Production of Diagnostic Pigment by Phenoloxidase Activity of Cryptococcus neoformans

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Cryptococcus neoformans produces brown pigmented colonies when grown on agar media made from an extract of potatoes and carrots, broad beans (Vicia faba), or Guizotia abyssinica seeds. Since other yeasts do not produce the pigment, these media are useful as differential isolation media for C. neoformans. Similar specific pigment was produced by C. neoformans on chemically defined agar media which contained six different substrates of phenoloxidase (o-diphenol: oxygen oxidoreductase EC 1.10.3.1) an enzyme which catalyses the oxidation of o-diphenols to melanin. Substrates were incorporated singly into the media and included l-3,4-dihydroxyphenylalanine (L-DOPA), chlorogenic acid, protocatechuic acid, catechol, norepinephrine, and 3-hydroxytyramine hydrochloride (dopamine). No pigment was produced on media without substrate. Phenoloxidase activity in (NH₄)₂SO₄ precipitates of C. neoformans cell-free extract was assayed by measuring increases in absorbance at 480 nm produced in solutions of L-DOPA. This reaction showed oxygen uptake and was effectively inhibited by copper chelators, but not by catalase. The enzyme also oxidized the five other substrates which induced pigment formation. Electron micrographs of cells incubated in L-DOPA showed deposition of the pigment in the cell wall.

Cryptococcus neoformans grows on standard mycological media such as Sabouraud glucose agar as a white colony which becomes light tan in color only upon incubation of from 4 to 8 weeks. In 1962 Staib observed that C. neoformans produced brown colonies when grown on a medium prepared from seeds of Guizotia abyssinica (23, 24). Because other yeasts remained white on this medium, pigment formation became a useful criterion for differential isolation of C. neoformans (25-27, 30). Similar differential media based on Guizotia seed were later announced by Shields and Ajello (21) and Botard and Kelley (1). More recently, an equally selective medium made from an extract of potatoes and carrots was developed in our laboratory, and an extensive survey of yeast-positive clinical specimens and culture collection yeasts showed the brown pigment produced on this medium also to be specific to C. neoformans (8).

In view of the value of this differential pigment to diagnostic laboratories, a study of the mechanism of its formation was undertaken. The possibility that the pigment produced by C. neoformans is a melanin was suggested by reports that potato used to prepare the potato-carrot agar contains tyrosine (15) and chlorogenic acid (7) which act as substrates for the potato phenoloxidase (synonymous with tyrosinase or o-diphenol: oxygen oxidoreductase, EC 1.10.3.1) to produce melanin responsible for the blackening of cut potato tubers. It was believed that these substrates might also be utilized by C. neoformans to produce pigment. According to Lerner (10), melanin is synthesized by the oxidation of tyrosine and 3,4-dihydroxyphenylalanine (DOPA) by phenoloxidase to various-colored quinone intermediates which polymerize to form high-molecular-weight compounds usually brown to black in color. Phenolic compounds other than L-DOPA and tyrosine can also be oxidized by phenoloxidase to produce other categories of melanins (13). This investigation was presented in part at the 10th International Congress for Micro-
MATERIALS AND METHODS

Pigment production on agar media. To determine whether C. neoformans could utilize known substrates of phenoloxidase to produce pigment, seven different substrates including l-DOPA, chlorogenic acid, protocatechuic acid, catechol, norepinephrine, 3-hydroxytyramine (dopamine), and tyrosine were incorporated singly into a chemically defined medium according to McVeigh and Morton (12) at a concentration of 0.1 mM. The medium was modified to contain 0.001 M CuSO₄, 5H₂O. Cystine was eliminated because some substrates underwent autoxidation in its presence. The pH was adjusted to 6.5 with 1.0 N NaOH. Because Gugenheim found large quantities of l-DOPA in broad beans (V. faba) (6), on agar medium was also prepared containing per liter: infusion from 50.0 g of beans; 5.0 g of glucose; and 5.0 ml of a 1% filter-sterilized solution of thiamine hydrochloride.

