Heterogeneity of Phenol Oxidases in Cryptococcus neoformans

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Received 16 April 1992/Accepted 20 May 1992

Phenol oxidase enzymes, linked to virulence in Cryptococcus neoformans, were prepared from broken cells. More enzyme activity was found in the ultracentrifugation supernatant; less was found in the membrane fraction. Phenol oxidases were located in acrylamide gel electropherograms by activity staining with L-dihydroxyphenylalanine (DOPA). Mobility differences between soluble and solubilized membrane-bound phenol oxidases were not found. Comparison of enzymes produced at 25 and 37°C revealed that the enzyme had lower activity and lower mobility at 37°C. The mobility of 25°C phenol oxidases from strains of C. neoformans var. gattii was lower than that of those from C. neoformans var. neoformans. Half of the phenol oxidase produced at 25°C was bound by concanavalin A, while that produced at 37°C was not bound. However, glucose starvation of cultures at 25°C overnight resulted in increased amounts of enzyme which did not bind to concanavalin A. A given strain of C. neoformans produces different species of phenol oxidase under different culture conditions.

Melanization has been linked to virulence in the pathogenic fungus Cryptococcus neoformans (6, 7, 12). Synthesis of the pigment melanin is accomplished by phenol oxidase enzymes, which catalyze the oxidation of various catechols to unstable intermediates. The immediate products autocopolymerize to melanin. Phenol oxidases are thus of great interest to those studying the pathogenesis of infection. However, chemical and biological studies of melanization in this species have only begun. Interestingly, albino mutants have been obtained either by direct isolation of nonpigmented colonies or by isolation of oxidant-sensitive mutants, implying an antioxidant role for melanin (4). These genetic studies were inconsistent in that, while phenol oxidase mutants selected for albinism exhibited extremely low or undetectable enzyme levels (6, 12), those selected for oxidant sensitivity exhibited intermediate levels (approximately 10% of the wild-type level [4]). This discrepancy suggested the existence of isoenzymes or other complexities in the synthesis of melanin. A further indication of complexity is the marked decrease in phenol oxidase activity in cultures grown at 37°C, curious behavior for a putative virulence factor in a parasite of mammals (5).

It was therefore desirable to gain an overview of cryptococcal phenol oxidases and to describe their regulation prior to embarking on detailed studies of individual melanizing enzymes. In this report, we describe a survey of cryptococcal melanin-producing enzymes which is based largely upon activity staining after nondenaturing gel electrophoresis. In order to allow an adequate amount of variability, we have studied different varieties within the species, different strains within varieties, and the effect of cultivation temperature on a few strains.

MATERIALS AND METHODS

Yeast strains. C. neoformans var. neoformans strains 415 and 416 originated as strains B-3501 and B-3502, respectively, from J. Kwon-Chung (National Institutes of Health), and strains 125 and 127 originated from strains 7472 and 28958, respectively, from the American Type Culture Collection. Strain 438 was isolated from cerebrospinal fluid from a nonimmunocompromised patient with cryptococcosis (5). C. neoformans var. gattii strains 9403, 9447, and 9498 were obtained from the culture collection of Smith Shadomy. All C. neoformans var. neoformans strains except for 438 were confirmed as serotype D by slide agglutination with eight-factor sera (3). Strain 438 was serotyped as A, while the three strains of C. neoformans var. gattii were all confirmed as serotype B.

Preparation of phenol oxidases. Each strain, grown and stored on brain heart infusion agar slants, was inoculated into 10 ml of 2% glucose and 2% yeast extract and incubated with agitation at 25 or 37°C for 24 h. The culture (0.6 ml for a 37°C culture or 0.3 ml for a 25°C culture) was inoculated into 145 ml of asparaginol salts medium (per liter: glucose, 3 g; asparagine, 1 g; MgSO4, 0.5 g; KH2PO4, 3 g; and thiamine, 1 mg) and incubated for 24 h with agitation. Cells were harvested by centrifugation at 23,000 x g for 20 min at 4°C and washed once with the above medium without glucose. The washed cells were suspended in the glucose-free medium, incubated at the selected growth temperature for 5.5 h with agitation, and harvested and washed with 1 mM sodium phosphate buffer, pH 7.0.

The cells were broken mechanically as follows (14). Cells were placed in a 50-ml ice-jacketed Beadbeater (Biospec Products) with 0.5-mm-diameter glass beads added almost up to the top, and phosphate buffer was added to displace all the air. Intervals of agitation (1 min) were interspersed with 1-min pauses for cooling. Microscopic observation indicated that five 1-min treatments generally provided good breakage. The broken cell suspension was ultracentrifuged at 100,000 x g for 60 min.

