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Regulation and Solubilization of *Candida albicans* Chitin Synthetase

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A cytoplasmic component which inhibited the activation of chitin synthetase was studied in the dimorphic fungus *Candida albicans*. The inhibitor was found to be heat stable and trypsin sensitive and was only effective when incubated with a vacuolar protease, an activator of chitin synthetase, before the activation of chitin synthetase. In addition, the particulate chitin synthetase from the yeast form of *C. albicans* was solubilized by a sodium cholate-digitonin extraction and subsequently was purified approximately 30-fold by Sepharose column chromatography and Amicon XM 100 filtration. Activity of the soluble enzyme was increased by the addition of trypsin or phosphatidyl serine. The molecular weight of the enzyme was estimated to be 400,000.

The regulation of chitin synthetase was studied in both yeast and pseudohyphae of the dimorphic fungus *Candida albicans*. Previous work from this laboratory (1) has established that the activity of chitin synthetase can be increased by preincubating the enzyme with trypsin as well as with a vacuolar protease isolated from yeast-phase cells.

The vacuolar protease, obtained from lysed yeast-phase protoplasts, stimulated the activity of chitin synthetase from both yeast and pseudohyphal forms (1). Cabib has postulated that regulation of chitin synthetase in *Saccharomyces cerevisiae* also involves a cytoplasmic (soluble) protease which is believed to inhibit vacuolar protease activity (2). Our data also indicated that chitin synthetase was bound to the plasma membrane.

In an attempt to determine whether or not a similar regulatory process occurs in *C. albicans*, experiments were performed to investigate the role of a proteinaceous, cytoplasmic component on the activation of chitin synthetase by the yeast vacuolar protease. In addition, this study also describes the solubilization, partial purification, and properties of chitin synthetase from yeast-phase cells.

MATERIALS AND METHODS

The preparation of protoplasts, extraction of activating factor, and enzyme assay mixture were described previously (1).

Preparation of cytoplasmic inhibitor. One gram (wet weight) of protoplasts from pseudohyphal or yeast forms of *C. albicans*, suspended in 0.5 ml of 0.2 M sodium phosphate-0.1 M sodium citrate buffer (pH 6.5) containing 1.0 M mannitol, was sonicated in 1.5 ml of imidazole buffer (0.5 M imidazole-chloride, 2.0 mM $MgSO_4$, pH 6.5). The sample was centrifuged at 80,000 × *g* for 30 min, and the supernatant was collected. In some experiments the supernatant was heated in a boiling-water bath for 3 min, and the coagulated protein was removed by centrifugation (2,000 × *g*, 5 min). The clear supernatant was used as a source of inhibitor (9). Protein content was quantitated by the Lowry procedure (7). The inhibitor was incubated (various concentrations) with the enzyme (20 μl) and activator (7 μg of protein in 3.0 μl) before the addition of substrate (activation stage; 30°C, 30 min). Also, the inhibitor was added after the activation of chitin synthetase (assay stage). The chitin synthetase reaction was initiated by the addition of 1 nmol of substrate (10 μl), UDP-*N*-acetyl[^{14}C]glucosamine (UDP-[^{14}C]GlcNAc; specific activity, 28.53 mCi/mmol). All reaction mixtures were incubated for an additional 60 min (30°C) at pH 6.5 (1). The total volume of each assay mixture was 73 μl.

Solubilization and purification of chitin synthetase. Yeast protoplasts (9 g, wet weight) were sonicated, centrifuged at 80,000 × *g* for 30 min, and washed as previously described (1). The protoplast pellet (9 g) was suspended in 9.0 ml of 0.05 M Tris-chloride (pH 7.5) containing 2 mM $MgSO_4$ and homogenized with a Teflon hand homogenizer. To 8.5 ml of this suspension was added 2.1 ml of a 2.5% sodium cholate (Sigma Chemical Co.) solution contained in buffer (0.05 M Tris-chloride [pH 7.5], 2 mM $MgSO_4$). This suspension was incubated at 0°C for 30 min, centrifuged at 80,000 × *g* for 30 min, and washed three times with the Tris-hydrochloride-$MgSO_4$ buffer. Preliminary assays indicated that the pellet still retained most of the enzyme activity. To the pellet 14.3 ml of a 1% digitonin (Sigma Chemical Co.) solution contained in buffer (25 mM Tris-chloride [pH 7.5], 5 mM $MgSO_4$, 0.2 M NaCl) was added, shaken at 25°C for 45