A total of 100 yeasts was streaked on agar slopes of the chemically defined and bean media and incubated at 25 C. The yeasts included 38 strains of C. neoformans (isolated in most instances from patients in the Montreal area), 5 strains of C. diffusus, 2 strains of C. laurentii, 1 strain of C. albidus, 40 strains including 18 different species of Candida, and several other yeasts including Saccharomyces, Schizosaccharomyces, Hansenula, Sporobolomyces, Torulopsis, Trichosporon, and Rhodotorula. The development of pigment in single colonies of C. neoformans grown on chemically defined agar medium containing l-DOPA was compared with that on potato-carrot agar by growing approximately 50 colonies of yeast on 20-ml agar medium in 250-ml Erlenmeyer flasks.

Enzyme preparation. Cultures for phenoloxidase assay were grown in either a yeast extract medium containing per liter: powdered yeast extract, 3 g; peptone, 5 g; and glucose, 10 g; or in the chemically defined medium of McVeigh and Morton (12) as modified above. Cultures were grown in 200 ml of liquid medium in 500-ml Erlenmeyer flasks on a shaking incubator at 25 C. At 24 hr (early logarithmic phase) cells were separated from the culture medium and washed twice with cold 0.85% NaCl by centrifugation at 4 C and 6,000 × g. Cells harvested from 3,600 ml of medium were pooled to form 25 to 30 ml of a very heavy suspension containing approximately 1.8 × 10⁸ cells per ml. Twenty-five milliliters of the suspension was added to 50 g of glass beads (0.45 to 0.50 mm) in a 75-ml glass homogenizer flask, and the suspension was homogenized for 12 min in a Braun Mechanical Cell Homogenizer, Model MSK. To avoid heating of the homogenate, the flask was cooled with liquid CO₂, and homogenization time was broken into six 2-min intervals. At 12 min, microscopic examination of the homogenate showed mostly broken and ghost cells, and viable counts revealed approximately 0.02% viable cells. Glass beads were separated from the homogenate by filtering the mixture on a Buchner funnel through Whatman no. 1 filter paper. The glass beads were washed with an additional 25 ml of cold saline, and the resulting filtrate was centrifuged at 20,000 × g to produce a cell-free extract containing 3.0 to 6.0 mg of protein per ml. Twenty-five milliliters of this extract was fractionated with saturated (NH₄)₂SO₄ to produce precipitates at 0 to 45%, 45 to 60%, and 60 to 70% saturation. The precipitates were each dissolved in 5 ml of 5 mM KH₂PO₄-Na₂HPO₄ buffer, pH 6.8, and dialyzed against six 1,500-ml changes of the same buffer over a period of 36 hr at 4 C. Freezing did not affect enzyme activity, and samples were stored at −20 C. Protein concentration was determined by the phenol method according to Oyama and Eagle (16).

Enzyme assay. The phenoloxidase assay was that used by Prabhakaran (19). Oxidation of DOPA by phenoloxidase results in the production of two intermediate chromophores, dopachrome and indole 5,6quinone, which are characterized by their absorbance maxima at 480 and 540 nm, respectively (13). Cell-free extracts of C. neoformans were incubated with a solution of l-DOPA (buffered with 0.2 ml of 0.5 M KH₂PO₄-Na₂HPO₄ buffer, pH 6.8, in a reaction mixture volume of 3.2 ml) in 25-ml Erlenmeyer flasks and a shaking water bath at 25 C. Increases in absorbance of the reaction mixture were read at intervals of 5 min at 480 nm on a Bausch & Lomb Spectronic 20 spectrophotometer. Readings were corrected for increases in absorbance which occurred in substrate plus heat-inactivated enzyme controls. (When whole, washed cells were assayed for activity incubation periods were 1 to 2 hr, and controls consisted of substrate plus heat-killed cells.) Oxygen uptake in the reaction between C. neoformans phenoloxidase and l-DOPA (incubated as for the enzyme assay) was studied by using a Warburg apparatus.

Peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) has also been observed to catalyze the oxidation of DOPA and other phenolic compounds to melanin (11, 29). This enzyme uses H₂O₂ rather than molecular oxygen as a hydrogen acceptor and is inhibited by catalase (29). To distinguish the C. neoformans enzyme from a peroxidase, catalase was added to the reaction mixture (DOPA, 8.0 mM; phenoloxidase, 100 μg of protein per ml; catalase, 0.1 mg per ml).

