Melanin Complex from Medicinal Mushroom

_Inonotus obliquus_ (Pers.: Fr.) Pilát (Chaga) (Aphylloporomyctideae)

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ABSTRACT: The production of melanin complex of _Inonotus obliquus_ (Pers.: Fr.) Pilát (Chaga in Russian) in submerged conditions was studied. It was demonstrated that copper ions (0.008%), pyrocatechol (1.0 mM), and tyrosine (20.0 mM) stimulated this process. It has been estimated that melanin of _I. obliquus_ has antioxidant and geneprotective effects. The investigation of the element composition of the pigment shows that it contains 38.2% C, 5.54% H, and trace amounts of N. The pigment was characterized by the following properties: extinction coefficient _E_250 (A = 250 nm) = 0.02; content of COOH groups = 0.93%, CO groups = 1.05%, OCH_3 groups = 0.96%, total OH groups 16.9% including aliphatic groups 15.05% and phenolic groups 1.25%. The data obtained in our work demonstrate high antioxidant and geneprotective effects of _I. obliquus_ melanin on peroxidase-catalyzed oxidation of aminoalcohols. These properties of melanin of _I. obliquus_ may be used for the development of anticarcinogenic preparations.

KEY WORDS: melanin complex, production, conditions, cultural liquid, mycelium, _Inonotus obliquus_

INTRODUCTION

_Inonotus obliquus_ (Pers.: Fr.) Pilát (Chaga fungus in Russian), a white-rot fungus, has been known in Russia as a medicinal fungus in folk medicine since the sixteenth or seventeenth century (Shivrina, 1965; Denisova, 1998). _I. obliquus_ has antiinflammatory, antitumor, immunomodulating, hepatoprotective, hypoglycemic, and tonic activities (Shivrina, 1965; Fedorov, 1973; Hobbs, 1996; Denisova, 1998; Mizuno et al., 1999; Wasser and Weis, 1999; Shin et al., 2000). The study of melanin complex production of _I. obliquus_ in submerged conditions and some its properties was the aim of our work.

MATERIALS AND METHODS

The studied strain of _Inonotus obliquus_ 32 was obtained from the culture collection of the Institute of Microbiology of the National Academy of Belarus, Minsk.

ABBREVIATIONS

BD: benzidine; DA: o-dianisidine; DMDB: 3,3'-dimethylbenzidine; HP: horseradish peroxidase; TMB: 3,3',5,5'-tetrabenzidine.

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Mycelium of this strain was grown in submerged culture on glucose-peptone medium (g/L): glucose, 10; peptone, 3; KH₂PO₄, 1; K₂HPO₄, 1; MgSO₄·7H₂O, 0.25; corn extract, 20 mL; deionized water, 1000 mL; pH 5.5.

After preparation, the medium was sterilized by autoclaving for 20 minutes at 121 °C. Mycelium was grown in 5-L flasks using the submerged cultivation technique at 25–27 °C for 10 days.

Mycelium was separated from the growth medium by filtration and washed with distilled water.

Melanin complex (pigment) was extracted from the mycelium by 2% NaOH over 2 hours on a boiling water bath. The extract was cooled. Then concentrated HCl was added to adjust the extract to pH 2.0. The coagulable pigment was separated from the liquid by centrifugation at 6000g for 15 minutes. The sediment was dissolved in 2% NaOH.

The pigment was purified by gel filtration on the sorbent Toyopearl HW-65 (Japan) in 0.01 N NaOH and lyophilized.

The amount of pigment was calculated from a calibration curve obtained by transmitted light photometry at 490 nm. Pigment was also determined in the culture liquid by direct photometry after separation of the mycelium.

Gel chromatography was performed in a Sephadex G-75 column (Pharmacia, Sweden) (Determan, 1967; Smychnic and Bambalov, 1980).

Melanin pigments were identified by qualitative reactions with KMnO₄, H₂O₂, and FeCl₃ (Elinov and Yurlova, 1976; Lyakh, 1981). Functional groups and the elemental composition were determined according to described methods (Zakis, 1987).

The absorption of the UV and visible light of 0.001–0.1% solutions of alkali-soluble pigments was estimated on a Specord M-40 (Germany) spectrophotometer, IR spectra on a Specord M-80 (Germany) spectrophotometer, and EPR spectra on a Varian E-112 (USA) radiospectrometer with MgO as standard.

The activity of α-diphenoloxidase (EC 1.14.18.1) was determined spectrophotometrically by measuring the optical density of the reaction products formed during oxidation of pyrocatechol over a fixed time period (Ermakov, 1987).