min (150 rpm), and centrifuged at 80,000 × g for 30 min. The digitonin extraction solubilized most of the chitin synthetase so that further purification was performed with the digitonin supernatant. To determine whether activation of the soluble enzyme by trypsin could occur, 14.3 ml of enzyme supernatant was incubated with 70 μl of trypsin (0.3 mg/ml, 15 min, 30°C). The reaction was terminated by the addition of 70 μl of soybean trypsin inhibitor (0.45 mg/ml). The activated enzyme (14.3 ml) was placed on a Sepharose CL-6B (Pharmacia Chemicals) column (2.5 by 50 cm) and eluted with buffer (25 mM Tris-chloride (pH 7.5), 5 mM $MgSO_4$, 0.1% digitonin, 0.02% sodium azide), and 5-ml fractions were collected and assayed for enzyme activity. The active fractions were pooled and concentrated to approximately one-half the starting volume on an Amicon XM100A (Amicon Corp.) filter. Nontrypsinized enzyme (40 μl) was also reacted with 0 to 30 μl of phosphatidyl serine (3.0 mg/ml of $MgSO_4$-imidazole buffer, pH 6.5). All reaction mixtures were brought to a constant volume with buffer.

Soluble chitin synthetase assay. Forty microliters of the soluble chitin synthetase preparation (in Tris-hydrochloride-$MgSO_4$-NaCl buffer, pH 7.5) was preincubated with trypsin (0.4 μl, 0.15 mg/ml, 15 min, 30°C). The activation was terminated by adding soybean trypsin inhibitor (0.4 μl) as described above. The chitin synthetase reaction was initiated by the addition (10 μl) of substrate (1 nmol of UDP-[^{14}C]GlcNAc; specific activity, 28.53 mCi/mmol) and stopped by adding 5 μl of glacial acetic acid. Portions of the reaction mixture (30 μl) were applied to strips (1.5 by 9.8 cm) of Gelman ITLC-SG chromatographic sheets (Fisher Scientific Co.) 1.5 cm above the bottom edge (6). The strips were dried with warm air and developed ascendingly in a solvent system of 66% ethanol containing 0.1 M ammonium acetate. After developing for 15 min to a distance of 9 cm, the strips were removed and dried again with warm air. In this solvent system the reaction production ([^{14}C]chitin) remained at the origin, whereas UDP-[^{14}C]GlcNAc moved with the solvent front. A 2.3-cm segment of each strip, from 1.3 cm above to 1.0 cm below the starting line, was cut out and inserted into a scintillation vial. Scintillation liquid [0.1 g of 1,4-bis-[2]-(5-phenyloxazolyl)benzene and 5 g of 2,5-diphenyloxazole dissolved in 1.0 liter of scintillation-grade toluene] was used to measure radioactivity.

To determine whether or not the product which remained at the origin was [^{14}C]chitin, the products of the assay were treated in two ways: (i) the reaction mixture was digested with 1 N HCl and 1 N KOH as previously described (1); (ii) the reaction mixture (1 ml) was shaken with 5 mg of chitinase (Sigma Chemical Co.) for 16 h at 30°C, 150 rpm. Controls (no chitinase) were incubated similarly. After acid-alkali or chitinase digestion, the reaction products were assayed for [^{14}C]chitin by paper chromatography. With yeast-phase cells, acid-alkali extraction solubilized only 4% of the radioactivity (96% remained at the origin), whereas chitinase digestion solubilized 85% of the radioactivity (15% remained at the origin).

Molecular weight determination. Three proteins of different molecular weight, collagenase (109,000), urease (482,000), and ferritin (750,000) (Sigma Chemical Co.), were passed through the Sepharose CL-6B column. Elution fractions (5 ml) were collected and assayed for chitin synthetase, and absorbance was measured at 280 nm with a Beckman spectrophotometer. A standard curve was obtained by plotting the log of molecular weight versus elution volume. The molecular weight of activated chitin synthetase was extrapolated from this standard curve.