The activity of C. neoformans phenoloxidase on six additional substrates was also tested. Substrates were incubated with enzyme as in the assay described above, and the absorbance maxima of the reaction products were read between 700 and 340 nm on a Beckman DK-2A ratio-recording spectrophotometer. Reaction mixtures were read against controls consisting of substrate plus heat-inactivated enzyme. Increases in absorbance produced in these various substrates by the C. neoformans enzyme were read on a Spectronic 20 at wavelengths corresponding to the absorbance maxima of the products formed.

Electron microscopy. Early logarithmic phase cells were washed with 0.85% saline and incubated for 8 hr at 25 C in a shaking water bath with 4.0 mM l-DOPA, pH 6.8. Controls were incubated in buffer at the same concentration and pH as in the test. Cells were fixed in glutaraldehyde-osmium, according to Edwards (4), and embedded in Spurr...
low-viscosity embedding medium (22). Ultrathin sections were cut with diamond knives on an LKB ultratome and stained with 3% uranyl acetate (alcohol solution) followed by Reynolds lead citrate. Electron micrographs were taken on Kodak electron image plates with a Philips 300 electron microscope. 

**Chemicals.** L-DOPA and l-tyrosine were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. DL-norepinephrine hydrochloride, protocatechuc acid (3,4-dihydroxybenzoic acid), dopamine (3-hydroxytyramine hydrochloride), L-mimosine, DL-fluorophenylalanine, 1-phenyl-2-thiourea, diethyldithiocarbamic acid sodium salt, and catalase (Sigma C-100; 2× crystallized) were products of Sigma Chemical Co., St. Louis, Mo. Chlorogenic acid (3-[3,4-dihydroxycinnamoyl]quinic acid) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. and catechol from Matheson, Coleman & Bell, Norwood, Ohio. Spurr low-viscosity embedding medium was a product of Polysciences Inc., Warrington, Pa.

All experiments were carried out at least in duplicate.

**RESULTS**

All substrates incorporated into the chemically defined agar medium, with the exception of tyrosine, were observed to induce pigment formation in *C. neoformans* similar to that produced on potato-carrot and *Guizotia abyssinica* agar. No color was produced on media without phenoloxidase substrates. Increase in the concentration of L-DOPA caused an intensification of color to dark brown at 1.0 mM and to black at 10.0 mM. Color produced by *C. neoformans* differentiated it from the majority of other yeasts tested by 7 days and from all other yeasts tested by 14 days. Although some yeasts other than *C. neoformans* produced traces of brown color on some of the substrates tested, the color produced by *C. neoformans* was always of greater intensity and allowed good differentiation of *C. neoformans* from other yeasts. Comparison of color development on potato-carrot agar and the chemically defined medium containing DOPA showed that color developed more rapidly in *C. neoformans* on both media when it was grown in mixed cultures with *Candida albicans* than when it was grown alone. Although the color on DOPA medium was more intense than on the potato-carrot agar, it was observed to develop less rapidly. Although single colonies of *C. neoformans* could be differentiated from *C. albicans* in mixed cultures by 6 days on potato-carrot agar, good differentiation on the DOPA medium did not occur until 9 to 10 days.

Color produced by all strains of *C. neoformans* on the bean agar was similar to that produced on the chemically defined medium containing L-DOPA and clearly differentiated it from other yeasts tested.

Phenoloxidase activity was detected in both washed, whole cells and cell-free extracts of *C. neoformans*. Heat-inactivated cells and cell-free extracts produced little or no increases in absorbance at 480 nm in solutions of L-DOPA. Phenoloxidase activity was not affected by the presence or absence of DOPA in the culture medium. Whole cells and cell-free extracts of nonpigment-producing yeasts, including a *Candida albicans* and a *C. laurentii*, did not exhibit phenoloxidase activity.

Protein with the greatest amount of phenoloxidase activity in the *C. neoformans* cell-free extract was precipitated by (NH₄)₂SO₄ at a concentration of 45 to 60%. Little or no activity was detected in the 0 to 45% and 60 to 70% (NH₄)₂SO₄ precipitates. The 45 to 60% fraction generally had a specific activity four to five times greater than the crude extract (Table 1) and is here referred to as *C. neoformans* phenoloxidase. Because of the hazard involved in working with this pathogen and the consequent difficulty experienced in producing large amounts of cell-free extract, further purification was not attempted.

