-alkali-extracted hypha showing the localization of  $[^3\text{H}]\text{GluNAc}$  near the apex. Bar indicates 1.4  $\mu\text{m}$ .

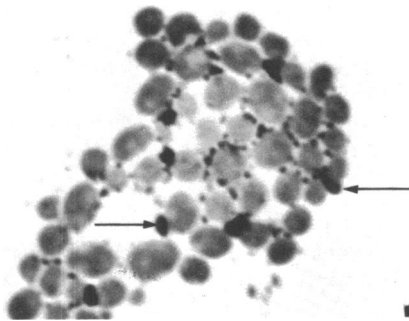


FIG. 3. Autoradiograph of whole yeast cells showing maximum localization of  $[^3\text{H}]\text{GluNAc}$  at the site of budding (see arrows). Bar indicates 1.4  $\mu\text{m}$ .

TABLE 1. Distribution of chitin synthetase in sonically treated protoplasts and the influence of trypsin on enzyme activity

Fraction	Form	nmol of $[^{14}\text{C}]\text{UDP-GluNAc}$ incorporated/mg of protein	
		- Trypsin	+ Trypsin
80,000 $\times$ g pellet	Yeast	0.271	1.75
	Hyphae	0.474	3.32
Supernatant	Yeast	0.01	0.011
	Hyphae	0.001	0.001

three times that of the crude sonically treated particles.

When protoplasts were pretreated with glutaraldehyde (1% final concentration), lysed, and then assayed, enzyme activity (counts per minute per milliliter) was similar to that of cells without pretreatment (Table 3). However, if protoplasts were lysed and then treated with glutaraldehyde and assayed, enzyme activity was 10% of control (no glutaraldehyde).

Since trypsin increased the enzyme activity of the sonically treated preparations, fractions of

*C. albicans* were tested for their stimulatory effects. In *Saccharomyces cerevisiae*, vacuolar fractions have been shown to contain an activating factor, which stimulates the activity of chitin synthetase (6). By similar procedures, we sought to isolate an activating factor from both the

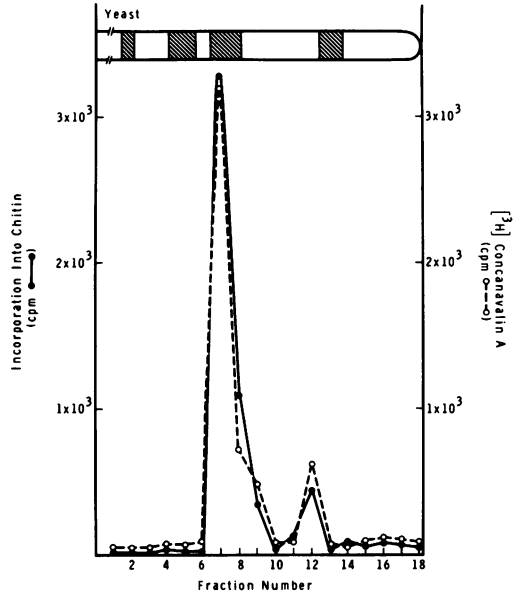


FIG. 4. Renografin density gradient of homogenized yeast protoplasts showing the association of chitin synthetase activity with  $[^3\text{H}]\text{Con A}$ .

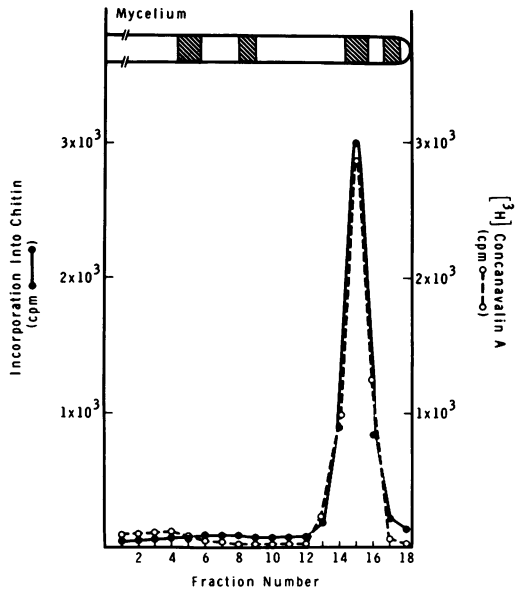


FIG. 5. Same as in Fig. 4 except homogenized hyphal protoplasts are represented.

