Antioxidant Activity of the Melanin Pigment Extracted from *Aspergillus nidulans*

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Melanins are pigments of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds. A number of fungi, including *Aspergillus nidulans*, produce pigments related or identical to melanin, which are located on cell walls or exist as extracellular polymers. The aim of the present study was to assess the antioxidant activity of synthetic melanin and of the pigment extracted from the mycelium and culture medium after growth of the highly melanized strain (MEL1) from *A. nidulans*. The ability of the melanin pigment to scavenge the oxidants HOCl and H_2O_2 was evaluated by inhibition of the oxidation of 5-thio-2-nitrobenzoic acid (TNB) using several melanin concentrations. The results showed that the pigment of the MEL1 strain competes with TNB for H_2O_2 and HOCl, inhibiting TNB oxidation in a concentration-dependent manner. For the HOCl oxidant, this inhibition was comparable to that of synthetic melanin, whose IC_{50} values were quite close for both pigments. Thus, our results suggest that the melanin from *A. nidulans* is a potential HOCl scavenger and may be considered a promising material for the cosmetic industry for the formulation of creams that protect the skin against possible oxidative damage.

Key words fungi; melanin; pigment; antioxidant activity; *Aspergillus nidulans*

Melanins are black or brown pigments of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds. They are found in organisms of all phylogenetic kingdoms, showing a broad spectrum of biological roles, including thermoregulation, chemoprotection, camouflage and sexual display.[1)]

These pigments are not essential for fungal growth and development, but have been reported to act as "fungal armor" because of the ability of the polymer to protect these microorganisms against harmful environmental conditions such as UV radiation, temperature extremes, hydrolytic enzymes, metals or plant defense mechanisms.[2—7)]

The effect of melanin enhancing the survival of fungi can be mainly due to its function as an extracellular redox buffer which can neutralize oxidants generated by environmental stress.[8)] Wang and Casadevall[9)] observed that melanized *Cryptococcus neoformans* cells survived the action of nitrogen- and oxygen-derived oxidants approximately 10-fold better than did non-melanized cells, suggesting that melanin contributes to virulence by protecting pathogens against free radicals generated immunologically. Studies on oxidative damage have shown that melanin in *Wangiella dermatidis* and *Alternaria alternata* appeared to confer cellular redox properties similar to those conferred by melanin upon *C. neoformans*.[10,11)] Hoogduijn *et al*.[12)] observed that melanin protects melanocytes and keratinocytes from the induction of DNA strand breaks by hydrogen peroxide, indicating that this pigment has an important antioxidant role in the skin.

In the last decades, skin cancer has been increasing alarmingly due to chronic exposure to UV light and certain environmental chemicals. The major cause of DNA damage induced by these agents is the production of reactive oxygen species (ROS) and free radicals.[13,14)] Substances acting as antioxidants, *i.e*., reactive oxygen and radical scavengers, protect cells from ROS-mediated DNA damage, which can result in mutation and subsequent carcinogenesis.[15,16)] Various studies have demonstrated that melanins act as antioxidants and suggest its use as a raw cosmetic material to minimize light- and toxin-induced tissue destruction.[1,17)] In this context, fungal melanin is of considerable biotechnological interest because it can be produced for low cost on a large scale compared with industrially-produced melanin, making its use as an antioxidative agent in cosmetic formulations economically advantageous. Taking this information into account, in the present study we assessed the antioxidant activity of the melanin pigment extracted from *A. nidulans* fungus in order to determine its possible utilization in the pharmaceutical industry.

RESULTS AND DISCUSSION

We first assessed the antioxidant activity of synthetic melanin, which was used as a control in the study because of its recognized activity as a reactive oxygen species (ROS) scavenger[5)] As shown in Tables 1 and 2, the oxidation of 5-thio-2-nitrobenzoic acid (TNB) by hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) resulted in a decrease of the absorbance to 412 nm compared to TNB alone, whose absorbance value was 0.491 ± 0.0438 (data not shown). However, in the presence of synthetic melanin there was an increase of absorbance at 412 nm proportional to melanin concentration (Tables 1, 2), indicating that the melanin reacts with H_2O_2 or HOCl and reduces the amount of TNB to be oxidized. In 100 μg melanin/ml, only 25.65% and 10.93% of TNB was oxidized, respectively, by H_2O_2 and HOCl (Tables 1, 2). Therefore, our results confirm the antioxidant properties of the synthetic melanin for the biological oxidants used.

As observed for the synthetic melanin, the incubation of H_2O_2 and HOCl with pigment extracted from the culture medium and from mycelium of the MEL1 strain also showed that this pigment inhibits TNB oxidation in a concentration-dependent manner, *i.e*., the lowest percentage of oxidized TNB was obtained in the presence of 100 μg melanin/ml (Tables 3—6). These results indicate that the pigment obtained

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Table 1. Effect of Synthetic Melanin on H_2O_2-Dependent TNB Oxidation

TNB plus H_2O_2 and melanin	A412[a)]	Oxidized TNB (%)
0 (μg/ml)	0.065±0.001	100
25 (μg/ml)	0.158±0.004	78.11*
50 (μg/ml)	0.199±0.006	68.47*
100 (μg/ml)	0.381±0.04	25.65*

a) values are the means±S.D. of three independent measurements. * Values were significantly different ($p<0.05$) from the control TNB plus H_2O_2 (100%).