*C. neoformans* phenoloxidase produced increases in absorbance of L-DOPA at 480 nm which were linear with time (Fig. 1). Enzyme (125 µg of protein per ml) was saturated by L-DOPA at 6.0 mM (Fig. 2). The *Kₐ*, determined from this data by the Lineweaver-Burk plot was 1.6 mM. Increases in absorbance at 480 nm of 8.0 mM solutions of L-DOPA were proportional to enzyme concentration between 50 and 200 µg of protein per ml. Little or no increase in absorbance due to autooxidation of L-DOPA was observed at all concentrations of this substrate used. The greatest absorbance increases due to autooxidation were produced in controls consisting of heat-inactivated enzyme.

| Fraction | Volume (ml) | Total protein (mg) | Activity (total units) | Specific activity (units/mg of protein) | Recovery (%) |
|----------|-------------|--------------------|------------------------|----------------------------------------|--------------|
| Crude extract 45 to 60% (NH₄)₂SO₄ precipitate | 25 | 110 | 2970 | 27 | 100 | 67.2 |

* One unit of activity is defined as an increase in absorbance at 480 nm of 0.01 produced in 3.2 ml of 2.0 mM L-DOPA in 30 min.
and 8.0 mM L-DOPA, and they were observed to be no greater than 0.02 per 15 min. Lower concentrations of L-DOPA exhibited less autoxidation. Maximal activity was obtained at 40 to 45°C at pH 6.8 (Fig. 3) and pH 6.8 to 7.2 at 25°C (Fig. 4). Autoxidation of 8.0 mM L-DOPA at elevated temperature and pH was greater than at 25°C and pH 6.8. Absorbance increases of controls at temperatures greater than 40°C (pH 6.8) and at pH 7.6 and 8.0 (25°C) were no greater than 0.02 per 10 min. Copper chelators were more effective inhibitors of the C. neoformans phenoloxidase than substrate analogues (Table 2). Oxygen uptake in the reaction between phenoloxidase and L-DOPA was linear with time (Fig. 5). DOPA incubated without enzyme showed only small amounts of oxygen uptake, whereas inactivated enzyme plus DOPA showed no oxygen uptake. Catalase was not observed to inhibit the oxidation of L-DOPA by C. neoformans phenoloxidase. The C. neoformans enzyme also produced colored intermediates when incubated with the five remaining substrates observed to induce pigment formation on agar media. Table 3 shows the absorbance peaks of the products formed with the different substrates and increases in absorbance produced at these wavelengths. No activity was observed with tyrosine.

Figure 6 shows the presence of an electron-dense layer in the cell wall of C. neoformans incubated in buffered L-DOPA which is not present in the controls incubated in buffer alone. Because melanin has been observed to be electron dense (5), this layer probably represents the deposition of melanin in the cell wall of the yeast.

**DISCUSSION**

It has been demonstrated here that C. neoformans produces specific brown pigment when grown on chemically defined agar media containing six different substrates of phenoloxidase, an enzyme which catalyses the oxidation of o-diphenols to melanin (10, 14). No pigment
Table 2. Effect of inhibitors on Cryptococcus neoformans phenoloxidase

| Inhibitor                  | Increase in absorbance at 480 nm | Inhibition (%) |
|----------------------------|----------------------------------|----------------|
|                            | Enzyme + DOPA                    | Enzyme + DOPA + inhibitor |
| Substrate analogues        |                                  |                 |
| Fluorophenylalanine        | 0.20                             | 0.21            | 0               |
| Mimosine                   | 0.20                             | 0.16            | 20              |
| Copper chelators           |                                  |                 |
| Phenylthiourea             | 0.19                             | 0.09            | 53              |
| Diethyldithiocarbamate     | 0.19                             | 0               | 100             |

*DOPA, fluorophenylalanine, mimosine, and phenylthiourea, 8.0 mM; diethyldithiocarbamate, 0.8 mM; phenoloxidase, 100 μg of protein per ml; time, 10 min.

Fig. 5. Oxygen uptake in the reaction of C. neoformans phenoloxidase on L-DOPA. L-DOPA, 8.0 mM; enzyme, 250 μg of protein per ml. Symbols: ●, DOPA plus enzyme; △, DOPA alone.

