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# Melanin Biosynthesis in *Cryptococcus neoformans*

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**Pigment production by *Cryptococcus neoformans* is virulence associated. Dopamine- and 3,4-dihydroxyphenylalanine–melanin products were identified after acidic permanganate oxidation, alkaline hydrogen peroxide oxidation, or hydrolysis with hydriodic acid. These data provide direct chemical evidence for the formation of eumelanin polymers by catecholamine oxidation by laccase alone followed by oxidative coupling of dihydroxyindole.**

Melanin, produced by the enzyme laccase, has been thought to be a major virulence factor in *Cryptococcus neoformans* since its first description by Staib (17). However, the chemical structure of *C. neoformans* pigment has not been established; only indirect studies implicate the presence of eumelanin-type pigments. Pigment production is dependent on the presence of *o*- or *p*-dihydroxybenzenes or diaminobenzenes (2), and the intermediate dopaminechrome involved in eumelanin formation is produced from purified laccase (20). The metal environment is similar to that of eumelanins as determined by electron paramagnetic resonance spectroscopy, although an unusually high carbon-to-nitrogen ratio in melanin particles prompted Wang et al. to conclude that the structure of *Cryptococcus* melanin differs from those of mammalian and other fungal eumelanins (19). Knowledge of the structure of *Cryptococcus* melanin is important not only for the accurate simulation of pigments for immunological experiments but also for the identification of possible ancillary enzymatic activities that may have major impacts on virulence mechanisms and their regulation in this important pathogen.

To understand the biosynthesis of eumelanin pigments in *C. neoformans* more fully, we used recently developed microanalytical high-performance liquid chromatography methods to chemically quantify and characterize cryptococcal pigment, based on the formation of specific degradative products (7, 8). Representative isolates of *C. neoformans*, namely, serotype A (H99) (14), serotype D (ATCC 34873) (9), and a set of isogenic *CNLAC1* and *cnlac1* strains (2E-TUC4 and 2E-TU4, respectively) (16), were each incubated on asparagine agar (pH 6.5) containing 100 mg of either dopamine (DA) or 3,4-dihydroxyphenylalanine (DOPA) per liter for 2 days at 30°C. Recombinant laccase was expressed with plasmid pPIC93 (Invitrogen, San Diego, Calif.) in *Pichia pastoris* (4) and used to generate pigment (isolate PIC93). (Recombinant laccase was purified by methods used to purify the native enzyme [20] and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which showed a single band of approximately 100 kDa, consistent with a glycosylated laccase-*Pichia* leader sequence fusion protein.) Laccase activities were determined (20) on cells induced on asparagine agar as described above and were as follows: 70.0 U/$10^7$ cells with H99, 31.3 U/$10^7$ cells with ATCC 34873, 82.0 U/$10^7$ cells with PIC93, 15.0 U/$10^7$ cells with 2E-TUC4, and $<1$ U/$10^7$ cells with 2E-TU4.

Solubilization of melanin from 10 million cells by Soluene-350 as described previously (8) produced an $A_{500}$ proportional to the amount of pigment observed in cryptococcal cells and proportional to laccase activity (Table 1). Notable was the low amount of solubilized pigment from PIC93 relative to the cryptococcal strains in spite of high laccase activity (82.0 U/$10^7$ cells). This may be due to the ability of the anionic cell wall and capsule of *C. neoformans* (13) to adsorb greater amounts of melanin pigments similar to the property of anionic carboxymethylcellulose beads (3). Laccase-negative 2E-TU4 produced a minimum of pigment at pH 6.5, but it did not produce pigment at a lower pH of 5.2, consistent with auto-oxidation of the catecholamine at the higher pH (10).

Acidic permanganate oxidation of *Cryptococcus* pigment was performed as described previously (7) on 5 million cells grown on DA agar. Pyrrole-2,3,5-tricarboxylic acid (PTCA), a specific breakdown product of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) polymeric units, was detected (7). Relative yields were less from strains having less laccase activity, with the laccase-negative strain 2E-TU having a minimal level of PTCA due to auto-oxidation (Table 1).

