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Melanin Complex from Medicinal Mushroom *Inonotus obliquus* (Pers.: Fr.) Pilát (Chaga) (Aphyllophoromycetideae)

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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 genoprotective 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: the extinction coefficient $E_{0.001\%}$ (λ = 465 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 15.65% and phenolic groups 1.25%. The data obtained in our work demonstrate high antioxidant and genoprotective effects of *I. obliquus* melanin on peroxidase-catalyzed oxidation of aminodiphenyls. 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 Byelorussia, Minsk.

ABBREVIATIONS

BD: benzidine; **DA**: *o*-dianisidine; **DMBD**: 3,3′-dimethylbenzidine; **HP**: horseradish peroxidase; **TMBD**: 3,3′,5,5′-tetramethylbenzidine.

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Mycelium of this strain was grown in submerged culture on glucose-peptone medium (g/L): glucose, 10; peptone, 3; KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 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 6000*g* for 15 minutes. The sediment was dissolved in 2% NaOH.

The pigment was purefied 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 cultural 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 $KMnO_4$, H_2O_2, and $FeCl_3$ (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 *o*-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 aminodiphenyls was performed in 0.1 M citrate-acetate buffer (pH 5.5). The reaction mixture (2 mL) contained 7×10^{-11} M horseradish peroxidase (HP) (Reanal, Hungary; RZ = 2.8), 0.5 mM substrate, and 0.1 mM H_2O_2. The reaction mixture was incubated at 30 °C for 4 minutes. The reaction was started by adding H_2O_2 and then monitored by the accumulation of colored products. Complete oxidation of aminodiphenyls in 4 minutes was taken as 100% (Kukulyanskaya and Kurchenko, 1997).

DNA λ phage was exposed with products of peroxidase oxidation of *o*-dianisidine (DA) in 0.1 M citrate-acetate buffer (pH 5.5) at 30 °C for 30 minutes. The reaction mixture contained 10^{-5} M DA, 2×10^{-8} M HP, and 4×10^{-3} M H_2O_2; 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 H_2SO_4, and HNO_3); bleaching by H_2O_2, $Na_2S_2O_4$, $KMnO_4$, and bromine water; the ability to interact with $FeCl_3$ 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 mela-

nin required 11 mmol/g $KMnO_4$. 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 $FeCl_3$ (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 (Lyakh, 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 $E_{0.001\%}$ (λ = 465 nm) was 0.02, the content of COOH groups = 0.93%, CO groups = 1.05%, OCH_3 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 2.93×10^{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 hydrogen 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 (Lyakh and Ruban, 1972). Investigation of the dynamics of melanin formation and enzymatic activities, especially *p*- and *o*-diphenoloxidases, showed that the maxi-

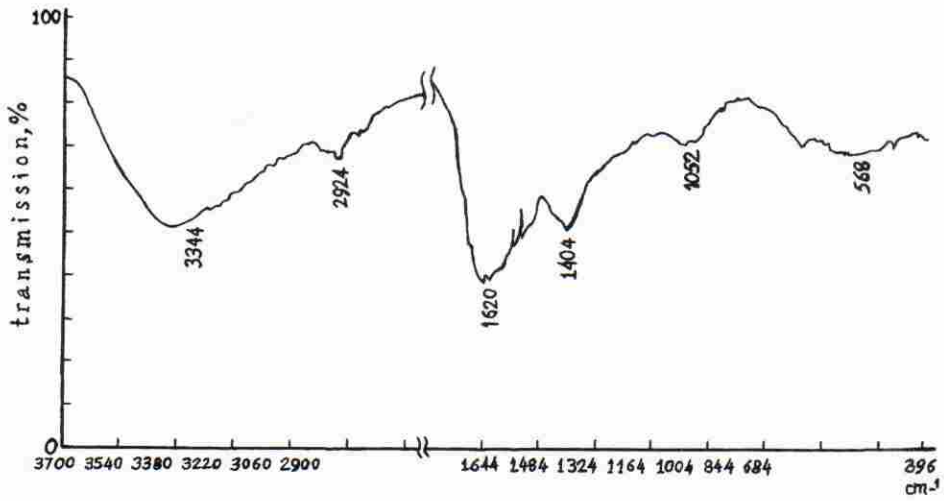

FIGURE 1. IR spectrum of the melanin isolated from *I. obliquus*.