Table 2. Effect of Synthetic Melanin on HOCl-Dependent TNB Oxidation

TNB plus HOCl and melanin	A412[a)]	Oxidized TNB (%)
0 (μg/ml)	0.069±0.001	100
25 (μg/ml)	0.110±0.003	90.26
50 (μg/ml)	0.128±0.005	85.98*
100 (μg/ml)	0.444±0.02	10.93*

a) Values are the means±S.D. of three independent measurements. * Values were significantly different ($p<0.05$) from the control TNB plus HOCl (100%).

Table 3. Effect of the Pigment Obtained from the Culture Medium for the MEL1 Strain on H_2O_2-Dependent TNB Oxidation

TNB plus H_2O_2 and pigment	A412[a)]	Oxidized TNB (%)
0 (μg/ml)	0.065±0.001	100
25 (μg/ml)	0.104±0.002	90.82
50 (μg/ml)	0.129±0.004	84.94*
100 (μg/ml)	0.215±0.02	64.70*

a) Values are the means±S.D. of three independent measurements. * Values were significantly different ($p<0.05$) from the control TNB plus H_2O_2 (100%).

Table 4. Effect of the Pigment Extracted from the Mycelium of the MEL1 Strain on H_2O_2-Dependent TNB Oxidation

TNB plus H_2O_2 and pigment	A412[a)]	Oxidized TNB (%)
0 (μg/ml)	0.065±0.001	100
25 (μg/ml)	0.095±0.002	92.94
50 (μg/ml)	0.107±0.005	90.11
100 (μg/ml)	0.163±0.004	76.94*

a) Values are the means±S.D. of three independent measurements. * Values were significantly different ($p<0.05$) from the control TNB plus H_2O_2 (100%).

Table 5. Effect of the Pigment Obtained from the Culture Medium for the MEL1 Strain on HOCl-Dependent TNB Oxidation

TNB plus HOCl and pigment	A412[a)]	Oxidized TNB (%)
0 (μg/ml)	0.069±0.001	100
25 (μg/ml)	0.298±0.02	45.6*
50 (μg/ml)	0.367±0.01	29.21*
100 (μg/ml)	0.459±0.02	7.36*

a) values are the means±S.D. of three independent measurements. * Values were significantly different ($p<0.05$) from the control TNB plus HOCl (100%).

from the MEL1 strain competed with TNB for H_2O_2 and HOCl and, therefore, had a scavenging activity on the oxidants tested. Studies with *A. fumigatus* and *A. nidulans* also showed that conidial pigments of both species are important for the protection of conidia against oxidative damage.[18,19)]

By comparing the results obtained with the two oxidants tested, it could be seen that the pigment of the MEL1 strain was a more efficient HOCl scavenger because the IC_{50} value

Table 6. Effect of the Pigment Extracted from the Mycelium of the MEL1 Strain on HOCl-Dependent TNB Oxidation

TNB plus HOCl and pigment	A412[a)]	Oxidized TNB (%)
0 (μg/ml)	0.069±0.001	100
25 (μg/ml)	0.211±0.02	66.27*
50 (μg/ml)	0.278±0.04	50.35*
100 (μg/ml)	0.471±0.01	4.51*

a) Values are the means±S.D. of three independent measurements. * Values were significantly different ($p<0.05$) from the control TNB plus HOCl (100%).

Table 7. Scavenging Activity of Synthetic Melanin and Pigment Obtained from MEL1 Strain on HOCl- and H_2O_2-Dependent TNB Oxidation

Scavenger	Oxidant	IC_{50}[a)]
Synthetic melanin	H_2O_2	57.91±0.87
Synthetic melanin	HOCl	56.89±0.34
MEL1 pigment (culture medium)	H_2O_2	121.87±1.12
MEL1 pigment (culture medium)	HOCl	31.16±0.26
MEL1 pigment (mycelium)	H_2O_2	186.17±4.91
MEL1 pigment (mycelium)	HOCl	40.57±0.36

a) Values are the means±S.D. of three independent measurements.

was much lower in the presence of HOCl than in the presence of H_2O_2 (Table 7). Jacobson *et al.*[11)] also observed that the melanin of *Cryptococcus neoformans* and *Wangiella dermatidisi* neutralized more HOCl than H_2O_2. Among the reasons for these results, the most important is that reaction of H_2O_2 with melanin requires an alkaline condition.[20)] This condition was not obtained here with the pigment from the MEL1 strain, whose reaction pH remained at 5.8 (data not shown) probably due to the acid conditions used for the extraction of this pigment.