The activity of p-diphenoloxidase (EC 1.10.3.2) was determined by using p-phenylenediamine hydrochloride (Ravin and Harvard, 1956).

The influence of copper ions, pyrocatechol, and tyrosine on the pigment production in mycelium and cultural liquid was studied. Copper ions were added to the glucose-peptone medium in concentrations of 0.001, 0.002, 0.003, 0.004, 0.008, 0.0012, 0.016, and 0.020%. Pyrocatechol and tyrosine were added to the glucose-peptone medium in concentrations of 0.1, 1.0, 5.0, 15.0, 20.0, and 25.0 mM.

The peroxidase-catalyzed oxidation of aminophenols was performed in 0.1 M citrate-acetate buffer (pH 5.5). The reaction mixture (2 mL) contained 7 × 10⁻⁹ M horseradish peroxidase (HP) (Reanal, Hungary; RZ = 2.8), 0.5 mM substrate, and 0.1 mM H₂O₂. The reaction mixture was incubated at 30 °C for 4 minutes. The reaction was started by adding H₂O₂ and then monitored by the accumulation of colored products. Complete oxidation of aminophenols in 4 minutes was taken as 100% (Kukulyanskaya and Kurchenko, 1997).

DNA λ phage was exposed with products of peroxidase oxidation of α-dianisidine (DA) in 0.1 M citrate-acetate buffer (pH 5.5) at 30 °C for 30 minutes. The reaction mixture contained 10⁻³ M DA, 2 × 10⁻⁶ M HP, and 4 × 10⁻³ M H₂O₂. DNA λ phage was added to the reaction mixture at a concentration of 12 µg/mL. Fungal melanin was added to the reaction mixture at concentrations from 2 to 400 µg/mL to study its antioxidant and genoprotective effects.

RESULTS AND DISCUSSION

The complex of physicochemical properties of the pigment produced by I. obliquus, such as the solubility in some solvents (NaOH, concentrated H₂SO₄, and HNO₃), bleaching by H₂O₂, Na₂S₂O₃, KMnO₄, and bromine water, the ability to interact with FeCl₃, is the basis of identification of this pigment as melanin.

Our studies showed that 0.1% solutions of the pigment were oxidized and bleached over 24 hours in the presence of 10% hydrogen peroxide. Addition of potassium permanganate changed the color of alkaline solutions from brown to green; the solution was then bleached, and a precipitate was formed. The bleaching of melai-
nin required 11 mmol/g KMnO₄. Addition of 2 mg/mL sodium dithionite (a strong reducing agent) to alkaline solutions of the pigment changed its oxidation degree, which was measured by change (from 0.0025 to 0.0028) in the slope tangent of the absorption spectrum in the range of 250–500 nm. Addition of FeCl₃ (0.5 mg/mL) to 0.01% alkaline solutions of the pigment (0.1 N NaOH) produced a floccular precipitate, which dissolved in the presence of an excess of ferric chloride.

The absorption spectra of melanin solution in UV and visible light were similar to spectra of fungal melanins (Lukh, 1981). However, the alkali-soluble fraction of melanin from I. obliquus displayed a lower optical absorption in visible light compared to melanins isolated from microfungi (Malama et al., 1996).

The investigation of the element composition of the pigment shows that it contains 38.2% C, 5.54% H, and trace amounts of N. The pigment was characterized by the following properties: the extinction coefficient $E_{1.00%}$ ($\lambda = 465$ nm) was 0.02, the content of COOH groups = 0.93%, CO groups = 1.05%, OCH₃ groups = 0.96%, total OH groups = 16.9% including aliphatic groups = 15.65% and phenolic groups = 1.25%.

The studied pigment had a slightly asymmetric singleton signal, which is characteristic of fungal melanins (Zhdanova and Vasilevskaya, 1998). The concentration of paramagnetic centres was $2.93 \times 10^{18}$ spins/g of the dry weight.

The molecular weight of melanin from I. obliquus was from 56 to 60 kDa. The obtained data indicated that the main part of the alkaline fraction of I. obliquus pigment is present in the fraction with molecular mass to 60 kDa. The pigment of I. obliquus also contained a small quantity of substances with the molecular mass 100–120 kDa.