TABLE 2. Specific activity of chitin synthetase associated with fractions collected from a Renografin density gradient

Fraction	Yeast		Hyphae	
	% Recovery	Sp act	% Recovery	Sp act
Crude particles	100.0	0.614	100.0	0.913
Band 1	1.5	0.235	2.1	0.110
Band 2	0.9	0.063	4.9	0.263
Band 3	57.0	2.100	54.0	3.150
Band 4	1.6	0.176	3.9	0.612

TABLE 3. Effect of glutaraldehyde on chitin synthetase activity<sup>a</sup>

Glutaraldehyde	<sup>14</sup> C]UDP-GluNAc incorporated (cpm × 10 <sup>4</sup> /ml)	
	Yeast	Hyphae
None	3.040	2.866
Before lysis	2.237	1.802
After lysis	0.329	0.223

<sup>a</sup> Protoplasts of both yeast and hyphae were pre-treated with 1% glutaraldehyde. After 30 s at 0°C, 20 volumes of 1.0 M mannitol in phosphate-citrate buffer was added. Protoplasts were centrifuged at 500 × g, washed, and then sonically treated and assayed for enzyme activity. Other protoplasts of yeasts and hyphae were sonically treated and then treated with 1% glutaraldehyde (30 s, 0°C), diluted with 0.05 M imidazole containing 2 mM MgSO<sub>4</sub>, centrifuged at 80,000 × g and assayed (9).

yeast and hyphae. A vacuolar fraction was obtained from protoplasts following a metabolic lysis. These vacuoles, which varied in size when examined by phase microscopy, possessed protease activity as determined by a hide-powder azure assay (6). Preliminary studies indicated that a concentration of 1.0 μg of protein from yeast vacuoles per 230 μg of enzyme gave a maximum stimulation of enzyme activity for both yeast and hyphae enzyme preparations (Table 4). Higher concentrations of vacuole protein tended to decrease activity below control (no activating factor). Vacuolar fractions, which possessed protease activity, were also obtained from hyphae. However, no stimulation of activity was observed when this preparation was preincubated with either yeast or hyphae enzyme preparations (Table 4).

## DISCUSSION

The purpose of this study was to compare and contrast chitin synthetase in yeast and hyphae of *C. albicans*. Initial studies indicated that hyphal incorporation of [<sup>3</sup>H]GluNAc into an acid-alkali-insoluble fraction was 10-fold greater than in yeast-phase cells. These data agree with those of Chattaway et al. (7) who demonstrated

by chemical analysis that hyphae of *C. albicans* contained three times as much chitin as yeast cells. In addition, our data indicate that uptake of [<sup>3</sup>H]GluNAc was also greater (approximately 48%) in hyphae and, whereas 84% of the label taken up by hyphae was incorporated, only 11% was incorporated into an acid-alkali-insoluble fraction by yeast cells. Additionally, chitin synthetase activity was approximately two times greater in hyphae.

Autoradiography of yeast and hyphae was performed to determine the location of chitin deposition. With whole yeast cells, label was preferentially located at the site of budding as described by Cabib for *S. cerevisiae* (4). Acid-alkali-digested yeast cells did not seem to retain their cellular integrity and were, therefore, impossible to see under light microscopy. With acid-alkali-treated hyphae, [<sup>3</sup>H]GluNAc was observed in greatest amount at a point just below the tip of the hyphae.

