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Effect of Detergents on Membrane-Associated Glucan Synthetase from *Paracoccidioides brasiliensis*

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Yeast and mycelial particulate preparations of *Paracoccidioides brasiliensis* were subjected to the action of several detergents in an attempt to solubilize the glucan synthetase present in these preparations. This was achieved more successfully in the yeast membranes than in the mycelial ones. The enzymatic activity was greatly stimulated in the insoluble fractions upon treatment with some of the detergents used. The results suggest that the yeast and mycelial phases of *P. brasiliensis* may differ in the structures of their membranes and also in the characteristics of their glucan synthetases.

One approach toward understanding fungal morphogenesis is to study the formation of major structural components. Regulations of syntheses of these materials should reflect the morphogenetic regulations of the corresponding structures. Accordingly, chitin synthetases have been studied in several fungi in which chitin seems to play an important role in morphogenesis (1, 5).

In the case of *Paracoccidioides brasiliensis*, a dimorphic pathogenic fungus, the morphogenesis of yeast and mycelial phases may be related to the presence of an α-glucan in the former and of a β-glucan in the latter (6). Moreover, these glucans seem to play an important role in the expression of *P. brasiliensis* virulence (11). However, the mechanisms which control fungal glucan synthesis have received limited study. Shematek et al. (12) reported on *Saccharomyces cerevisiae* β-1,3-glucan synthetase (EC 2.4.1.34), and San-Blas (10) studied a similar enzyme from both forms of *P. brasiliensis*. In both studies, characterization was limited to crude membrane preparations.

In the present work, we attempted the separation of *P. brasiliensis* glucan synthetase from the membrane fraction by the use of detergents, which are frequently used to solubilize membrane enzymes (7). We also report on the comparative activities of glucan synthetases extracted from the yeast and mycelial forms of *P. brasiliensis*.

MATERIALS AND METHODS

Organism and growth conditions. *P. brasiliensis* strain IVIC Pb73 was maintained on Sabouraud liquid broth-modified (BBL Microbiology Systems, Cockeysville, Md.) agar slants. Broth cultures of both yeast and mycelial forms were made as previously described (10).

Membrane preparations. General procedures followed those previously described (10). Once the cells were harvested and broken in a Ribi cell fractionator (model RF-1; Ivan Sorvall, Inc., Norwalk, Conn.), walls were separated by centrifugation at 5,000 × *g* for 15 min. The supernatant was further centrifuged at 60,000 × *g* for 1 h, and the sediment containing the membrane fraction was retained. This membrane fraction was suspended in 0.05 M Tris-hydrochloride buffer (pH 7.0) to a final concentration of 40 mg of protein per ml.

Centrifugation in sucrose gradients. Membranous material was applied to the top of linear (20 to 40% [wt/vol]) sucrose gradients, and centrifugation was carried out at 64,000 × *g* for 3 h at 4°C in a Beckman SW25.1 rotor. The gradients were fractionated into 1-ml portions. Protein concentrations were monitored by absorbance at 280 nm, and glucan synthetase activities were estimated on 0.1-ml samples.

Detergents. The detergents used were Brij 35, Brij 58, Lubrol WX, Nonidet P-40, Tergitol 15-S-9, Tergitol NP10, Triton 770, Triton WR-1339, Triton X-100, Tween 20, Tween 40, Tween 60, and Tween 80 (Sigma Chemical Co., St. Louis, Mo.), and digitonin and saponin (BDH, Poole, England).

Treatment of membrane fractions with detergents. Individual detergents were added to the membrane preparations (1 mg of protein per ml) to a final concentration of 1% (wt/vol). Tris-hydrochloride buffer (0.05 M; pH 7.0) was used throughout. Mixtures were shaken at 4 or 37°C for 1 h and then centrifuged at 60,000 × *g* for 30 min. The enzymatic activity was tested in both the soluble and the insoluble fractions. This centrifugation step was performed either immediately after incubation with the detergent for 1 h or after storing the mixture for up to 40 h at −20°C. Four of the detergents (Brij 58, Lubrol WX, Triton 770, and Tween 60) were tested at several concentrations in the range 0 to 2% (wt/vol).