In order to assess for possible copolymerization of cysteine to form pheomelanins, reductive hydrolysis of *Cryptococcus* pigment with hydriodic acid (HI) from 5 million cells was performed (8). Aminohydroxyphenylalanine (AHP) is a specific indicator of pheomelanins (7), and its presence was confirmed in this study by subjecting various synthetic pheomelanins (obtained as described in reference 8) to reductive hydrolysis with HI (Table 1). AHP was not found in significant quantities from cryptococcal pigment, showing that cysteine is not incorporated in the form of pheomelanin (Table 1). Detectable amounts of aminohydroxyphenylethylamine (AHPEA) were found with cells grown on DA but not on DOPA. AHPEA has been proposed to be an indicator of cysteinyl-DA-derived units in melanin (1, 18) but has recently been found in DA-melanin as well (8). AHPEA found in this study is most likely due to DA-melanin formed from cells grown on DA, since (i) the ratio of AHPEA to $A_{500}$ from strain H99 (0.50) was similar to that of synthetic DA-melanin (0.40) and (ii) the ratio of the 3-amino isomer of AHPEA to the 4-amino isomer (0.80) from strain H99 was similar to that of synthetic DA-melanin (0.95) (8).

In order to clarify this issue further, additional studies which showed that AHPEA is formed from DA-melanin and that oxidation by alkaline $H_2O_2$ could be used to identify DA-melanin were undertaken (8). Oxidation by alkaline $H_2O_2$ was performed on 5 million cells by the method of Napolitano (11)

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TABLE 1. Chemical characterization of melanins in *C. neoformans* in comparison with that of synthetic melanins

| Melanin (quantity) | $A_{500}$ (1,000) after solubilization[a] | $H_2O_2/OH^-$ production of[b]: | | $KMnO_4/H^+$ production of PTCA[b] | HI hydrolysis production of[b]: | |
|---|---|---|---|---|---|---|
| | | PTCA | PDCA | | AHPEA | AHP |
| *C. neoformans* in DA medium ($5 \times 10^6$ cells) | | | | | | |
| 2E-TU4 | 2.8 | 2.3 | 0.4 | 0.3 | 0.4 | 1.1 |
| 2E-TUC4 | 6.8 | 3.1 | 1.9 | 1.3 | 1.9 | 0.9 |
| H99 | 133 | 9.4 | 35.2 | 4.5 | 66.3 | 1.1 |
| ATCC 34873 | 10.8 | 2.0 | 3.4 | 1.5 | 2.3 | 0.5 |
| PIC93 | 4.5 | 1.4 | 1.0 | 0.9 | 1.0 | 1.1 |
| *C. neoformans* in DOPA medium ($5 \times 10^6$ cells) | | | | | | |
| 2E-TU4 | 6.0 | 9.8 | 1.5 | 0.6 | <0.2 | 1.1 |
| 2E-TUC4 | 17.5 | 11.4 | 3.6 | 3.1 | <0.2 | 1.6 |
| H99 | 71.0 | 32.9 | 9.3 | 17.0 | <0.2 | 1.5 |
| ATCC 34873 | 9.8 | 6.6 | 1.4 | 3.9 | <0.2 | 1.3 |
| PIC93 | 3.0 | 1.3 | 0.0 | 1.9 | <0.2 | 1.1 |
| Synthetic (0.1 mg)[c] | | | | | | |
| DA-melanin | 769 | 283 | 377 | 186 | 304 | 0 |
| DOPA-melanin | 954 | 429 | 167 | 255 | 0 | 6 |
| DHI-melanin | 941 | 329 | 166 | 206 | ND[d] | ND |
| DHICA-melanin | 467 | 5,350 | <4 | 2,490 | ND | ND |
| DA + Cys-melanin[e] | 435 | 41 | 534 | 18 | 12,200 | 26 |
| 5-*S*-Cysteinyl-DOPA-melanin | 437 | 708 | 430 | 38 | <20 | 17,500 |