Our results also showed that the pigment extracted from the MEL1 strain is a potential HOCl scavenger because the magnitude of the protection it provides was comparable to that of the synthetic melanin, with IC_{50} value of the pigment from the MEL1 strain being quite close to that for synthetic melanin (Table 7). In biological systems, HOCl is regarded as the most toxic and abundant oxidant and capable of doing considerable tissue damage, because it rapidly attacks a wide range of physiologically relevant molecules (such as amines, amino acids, nucleotides) and can also generate other very reactive oxidizing species like singlet oxygen and hydroxyl radical.[21)] In this context, our results indicate that the melanin from *Aspergillus nidulans* can be viewed as a promising source for future practical applications in the cosmetic industry as a component of creams that protect the skin from possible oxidative damage.

EXPERIMENTAL

Strains and Growth Conditions The present study was carried out with highly melanized mutant (denominated MEL1) of *Aspergillus nidulans*. This strain was grown in 250 ml Erlenmeyer flasks containing 100 ml of liquid minimal medium[22)] using 55 mM glucose as the carbon source and 70 mM sodium nitrate as the nitrogen source; it was inoculated with approximately 10^6 conidia/ml and incubated at 37 °C in a rotary shaker at 220 rpm for 72 h. Thereafter, the mycelium was collected by vacuum filtration and the melanin

present in the mycelium and culture medium was then extracted.

Melanin Extraction The melanin in the culture medium was extracted by the method of Paim *et al*.[23] The culture solutions were acidified to pH 1.5 with 6 N HCl and allowed to stand overnight to precipitate the polymer. The melanin precipitates were recovered by centrifugation at 4500 ***g*** for 15 min and lyophilized. The amount of produced pigment was approximately 175 μg per ml of medium.

Extraction and purification of melanin from the mycelium were performed according the procedure described by Sava *et al*.,[24] with minor modifications. First, the mycelial mass was treated with 2 M NaOH, pH 10.5, for 36 h. Thereafter, the mixture was centrifuged at 4000 ***g*** for 15 min and the supernatant was acidified with 2 M HCl to pH 2.5, incubated for 2 h at room temperature, and centrifuged at 4000 ***g*** for 15 min. The precipitate obtained was purified by acid hydrolysis using 6 M HCl at 100 °C for 2 h to remove carbohydrates and proteins and treated with an organic solvent (chloroform, ethyl acetate and ethanol) to wash away lipids. The precipitate was then dried at room temperature, re-dissolved in 2 M NaOH and centrifuged at 4000 ***g*** for 15 min. The supernatant was precipitated by the addition of 1 M HCl, washed with distilled water and lyophilized. This procedure yielded about 94.5 μg of pigment per mg mycelial mass.

Antioxidant Activity The antioxidant activity of the melanin extracted from mycelium and culture medium after growth of the MEL1 mutant was determined by a simple and rapid method described by Ching *et al*.[25] This method is based on the ability of a substance (scavenger) to inhibit the oxidation of 5-thio-2-nitrobenzoic acid (TNB) to 5,5′ dithio-2-nitrobenzoic acid (DTNB) in the presence of the oxidants HOCl and H_2O_2. The ability of the pigment to scavenge HOCl and H_2O_2 was determined by the concentration of TNB, as quantified by absorbance at 412 nm.[25]

TNB was synthesized by adding 20 mM sodium borohydride and then added to a 1 mM DTNB solution in 50 mM potassium phosphate buffer, pH 6.6, containing 5 mM EDTA. The solution was incubated at 37 °C for 30 min and TNB concentration was determined by absorbance at 412 nm using the molar absorption coefficient of 13600 $M^{-1}\,cm^{-1}$.

In the assay for hypochlorous acid scavenging, HOCl was first produced from sodium hypochlorite at pH 12 in potassium phosphate buffer 10 mM pH 7.4 containing NaCl 0.85%; HOCl concentration was determined spectrophotometrically at 295 nm using the molar absorption coefficient of 350 $M^{-1}\,cm^{-1}$. For assay, 20 μM HOCl was incubated for 5 min in the presence or absence of the scavenger (melanin pigment) and 40 μM TNB solution (described above) was then added in a final volume of 1 ml. The synthetic melanin (Sigma ref. 8631) and the melanin extracted from the MEL1 strain were used at concentrations of 25, 50 and 100 μg/ml.

In the assay for hydrogen peroxide scavenging, the solution of H_2O_2 was previously prepared in milli-Q water and the H_2O_2 concentration was calculated by absorbance measured at 230 nm using the molar absorption coefficient of 80 $M^{-1}\,cm^{-1}$. For the assay, 80 μM H_2O_2 solution was incubated for 10 min in the presence or absence of the scavenger (melanin pigment) and 40 μM TNB solution (described above) was then added in a final volume of 1 ml. The synthetic melanin (Sigma ref. 8631) and the melanin extracted from the MEL1 strain were used at concentrations of 25, 50 and 100 μg/ml.

The IC_{50} (inhibitory concentration, 50%) was determined using linear regression analysis of the dose–response curves (melanin concentration *versus* absorbance at 412 nm).

Statistical Analysis The parameters were reported as mean$\pm$S.D. Statistical analysis was performed by analysis of variance (ANOVA). Differences were considered significant at $p<0.05$.

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