Table 3. Oxidation of phenolic compounds by Cryptococcus neoformans phenoloxidase

| Substrate          | Absorbance maxima of products (nm) | Increase in absorbance |
|--------------------|-----------------------------------|------------------------|
| L-DOPA             | 480                                | .34                    |
| Catechol           | 405                                | .69                    |
| Chlorogenic acid   | 385                                | .12                    |
| Protocatechuic acid| 355                                | .40                    |
| Norepinephrine-HCl | 480                                | .24                    |
| Dopamine-HCl       | 480                                | .38                    |
| Tyrosine           | nil                                | nil                    |

*Substrates, 2.0 mM; phenoloxidase, 100 μg of protein per ml; pH 6.8, except for chlorogenic acid (pH 6.0); time, 30 min.

According to Mason (14), naturally occurring melanins are insoluble and chemically intractable. Attempts by Strachan et al. (28) to extract the pigment from C. neoformans have shown that it could not be extracted by common organic solvents, by acid (CH₃COOH or 6 N HCl), or by alkali (2 N NaOH). It was not solubilized when pigmented yeast cells were kept in CH₃OH-HCl in a sealed tube at 100 °C for 3 hr. Strachan concluded that the pigment appeared to be a polymeric compound.

The C. neoformans phenoloxidase was observed to have wide substrate specificity and is thus more similar to the phenoloxidase from mushrooms and Mycobacterium leprae (17, 18, 20) than to mammalian tyrosinase which oxidizes only tyrosine and DOPA (2). It is similar to phenoloxidase from other sources (M. leprae and mushroom and mammalian phenoloxidases) in that it was more effectively inhibited by copper chelators than by substrate analogues (19, 20). The activity of the C. neoformans enzyme could be distinguished from that of a peroxidase, because it exhibited linear oxygen uptake in its reaction with L-DOPA and was not inhibited by catalase.

Since our first report on the nature of pigment formation by C. neoformans, Korth and Pulverer (9) also have reported that phenolic compounds, including 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), DOPA, and 3,4-dihydroxy-cinnamic acid (caffeic acid), incorporated into a glucose yeast extract agar will induce pigment formation in C. neoformans. The pigment formed was similar to that formed on media containing extracts of G. abyssinica seed. No pigment was formed from monohydroxy phenols. Analysis of G. abyssinica seed revealed the presence of o-diphenols, which were produced on media without substrate. C. neoformans was also shown to possess phenoloxidase activity which oxidized the six o-diphenol substrates. Because o-diphenol substrates occur in potato (3, 7), carrot (3), Vicia faba seed (6), and G. abyssinica seed (9, 28), C. neoformans also may be utilizing these compounds to synthesize pigment when grown on media incorporating extracts of these vegetables.
FIG. 6. A, Ultrathin sections of C. neoformans (showing deposition of pigment in cell wall) incubated in phosphate-buffer 4.0 mM L-DOPA. B, Control incubated in phosphate buffer. Cell wall, (cw); plasma membrane, (pm); ×40,000.
Korth and Pulverer concluded were either alkyl-, or methyl-, and vinyl-substituted protocatechuic acid or methyl-substituted dihydroxybenzoic acid. Strachan et al. (28) have recently isolated caffeic acid from G. abyssinica seed and have observed that caffeic acid, the methyl ester and diacetate of caffeic acid, 3-hydroxytyramine, 3, 4-dihydroxybenzoic acid (protocatechuic acid), 3, 4-dihydroxyphenylethanolamine (norepinephrine), and other select other groups. Strachan et al. (28) also isolated brown pigmentation from C. neoformans. Nine other phenolic compounds, including tyrosine, did not induce coloration. Strachan concluded that coloration is dependent upon the hydroxyl groups in the 3, 4 positions of the phenyl ring. The six phenolic substrates observed by us to induce pigment formation also had hydroxyl groups in these positions.

Studies are currently being undertaken to select the most suitable substrate of the C. neoformans phenoloxidase to be incorporated into diagnostic media for the differential isolation of this pathogen from specimens such as sputum, urine, or stool in which Candida and other yeasts frequently grow.

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