After centrifugation, phenol oxidase activities in the supernatant (crude soluble phenol oxidase) and in the resuspended pellet (particulate phenol oxidase) were assayed (11). Enzyme (0.1 ml) was incubated with 0.1 ml of 10 mM

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Phenol oxidases were solubilized with digitonin and 0.8 ml of 50 mM phosphate buffer, pH 7.0, at 30°C for 2 h. The reaction was stopped by the addition of 10 μl of 1 M KCN, and the A₄₈₀ was measured. One unit of activity was taken to represent an increase of 0.001 A₄₈₀ unit in 120 min. Membrane-bound (particulate) phenol oxidases were solubilized with digitonin as described by Polacheck et al. (11). Briefly, 1% digitonin was added to the same volume of membrane suspension. The suspension was homogenized in a glass homogenizer and incubated at room temperature for 45 min with stirring. After ultracentrifugation at 100,000 × g for 1 h, the new pellet was again resuspended in buffer. Phenol oxidase activities in the supernatant and in the additionally resuspended pellet were assayed. The supernatant (solubilized particulate phenol oxidase) was simultaneously dialyzed against 1 mM phosphate buffer and concentrated with a Micro-ProDiCon (Bio-Molecular Dynamics, Beaverton, Ore.).

**Activity staining after nondenaturing gel electrophoresis.** The phenol oxidase samples were applied to a 10% polyacrylamide gel, using the Laemmli system (8) without sodium dodecyl sulfate. The slab gel was run at a constant current of 18 mA. After electrophoresis, the gel was immersed in freshly prepared 1 mM L-dihydroxyphenylalanine (l-DOPA) dissolved in pH 6.0 citrate phosphate buffer (0.1 M citric acid [17.9 ml], 0.2 M dibasic sodium phosphate [32.1 ml], diluted to a final volume of 100 ml) at room temperature overnight with gentle agitation.

**Concanavalin-agarose chromatography.** Crude soluble phenol oxidase was applied to a column (8 by 20 mm) of concanavalin A-agarose (Miles-Yeda, Ltd.). After the column was washed with 1 mM phosphate buffer (pH 7.0), the bound fraction was eluted with buffer containing 10% methyl-α-D-mannopyranoside until the effluent contained no phenol oxidase activity (between 5 and 10 column volumes of eluant).

### RESULTS

**Phenol oxidases in C. neoformans var. neoformans grown at 25°C.** The phenol oxidase activities in the supernatant and particulate fractions of five strains cultured at 25°C are shown in Table 1. In all cases, the activity of the soluble enzyme was greater than the activity of the particulate enzyme. On average, more than half of the particulate enzyme could be solubilized with digitonin; when this fraction was added to the larger, initially soluble fraction, it was calculated that between 86 and 112% of the total detectable enzyme was obtained in solution. These economics were helped by the finding that detergent tended to increase the activity of the phenol oxidase (10). Extracted samples were subjected to nondenaturing gel electrophoresis and activity staining (Fig. 1). Black melanin spots revealed the final positions of phenol oxidases. Each strain cultured at 25°C exhibited a major enzyme species with a common mobility, characteristic of all strains, although minor components with much lower mobilities were often present. The latter are believed to represent multimeric species of enzyme (unpublished data). No significant differences in the mobilities of soluble phenol oxidases and solubilized particulate phenol oxidases were detected in any C. neoformans var. neoformans strain.

**Comparison of phenol oxidases produced at 25 and 37°C.** It is known that phenol oxidase activity in a 37°C culture is usually much lower than it is in a 25°C culture (5). In order to detect possible qualitative temperature-linked differences, strains 415, 416, and 438 were cultured at both temperatures, phenol oxidases were extracted, and their electropherograms were compared (Fig. 2). In all three strains, the mobility of the phenol oxidases produced at 37°C was lower than that of enzymes produced at 25°C. (The amount of soluble phenol oxidase produced at 37°C by strain 415 was too small to visualize by activity staining and impossible to concentrate sufficiently for staining because of the presence of viscous capsular polysaccharides. Therefore, only the solubilized particulate enzyme produced at 37°C is shown for strain 415.) These results demonstrated a qualitative as well as quantitative difference between the 25 and 37°C enzymes.

Binding to concanavalin A by the soluble enzymes from strains 415 and 416 produced at the different temperatures is shown in Table 2. The phenol oxidase made at 37°C did not show any detectable activity staining.

**FIG. 1.** Electrophoretic comparison of phenol oxidases from five wild-type Cryptococcus strains. S, soluble enzyme; P, solubilized particulate enzyme.
bind to concanavalin, while half of that made at 25°C did bind to it. On the other hand, when strain 415 was starved for glucose for 12 h at 25°C, several times as much enzyme was produced (data not shown), but most of the enzyme did not bind to concanavalin A (Table 2). These results suggest that glycosylation of enzyme is affected both by the culture temperature and by the availability of glucose.

Comparison of phenol oxidases from C. neoformans var. neoformans and C. neoformans var. gattii. Various strains of C. neoformans var. gattii were cultured on bird seed agar (9) at 25°C, and the strains which showed a brown color were used to prepare soluble phenol oxidases for activity staining. Enzymes from five strains of C. neoformans var. neoformans and three strains of C. neoformans var. gattii are shown in Fig. 3. The mobility of the phenol oxidases from C. neoformans var. gattii was lower than that of the enzymes from C. neoformans var. neoformans.