IR spectra of absorption are important spectral characteristics of melanin pigments. The IR spectrum of the studied pigment had several bands typical of fungal melanins. Broad complex bands in the region of 3300–3000 cm⁻¹ indicate the presence of OH– groups linked by hydroxyl bonds and =N–H groups exhibiting valence vibrations at 3500–3300 cm⁻¹ in the form of a broad band. The most intensive band in the region of 1710–1580 cm⁻¹ was typical for melanins and caused the valence vibrations of the –C=O groups. The presence of broad bands in this spectrum did not allow us to obtain more detailed information but suggested the presence of carbonyl, ketone, aldehyde, and carboxyl groups (Fig. 1).

During submerged growth, I. obliquus accumulated melanin in both the mycelium and the culture liquid. It has been demonstrated that the degree of pigmentation of fungi depends on the activities of oxidative enzymes (Lukh and Ruban, 1972). Investigation of the dynamics of melanin formation and enzymatic activities, especially $p$- and $o$-diphenol oxidases, showed that the maxi-
mum yield of melanin was obtained on the ninth day of growth and was stable during the following period of growth (Fig. 2). The enzymatic activity of the fungal phenoloxidase complex considerably increased up to the ninth day of growth, similar to melanin production, and then increased more slowly (Fig. 2).

The data obtained in our work indicated that the concentration of hydrogen ions (pH) influenced the *I. obliquus* pigment formation and enzymatic activity. The maximum enzymatic activity and melanin yield were observed at pH 7.0–8.0 of the studied medium.

Since the enzymes of the phenoloxidase complex responsible for the synthesis of melanin precursors are copper-dependent (Gadd and Griffiths, 1980), we studied the effect of copper ions on pigment formation. Analysis of data given in Table 1 showed that the increase of copper ions concentration in the medium up to 0.003% increased the pigment production of mycelia 7 times, and of the culture liquid almost 4 times. A further increase of the copper ions concentration inhibited the growth of the fungus and melanin synthesis (Table 1). It has been demonstrated that the highest production of biomass did not correlate with the maximal production of melanin in mycelia or in the culture liquid.

*I. obliquus* synthesized melanin on mineral media containing hydrocarbons. It used simple non-aromatic compounds to produce low-molecular-weight aromatic (phenol) precursors, which were further polymerized to form a high-molecular-weight pigment. We suggest that addition of possible melanogenesis precursors to the culture medium might stimulate pigment production. The results obtained in our work showed that pyrocatechol, tyrosine (Table 2), benzoic, and *p*-oxybenzoic

![FIGURE 2. Dynamics of melanin production by I. obliquus and activity of its phenoloxidase complex: (1) p-diphenoloxidase (conventional unit); (2) o-diphenoloxidase (units/g); (3) melanin (g/L).](image)

### TABLE 1

<table>
<thead>
<tr>
<th>Concentration of copper ions in medium, %</th>
<th>Biomass, g/L dry weight</th>
<th>Mycelia, mg/g of dry mass</th>
<th>Culture liquid, g/L</th>
<th>Total,* g/L</th>
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<td>0</td>
<td>6.2</td>
<td>14.52</td>
<td>0.24</td>
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<td>30.00</td>
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<td>40.13</td>
<td>0.50</td>
<td>0.70</td>
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</table>

* Calculated as sum of melanin from 1 L of culture liquid and melanin from biomass contained in 1 L culture liquid.
acids stimulated melanogenesis of *I. obliquus*, whereas phenol and 2-naphthol inhibited it. The production of biomass, as in the case with the copper ions, did not correlate with the melanin synthesis in the mycelia and the culture liquid. The optimal concentration of pyrocatechol, 5.0 mM, increased the total melanin yield 2 times. Unlike pyrocatechol, tyrosine increased the total yield of *I. obliquus* melanin nearly 10 times at a concentration of 20 mM. Such a considerable increase of melanin production is related to the high enzymatic activity of the fungal polyphenoloxidase complex: *I. obliquus* had higher activities of \( \alpha \)- and \( \rho \)-diphenoloxidases when it was cultivated in the medium with tyrosine than with pyrocatechol. However, the dynamics of the studied enzymes were similar.

It is known that antioxidant activity of natural and synthetic melamins is very high (Lyakh and Ruban, 1972; Lyakh, 1981; Kukulyanskaya and Kuruchenko, 1997). We investigated the antioxidant effect of melanin from *I. obliquus* using peroxidase-catalyzed oxidation of aminodiphenyls, which is a free-radical process. Benzidine (BD) and its methyl derivatives, 3,3'-dimethylbenzidine (DMBD) and 3,3',5,5'-tetramethylbenzidine (TMBD), were used as oxidized substrates. Similar to other aromatic amines, these compounds are indirect carcinogens: for their transformation into highly reactive metabolites, metabolic activation is required. Peroxidase-catalyzed oxidation of aminodiphenyls resulted in electrophilic products capable of interacting with nucleophilic groups of macromolecules, acting as direct carcinogens (Averyanov et al., 1986; Kukulyanskaya and Kuruchenko, 1997).