[a] Absorbance unit times 1,000 per $5 \times 10^6$ cells or per 0.1 mg of melanin dissolved in 1 ml of Soluene-350 plus water (9:1). Values are averages of two determinations.
[b] Values are expressed in nanograms per $5 \times 10^6$ cells or per 0.1 mg of melanin. Except for AHP values (single determinations), values are averages of two determinations.
[c] Data cited are from reference 8.
[d] ND, not determined.
[e] Prepared from a mixture of DA (10 mM) and cysteine (5 mM). The N/S ratio indicates a DA/Cys-DA molar ratio of ca. 1:3 (18).

as modified by Ito and Wakamatsu (8). $H_2O_2$ oxidation of *Cryptococcus* cells produced, in addition to PTCA, pyrrole-2,3-dicarboxylic acid (PDCA), a specific degradation product of 5,6-dihydroxyindole (DHI) units. High PDCA/PTCA ratios from pigment obtained from growth on DA agar (Table 1) indicate that AHPEA derived from *Cryptococcus* is from DA-melanin and not cysteinyl-DA-melanin.

To assess DOPA decarboxylase and dopachrome tautomerase activities, strains of *Cryptococcus* were incubated in the presence of DOPA as described above. High PTCA/PDCA ratios obtained after oxidation by alkaline $H_2O_2$ (Table 1) showed that DOPA-melanin was produced without DOPA decarboxylation. This result differentiates melanogenesis in *Cryptococcus* from that in other systems, such as *Drosophila melanogaster*, where DOPA decarboxylase activity is required for effective melanogenesis and protection from parasites (12). In addition, the similarity of DOPA-melanin of *C. neoformans* to synthetic DOPA-melanin argues against ancillary dopachrome tautomerase activity in the fungus. This supports a Mason-Raper-derived scheme of melanogenesis (Fig. 1) wholly dependent on laccase (15). Finally, lack of AHP after HI hydrolysis of cells grown on DOPA medium shows that, unlike in mammalian systems, these *Cryptococcus* strains do not incorporate cysteine from intrinsic sources into melanin. We found nothing to suggest any unusual structure in the melanin polymer, in contrast to the structure suggested by a high C/N ratio found in an elemental analysis of melanin "ghosts" by Wang et al. (19). This may be a strain-related phenomenon, or the previously reported high C/N ratio may have resulted from impurities which are difficult to remove from melanin (5) or from the introduction of impurities during isolation procedures (6).

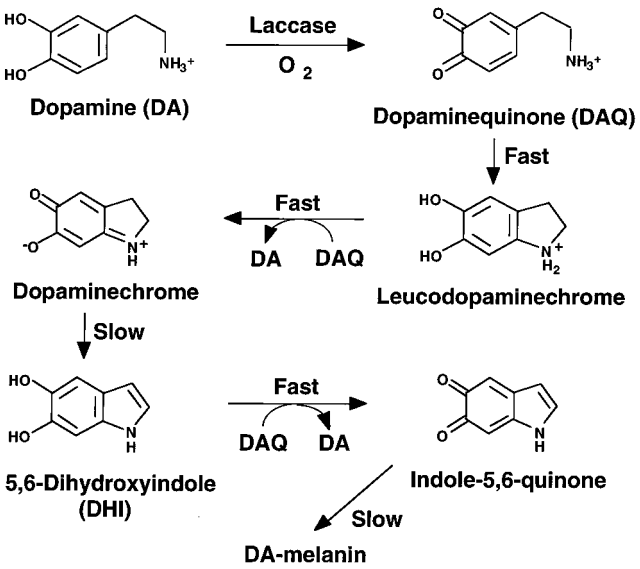


FIG. 1. Melanogenesis from DA in *C. neoformans*.

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