DISCUSSION

Melanization on medium containing diphenols was first proposed for use in the identification of C. neoformans (2, 9, 13), but the phenomenon has also been related to the virulence of this fungus (6, 7, 12). In a prior study of extracted crude cryptococcal phenol oxidase (11), the enzyme was reported to be membrane bound. In contrast, we found that phenol oxidase activity was located primarily in the soluble fraction. The reason for the disagreement is not clear, although it may have been due to small differences in the conditions used for disruption and extraction. We considered a "salting-out" effect, since our preparation was made with extremely dilute (1 mM) extraction buffer instead of the 50 mM concentration used formerly (11). However, when we resubstituted 50 mM extraction buffer, predominately soluble enzyme resulted (not shown). In partial agreement with the earlier results, we always found a small proportion of phenol oxidase in the particulate fraction; we were also able to solubilize this fraction with digitonin. Moreover, solubilized particulate enzyme appeared to be identical to soluble enzyme on electrophoresis (Fig. 1 and 2). Consequently, we doubt that our finding of predominantly soluble enzyme contradicts the earlier work in any fundamental way; it remains possible that the enzyme is actually membrane bound in vivo.

The method for activity staining electrophoresed phenol oxidases has been adapted to the cryptococcal system. In preliminary experiments, we discarded several substrates (catechol, norepinephrine, N,N-dimethyl-p-phenylenediamine, N,N,N',N'-tetramethyl-p-phenylenediamine, and o-phenylenediamine) before choosing L-DOPA, which revealed the enzyme most clearly. Citrate phosphate buffer, pH 6.0, previously used for activity staining of laccase from Aspergillus nidulans with N,N-dimethyl-p-phenylenediamine (1), gave a relatively clear background for activity staining of the C. neoformans phenol oxidase when used with L-DOPA. Ammonium persulfate, used for polymerization of the gel, seemed to contribute to high backgrounds. Using the gel on the day following preparation helped to reduce background staining.

It was found that a given strain produces different phenol oxidases under different conditions. Not only was the activity of phenol oxidase in 37°C cultures lower than that of phenol oxidase in 25°C cultures (5), but the mobility was also lower, suggesting that a chemically distinct species is made at the elevated temperature. It is curious that only one or the other species of phenol oxidase was seen. If strains were cultivated at additional temperatures, conceivably both species of enzyme, additional species, or one smeared spot might be seen. Another difference between the isozyymes seemed to consist in glycosylation, since the enzyme produced at 37°C did not bind concanavalin A, whereas half of the enzyme produced at 25°C did. Moreover, when a culture of strain 415 was starved for glucose for 12 h at 25°C, the affinity of its phenol oxidase for concanavalin A was

![FIG. 2. Electrophoretic comparison between phenol oxidases produced at 25 and 37°C. S, soluble enzyme; P, solubilized particulate enzyme.](image)

![FIG. 3. Electrophoretic comparison of soluble phenol oxidases from C. neoformans var. neoformans and C. neoformans var. gattii.](image)

### TABLE 2. Affinity of phenol oxidases for concanavalin A

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth temp (°C)</th>
<th>Activity applied (U)</th>
<th>Activity (U) not bound (% of input)</th>
<th>Activity (U) eluted by MEMAN* (% of input)</th>
</tr>
</thead>
<tbody>
<tr>
<td>415</td>
<td>25</td>
<td>12,915</td>
<td>7,746 (60)</td>
<td>7,913 (61)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1,300</td>
<td>1,078 (83)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>25, starvedb</td>
<td>2,730</td>
<td>3,720 (136)</td>
<td>330 (12)</td>
</tr>
<tr>
<td>416</td>
<td>25</td>
<td>6,500</td>
<td>4,712 (72)</td>
<td>3,150 (48)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>5,600</td>
<td>6,877 (123)</td>
<td>711 (13)</td>
</tr>
</tbody>
</table>

* MEMAN, methyl-a-D-mannopyranoside.

b Cells were starved for glucose for 12 h.
changed, and 88% of the enzyme produced under this condition did not bind. Thus, glycosylation is a second basis for heterogeneity in phenol oxidases. Perhaps one species of enzyme has high-mannose-type oligosaccharides, while the other does not. It seems unlikely, however, that the mobility differences observed in polyacrylamide gel electrophoresis simply reflect the presence or absence of glycosylation; these mobility differences are more likely due to other chemical differences. If simple glycosylation were the basis for the difference, cultures exhibiting equal proportions of concanavalin-binding and non-concanavalin-binding enzyme should be resolved by gel electrophoresis into two equal spots with mobilities characteristic of the 25 and 37°C enzymes. In fact, this behavior was never observed; occasional second spots were always minor components (Fig. 1), with mobilities even lower than that of the 37°C enzymes (Fig. 2). The biological function of carbohydrate on this enzyme remains unknown, as does the function of the regulation that we have observed.

REFERENCES