Protein was measured by the procedure of Lowry et al. (7), with bovine serum albumin as a standard. Enzyme activity was expressed as nanomoles of UDP-[^{14}C]GlcNAc incorporated per milligram of protein.

RESULTS

As previously reported (1), chitin synthetase in *C. albicans* was exclusively associated with the 80,000 × g pellet of sonicated protoplasts. The regulation of chitin synthetase obtained from sonicated protoplasts was investigated by using a supernatant fraction from the 80,000 × g centrifugation. Additions of increasing amounts of boiled or nonboiled 80,000 × g supernatant to the enzyme assay which contained yeast activating factor resulted in a dramatic decrease in chitin synthetase activity in both yeast and pseudohyphal enzyme preparations (Fig. 1 and 2). As shown, the boiled preparations from both yeast and pseudohyphae had a greater effect than the nonboiled ones. The boiled preparations from both growth forms lost their activity when incubated with trypsin (data not shown), indicating that the active component was a protein.

Mixing experiments were done to determine whether the 80,000 × g supernatant inhibitor acted upon the activating factor or directly upon the enzyme. As shown in Tables 1 and 2, the incubation of soluble inhibitor with activating factor before assay of the enzyme slightly inhibited the stimulatory effect of the activating factor (compare lines 2 and 4), whereas the inhibitor was without effect after activation had already occurred (compare lines 3 and 5). This effect was seen with both yeast (Table 1) and pseudohyphal (Table 2) inhibitor with yeast activating factor in both experiments. The yeast activating factor was used since pseudohyphal vacuolar preparations lacked stimulatory activity (1).

The solubilization and purification of chitin synthetase was attempted with the yeast form of *C. albicans*. Since the enzyme is tightly bound to the plasma membrane (1), many detergents were tried to obtain a soluble preparation. The greatest solubilization occurred when treatment with 2.5% sodium cholate (0°C, 30 min) was followed with 1% digitonin (25°C, 45 min). After solubilization, enzyme activity was greatly en-

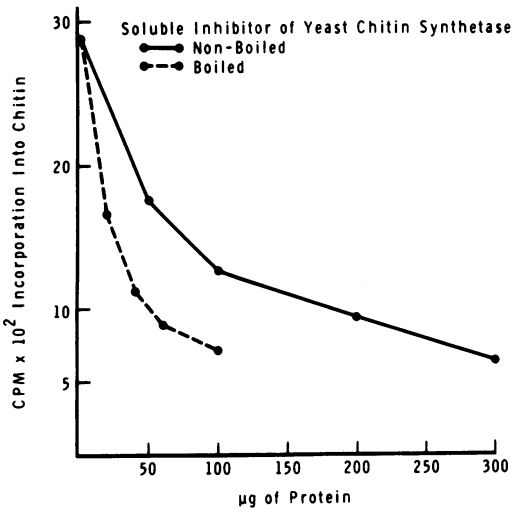

FIG. 1. *Effect of nonboiled and boiled yeast soluble (cytoplasmic) inhibitor on yeast chitin synthetase activity.*

hanced when trypsin was added. A small amount (0.06 µg) of trypsin, added as described for the particulate preparations of chitin synthetase, increased the activity of the soluble enzyme fivefold. In addition, the activity of the solubilized enzyme was stimulated to a smaller extent when phosphatidyl serine was added to the reaction mixture (Fig. 3). The greatest stimulation was observed with 9 µg of phosphatidyl serine, although some stimulation was observed at other quantities (up to 60 µg).

Subsequently, the trypsin-activated enzyme was placed on a Sepharose CL-6B column. Five-milliliter fractions were collected by eluting with a Tris-chloride–digitonin-$MgSO_4$-NaCl buffer and assayed for chitin synthetase activity. Fractions showing activity were pooled and concentrated to one-half of the original volume by ultrafiltration with an Amicon XM100A filter. After Sepharose chromatography and Amicon ultrafiltration, the enzyme was purified approximately 33-fold (specific activity of crude 80,000 × *g* pellet, 0.365; specific activity of Amicon concentrate, 12.18; specific activity is the number of nanomoles of UDP-[^{14}C]GlcNAc incorporated per milligram of protein per hour).