Figure 3 shows that the melanin pigment of *I. obliquus* inhibited the peroxidase-catalyzed oxidation of BD and its methyl derivatives with different efficiencies. The pigment concentrations causing 50% decrease of the oxidation rates of DMBD and TMBD were more than 2 times and 5 times higher, respectively, in comparison to BD oxidation. Thus, the efficiency of inhibition of peroxidase-catalyzed oxidation of aminodiphenyls decreases with an increase of the number of methyl substituents in the order BD, DMBD, TMBD.

Electrophilic products which are formed during metabolic activation of aminodiphenyls can interact with nucleophilic groups of biopolymers, in particular DNA, with the formation of DNA-

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**Table 2**

**Influence of Pyrocatechol and Tyrosine on Melanin Production of *Inonotus obliquus***

<table>
<thead>
<tr>
<th>Concentration in medium, mM</th>
<th>Biomass, g/L</th>
<th>Mycelia, mg/g of dry mass</th>
<th>Cultural liquid, g/L</th>
<th>Total, g/L</th>
<th>Production of melanin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyrocatechol</td>
<td>Tyrosine</td>
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<td></td>
<td></td>
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<td>0.0</td>
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<td>0.74</td>
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<tr>
<td>15.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* Growth is absent.

* Calculated as sum of melanin from 1 L of culture liquid and melanin from biomass contained in 1 L of culture liquid.
DNA intermolecular links (Tarusova, 1986). We used peroxidase-catalyzed DA oxidation to study the influence of I. obliquus melanin on this process. The main reaction product, the bifunctional reagent diimine, caused DNA cross-linking (Kukulyanskaya and Kurenko, 1977):

\[
\text{AH}_2 + \text{H}_2\text{O}_2 + 2\text{DNA} \rightarrow \text{DNA-A-DNA} + 2\text{H}_2\text{O}
\]

where A is aminophenyl and HP is horseradish peroxidase.

The dependence of the initial rate of DA oxidation on the melanin concentration in the reaction mixture is shown in Figure 4. Melanin inhibited this process at concentrations exceeding 20 mg/mL. The pigment at a concentration of 90 mg/mL halved the rate of DA oxidation. These data show that the fungal melanin inhibited the free-radical process of DA metabolic activation in vitro through peroxidase-catalyzed oxidation and displayed antioxidant properties.

For estimating the genoprotective properties of melanin from I. obliquus, we studied the effect of the pigment on DNA λ phage damage by products of peroxidase-catalyzed DA oxidation. The rate of DNA damage depended on the initial DA concentration. At a concentration of 0.3 × 10⁻³, DA caused 100% DNA damage (Fig. 5). Figure 6 shows that melanin at a concentration of 6 µg/mL prevented 50% of the DNA damage; at 20 µg/mL the melanin completely prevented DNA damage.

Inhibition of the peroxidase-catalyzed oxidation of aminophenyls by melanin may be explained by several mechanisms: (1) Melanin interacts with hydrogen peroxide and decomposes it. (2) Melanin binds the enzyme and inactivates it. (3) Melanin interacts with intermediate free-

![Figure 3](image1.png)

**FIGURE 3.** Effect of melanin concentration (µg/mL) on the rate of aminophenyl oxidation (Vₚₜ) (1) benzidine; (2) 3,3'-dimethylbenzidine; (3) 3,3',5,5'-tetramethylbenzidine.

![Figure 4](image2.png)

**FIGURE 4.** Effect of melanin concentration (µg/mL) on the rate of DA oxidation (V₀).

![Figure 5](image3.png)

**FIGURE 5.** Effect of DA (×10⁻³ µM) concentration on the damage of DNA phage cross-linking (%).
radical products of aminodiphenyl oxidation, preventing their transformation to eliminating the end products of this reaction. (4) Melanin protects DNA against free-radical damage through formation of DNA-melanin complexes (Kukulyanskaya and Kuruchenko, 1997).

The data obtained in our work demonstrate high antioxidant and genoprotective effects of L. obliquus melanin on peroxidase-catalyzed oxidation of aminodiphenyls. These properties of melanin of L. obliquus may be used for the development of anticarcinogenic preparations.

REFERENCES