As noted with the chitin synthetase system of *S. cerevisiae* described by Cabib (4-6), chitin synthetase in *C. albicans* in both yeast and hyphae is associated with particulate fractions of sonically treated protoplasts, and specific activity of the enzyme is increased significantly by preincubating the enzyme preparations with trypsin. At this time, it is difficult to say with certainty whether or not the enzyme of *C. albicans* exists as a zymogen since purification has not been accomplished. As with *S. cerevisiae*, a vacuolar fraction with protease activity, obtained from yeast protoplasts, stimulated yeast and hyphae chitin synthetase activity approximately 45 to 60% above control (no vacuolar extract). However, even though the hyphal chitin synthetase was stimulated by trypsin, a vacuolar preparation from hyphae failed to enhance the activity of either the yeast or hyphal chitin synthetase, although both preparations were obtained under the same conditions and exhibited protease activity as determined by a hide-powder azure assay. The failure to demonstrate an activating factor from hyphae is puzzling since trypsin as well as yeast-activating factor enhanced enzyme activity from hyphae. Since chi-

TABLE 4. Effect of activating factor on chitin synthetase activity from yeast and hyphae of *C. albicans*

Activator form:	nmol of [ <sup>14</sup> C]UDP-GluNAc incorporated/mg of protein	
	Yeast	Hyphae
None	0.271	0.474
Yeast	0.439	0.691
Hyphae	0.216	0.359

tin synthesis ( $[^{14}\text{C}]\text{UDP-GluNAc}$  per milligram of protein) is much greater in hyphae, perhaps that activating factor is more often associated with the enzyme rather than in cell vacuoles. In yeast, chitin deposition is synchronized with cell division so that its activating factor is only utilized during a portion of the cell cycle. Therefore, yeast-activating factor could be more readily extractable.

Treatment of protoplasts with ConA before homogenization binds to and preserves the integrity of the plasma membrane (9). By utilizing this fact, we were able to obtain elution profiles from both yeast and hyphae protoplast homogenates in which enzyme activity coincided with  $[^3\text{H}]\text{ConA}$  activity. These data strongly indicate that the majority of chitin synthetase was associated with the plasma membrane. The difference in sedimentation rates of the plasma membrane from yeast and hyphae is similar to the data of Marriott (11) who described major differences in the chemical composition of the plasma membrane of each growth form. Our studies on the effect of glutaraldehyde on enzyme activity further suggest that chitin synthetase is located on the inner side of the plasma membrane. Enzyme activity was greatly reduced when protoplasts were treated with glutaraldehyde after sonic oscillation. However, when protoplasts were treated with glutaraldehyde before sonic oscillation, enzyme activity was only slightly below control (no glutaraldehyde). Our data are, thus, similar to those of Duran et al. (9) for *S. cerevisiae*.

Comparative studies of another dimorphic fungus, *Mucor rouxii*, have been carried out by Ruiz-Herrera and Bartnicki-Garcia (14). The enzymes from both yeast and hyphal forms had similar requirements for optimum activity. However, the stability of the hyphal (mycelial)-phase enzyme at 28°C was much lower than that of the yeast-phase enzyme. Enzyme activity of hyphal-phase cells was virtually lost within 2 h at 28°C, whereas the activity of the enzyme from yeast cells increased dramatically with time. These data indicated that the hyphal form of chitin synthetase was present primarily in an active state and that the prolonged incubation at 28°C resulted in a rapid degradation of the enzyme by endogenous proteolysis. Also, an exogenous acid protease did not increase the activity of the hyphal enzyme to any great extent. The yeast-phase chitin synthetase appeared to be zymogenic, however, based upon the increase in activity upon prolonged incubation at 28°C.

It would seem that the hyphal chitin synthetase in *C. albicans* is different from that of *M. rouxii* since our studies indicate that the activity of the enzyme is greatly stimulated by trypsin.

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