To determine the molecular weight of *C. albicans* chitin synthetase, we placed collagenase, urease, and ferritin on the Sepharose CL-6B column along with the solubilized enzyme. Fractions were collected as previously described, and a standard plot was obtained. From this curve, it was determined that trypsinized chitin synthetase had a molecular weight of 400,000.

DISCUSSION

As previously reported, chitin synthetase from *C. albicans* is bound to the plasma membrane (1). The enzyme probably exists as a zymogen since both trypsin and a vacuolar fraction possessing protease activity from *C. albicans* yeast-phase cells stimulated the activity of chitin synthetase from yeast and pseudohyphal cells (1).

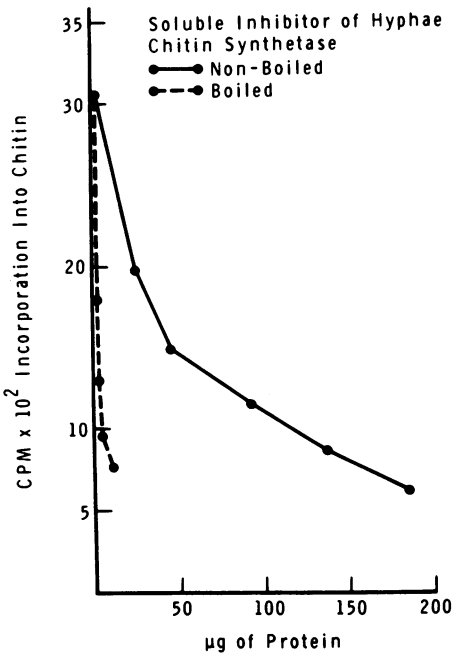

FIG. 2. *Effect of nonboiled and boiled pseudohyphal soluble (cytoplasmic) inhibitor on pseudohyphal chitin synthetase activity.*

TABLE 1. *Effect of yeast activating factor (AF) and inhibitor on yeast chitin synthetase activity*[a]

Addition during activation stage:		Addition during assay stage:	[^{14}C]GlcNAc incorporated (nmol/mg of protein per h)
Inhibitor	AF	Inhibitor	
−	−	−	0.068
−	−	+	0.065
−	+	+	0.110
+	+	−	0.060
−	+	−	0.110
+	−	−	0.066

[a] Data represent average of two experiments. The 80,000 × *g* soluble fraction was either incubated with the activating factor and enzyme before the addition of substrate (activation stage), or after activation, during the incubation of enzyme with substrate (assay stage).

TABLE 2. *Effect of yeast activating factor (AF) and pseudohyphal inhibitor on pseudohyphal chitin synthetase activity*[a]

Addition during activation stage		Addition during assay stage	[^{14}C]GlcNAc incorporated (nmol/mg protein per h)
Inhibitor	AF	Inhibitor	
−	−	−	0.094
−	−	+	0.096
−	+	+	0.140
+	+	−	0.090
−	+	−	0.140
+	−	−	0.093

[a] Conditions were the same as described for Table 1, except that pseudohyphal inhibitor was added instead of yeast inhibitor.

The separate location of chitin synthetase and the vacuolar protease (activating factor) has been described for other fungi, including *S. cerevisiae* (2, 9). A third component of the system, described as a soluble, proteinaceous inhibitor, has also been described by Cabib and his coworkers for *S. cerevisiae* (2, 9) and for *Mucor rouxii* (8). With *C. albicans*, the 80,000 × *g* supernatant from either yeast or pseudohyphae inhibited chitin synthetase activity (see Fig. 1 and 2). The active component in this crude extract was found to be trypsin sensitive and heat stable. In addition, the inhibitor was found to decrease chitin synthetase activity by inhibiting the activating factor. This observation was obtained by experiments in which the inhibitor was preincubated with enzyme, with or without activating factor. The stimulation of enzyme activity by the activating factor was not observed if the inhibitor was included in the preincubation mixture, whereas the activity of the enzyme itself (without activator) was unchanged regardless of the presence or absence of the inhibitor. Furthermore, once activation of the enzyme by the activating factor occurred, the addition of the inhibitor to the assay mixture did not reverse the stimulatory effect. Thus, the soluble inhibitor acts upon the activating factor rather than the enzyme itself. These observations support the data of Cabib (2–4, 9) as described for *S. cerevisiae*. It should be mentioned that recently Wolf et al. (10) have described mutants of *S. cerevisiae* which lack activating factor activity (also referred to as proteinase B). These same mutants showed normal phenotype with no impairment in budding.