Enzyme assays. Glucan synthetase assay was done

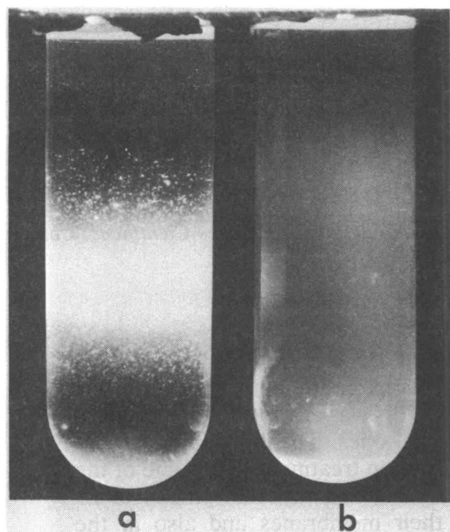

FIG. 1. Sucrose density gradient (20 to 40% [wt/vol]) of membrane fractions from *P. brasiliensis* Pb73. (a) Yeast form; (b) mycelial form.

by the method previously described (10), with uridine diphosphate-[^{3}H]glucose (specific activity, 4.85 Ci/mmol; New England Nuclear Corp., Boston, Mass.) as the sugar nucleotide precursor. Soluble fractions were concentrated by lyophilization. Reaction products were separated by paper chromatography (10).

Enzymatic digestion of reaction products. To determine partially the structure of the glucan formed in each case, 10 μg of laminarinase (exmollusca) (a β-1,3-glucanase; EC 3.2.1.6; Calbiochem, La Jolla, Calif.) was added to a mixture prepared as described above which had been previously incubated for 2 h to allow synthesis. Samples (0.05 to 0.1 ml) were withdrawn and spotted as before. The decrease in radioactivity (if any) at the origin was taken as the degree of digestion caused by the enzyme.

RESULTS

Particulate preparations from yeast and mycelial forms behaved differently on sucrose density gradients (Fig. 1). The yeast preparation yielded two fractions, a very thin band localized at 22% sucrose and a broad band in the region of 30 to 35% sucrose. The former was inactive, whereas the latter contained 95% of the glucan synthetase activity of the crude preparation. The mycelial glucan synthetase activity migrated to the bottom of gradients even when expanded to 50% sucrose.

When yeast membrane preparations were treated with any of 13 different detergents for 1 h at 4°C (Table 1), the activity of glucan synthetase always increased in the insoluble fraction, but the enzyme was not released to the supernatant. When the detergent was left to act for a further 40 h at −20°C (results not shown), partial solubilization was observed in several, but not all, cases, mainly with Brij 58 and Lubrol WX. In this experiment, an apparent increase in enzymatic activity was also observed in most of the insoluble fractions treated with detergents. Electron microscopic observations indicated that the longer the exposure of membranes to detergents, the more dissociated these membranes became.

Four of the detergents (Brij 58, Lubrol WX, Triton 770, and Tween 60), over the concentration range 0 to 2%, were chosen for more detailed study (Table 2). In general, the highest enzymatic activity was observed at detergent concentrations between 1 and 2%. Increases in enzymatic activities did not correlate with the total amount of protein solubilized.

Contrary to the results found for yeast membranes, none of the detergents tested (with the exception of Triton 770) released the glucan synthetase from mycelial preparations, although most of the detergents increased activities within the membrane preparations (Table 1). Storage of the preparations in the presence of detergent for 40 h at −20°C (not shown) resulted in increased activities.

As before, the same four detergents were chosen to observe their effect at several concentrations on mycelial preparations (Table 2). Throughout the whole range of detergent concentrations (0 to 2%) and times of exposure tested, glucan synthetase activity was detected in the soluble fraction obtained after Brij 58 or Triton 770 treatment.

Glucan synthetase activities in preparations decreased upon the addition of urea. Urea at 1 M gave about 87% inactivation, and 5 M urea

TABLE 1. Extraction of membrane preparations from *P. brasiliensis* Pb73

Detergent (1% [wt/vol])	% Incorporation of [^{3}H]glucose into glucan			
	Yeast form		Mycelial form	
	Insoluble fraction	Soluble fraction	Insoluble fraction	Soluble fraction
Brij 35	9.6	0.1	15.0	0.1
Brij 58	7.1	0.6	18.6	0.4
Lubrol WX	6.3	0.2	16.2	0.3
Nonidet P-40	4.5	0.6	15.4	0.5
Tergitol 15-S-9	4.1	0.4	12.7	1.0
Tergitol NP10	4.3	0.3	12.5	1.3
Triton 770	19.3	0.2	19.8	2.3
Triton WR-1339	5.7	0.1	6.9	0.2
Triton X-100	5.8	0.1	13.0	0.4
Tween 20	5.6	0.8	10.4	0.8
Tween 40	6.6	0.4	9.9	0.2
Tween 60	10.6	0.3	11.0	0.2
Tween 80	7.0	0.2	11.0	0.2
None	5.1	0.03	9.3	0.1