Previous work, with the crude, particulate preparation indicated that trypsin increased enzyme activity six- to sevenfold. However, whether or not the enzyme existed in an unstimulated state (zymogen) on the plasma membrane was uncertain. It could be speculated that trypsin did not act directly on the enzyme but on some membrane component enabling substrate and enzyme to work more efficiently. To determine if trypsin acted directly on the enzyme, solubilization was attempted. It was determined that a combination of sodium cholate and digitonin released the enzyme from the membrane and that trypsin equivalently activated the soluble enzyme as it did the particulate synthetase. Solubilization has also recently been obtained by Duran and Cabib with *S. cerevisiae* chitin synthetase (5). Trypsin, likewise, stimulated enzyme activity. In addition, phosphatidyl serine (3 μl; 3.0 mg/ml) also increased activity by approximately 40%. It appears that for maximal activity, chitin synthetase requires a lipid associated with it. The activated chitin synthetase was also estimated to have a molecular weight of 400,000. From these results, it was concluded that chitin synthetase exists as a zymogen on the yeast plasma membrane of *C. albicans*. The same situation probably exists for the pseudohyphae. However, chitin synthetase from this morphological form has not been solubilized.

The results obtained on the chitin synthetase system in *C. albicans* appear to support the hypothetical scheme for the initiation of chitin synthesis as developed in *S. cerevisiae* (2–4, 9). The separate location of zymogen, activating factor, and inhibitor in the chitin synthetase system illustrates the importance of compart-

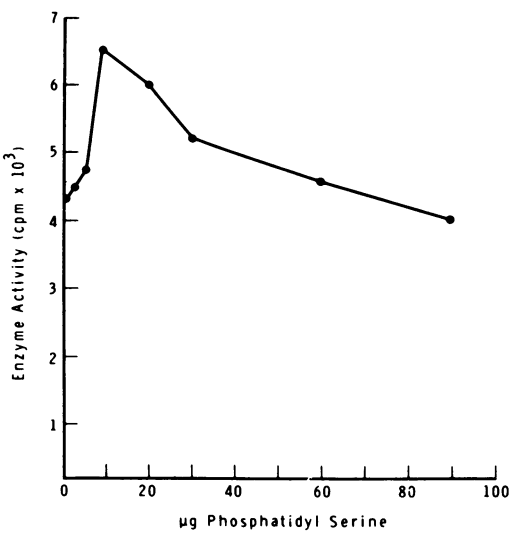

FIG. 3. *Effect of phosphatidyl serine on soluble chitin synthetase activity.*

mentation in complex biological processes. The model for chitin deposition as described by Cabib (2) is as follows: chitin synthetase, a zymogen, is distributed uniformly on the plasma membrane. The vacuoles within the cell, carrying activating factor, coalesce with the plasma membrane at the site of chitin deposition permitting the activation of zymogen in a restricted area. The inhibitor acts as a safety device to inactivate any vacuolar protease that spills into the cytoplasm preventing random stimulation of the enzyme. Our reported results on the zymogen, activating factor, and inhibitor from yeast and pseudohyphal forms of *C. albicans*, lend support to the Cabib hypothesis. Although the characterization of zymogen, activating factor, and inhibitor provides an explanation for the triggering of chitin synthesis in the *C. albicans* cell, it remains to be explained how the activation of a small percentage of the total synthetase occurs at a specific site and time. It seems that this would require some kind of subcellular organization which is not understood at this time.

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