TABLE 2. Extraction of membrane preparations from *P. brasiliensis* Pb73 over a range of detergent concentrations

Detergent (0.0 to 2.0% [wt/vol])	% Incorporation of [^{3}H]glucose into glucan					
	Yeast form			Mycelial form		
	% Protein solubilized	Insoluble fraction	Soluble fraction	% Protein solubilized	Insoluble fraction	Soluble fraction
Brij 58	14.6–24.4	10.1–23.8	0.3–0.7	17.9–24.4	13.8–30.9	0.4–7.4
Lubrol WX	16.7–20.6	11.7–25.1	0.6–11.9	16.7–23.6	10.1–37.8	0.6–0.7
Triton 770	17.4–42.3	9.2–36.5	0.0–0.1	18.6–48.6	14.3–16.3	0.4–12.6
Tween 60	14.6–34.4	10.5–21.9	0.0–2.2	17.9–23.6	16.0–31.4	0.6–2.0

caused virtually total inactivation. The mechanism is presumed to be protein denaturation. Saponin and digitonin, at 1% concentration, decreased enzymatic activity (54 and 63%, respectively). Sonication for up to 1 min neither affected the synthetase activity in the membrane nor rendered the enzyme more soluble.

Partial determination of glucan structure was carried out in the reaction products obtained in the presence or absence of any of the detergents listed in Table 2. In all cases, [^{3}H]glucose was released (75 to 85%) after treatment with laminarinase.

DISCUSSION

The object of this study was to investigate the dissociation of *P. brasiliensis* glucan synthetases from their membrane environment, in which they seem to be intimately bound. As seen in Fig. 1, the sedimentation profiles of yeast and mycelial membrane preparations varied, without improving purification of the enzyme.

Both membrane preparations differed in their responses to the detergents used. Nonionic detergents of the polyoxyethylene type, such as Triton X-100, Tergitol NP10, and Nonidet P-40, had no apparent effect on the structural integrity of yeast membranes while rendering the mycelial membranes more active without solubilizing the synthetase. The detergents of the Brij series, which are linear primary alcohol polyoxyethylene compounds, were more effective in inactivating the enzyme of both yeast and mycelial preparations, while at the same time favoring the partial solubilization of the yeast membrane. The Tween detergents are polyoxyethylene sorbitan monofatty acids. Of the four tested, Tween 60 induced a higher synthetase activity in the insoluble yeast and mycelial membranes, particularly when the membranes were treated for 40 h. Electron microscopic observations indicated dispersion of the yeast membrane upon long exposure to the detergents, accompanied by induction of a higher enzymatic efficiency, perhaps through a better accessibility of the substrate to the enzyme. Although dispersion of mycelial membranes, as seen with yeast preparations, was not observed, there were changes in their appearance upon treatment with the detergent.

The influence of charged groups in the basic nonpolar detergent molecule was tested with Triton WR-1339 and Triton 770, which are sulfonated and sulfated polyoxyethylene alkyl phenols, respectively. Although the former was ineffective toward stimulation or solubilization of synthetase, the latter stimulated the enzymatic activity in the insoluble fraction, inducing at the same time a partial solubilization of the enzyme at concentrations higher than 1%, particularly in the mycelial fraction.

The presence of detergent in the reaction mixture, while influencing the efficiency of the enzyme, did not alter the final structure of the reaction product, as suggested by the extensive degradation provoked by laminarinase in glucans synthesized in the presence or in the absence of detergent.

The unsuccessful treatment of *P. brasiliensis* membranes with digitonin contrasts with the partial solubilization obtained in *Coprinus cinereus* and *Saccharomyces cerevisiae* chitin synthetases (2, 4) and *Phaseolus aureus* glucan synthetase (3). Ruiz-Herrera et al. (9) observed that *Mucor rouxii* chitosomes were dissociated by most detergents, digitonin being the only detergent tested which dissociated chitosomes with partial preservation of chitin synthetase activity. These results lead Hernández et al. (5) to suggest the important role of lipids, particularly sterols, in the function of chitin synthetase. Although detailed studies on lipids from *P. brasiliensis* membranes have not been performed, it is known that the total lipid contents of the yeast and mycelial phases differ qualitatively and quantitatively (8); these differences must influence the actual lipid composition of their membranes. This, in turn, would explain the different ability of the detergents used in this study to affect the yeast and mycelial preparations of *P. brasiliensis*.

These results and those reported before (10) suggest that glucan synthetases in *P. brasiliensis* have different characteristics in both forms of

the fungus, forming a complex system which, once better known, may provide some clues to the process of dimorphism in *P. brasiliensis*.

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