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 0.008% 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-

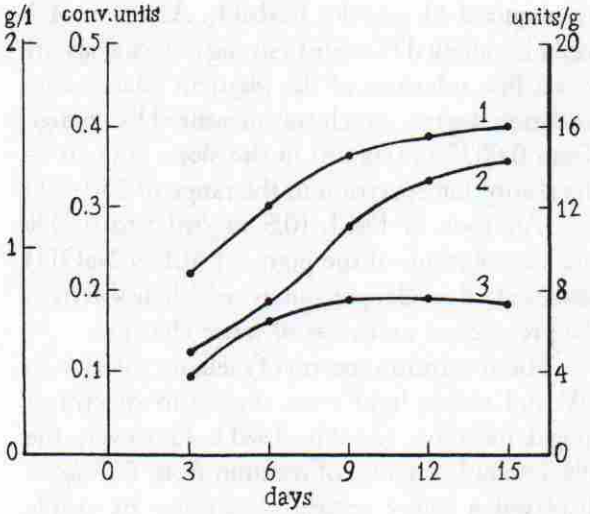

FIGURE 2. Dynamics of melanin production by *I. obliquus* and activity of its phenoloxidase complex: (1) *p*-diphenoloxidase (conventional unit); (2) *o*-diphenoloxidase (units/g); (3) melanin (g/L).

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

TABLE 1
Influence of Copper Ions on Melanin Production of *Inonotus obliquus*

Concentration of copper ions in medium,%	Biomass, g/L dry weight	Production of melanin		
		Mycelia, mg/g of dry mass	Culture liquid, g/L	Total,* g/L
0	6.2	14.52	0.24	0.33
0.001	5.8	15.69	0.30	0.39
0.002	8.0	17.40	0.34	0.48
0.003	6.7	30.00	0.39	0.59
0.004	6.4	42.57	0.68	0.95
0.008	4.8	104.90	0.90	1.40
0.012	4.0	80.40	0.70	1.02
0.016	4.5	67.29	0.65	0.95
0.020	5.0	40.13	0.50	0.70

* 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. obliques,* 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 polyphenol-oxidase complex: *I. obliquus* had higher activities of *o*- and *p*-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 melanins is very high (Lyakh and Ruban, 1972; Lyakh, 1981; Kukulyanskaya and Kurchenko, 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 oxidated substrates. Similar to other aromatic amines, these compounds are indirect carcinogens: for their transformation into highly reactive metabolities, 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 Kurchenko, 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–

TABLE 2
Influence of Pyrocatechol and Tyrosine on Melanin Production of *Inonotus obliquus*

	Production of melanin							
	Pyrocatechol				Tyrosine			
Concentration in medium, mM	Biomass, g/L	Mycelia, mg/g of dry mass	Cultural liquid, g/L	Total,* g/L	Biomass, g/L	Mycelia, mg/g of dry mass	Cultural liquid, g/L	Total,* g/L
0	3.12	16.08	0.35	0.40	8.75	16.00	0.26	0.40
0.1	4.16	16.84	0.74	0.81	9.50	15.76	0.75	0.80
1.0	4.36	29.87	0.84	0.97	3.90	23.26	0.81	0.90
5.0	1.98	40.43	0.72	0.90	3.58	116.66	0.97	1.39
10.0	4.47	38.00	0.68	0.85	5.56	241.07	1.03	2.37
15.0	—	—	—	—	6.74	288.09	1.50	3.44
20.0	—	—	—	—	7.60	300.48	1.88	4.16
25.0	—	—	—	—	7.58	290.00	1.80	4.00

—, 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.

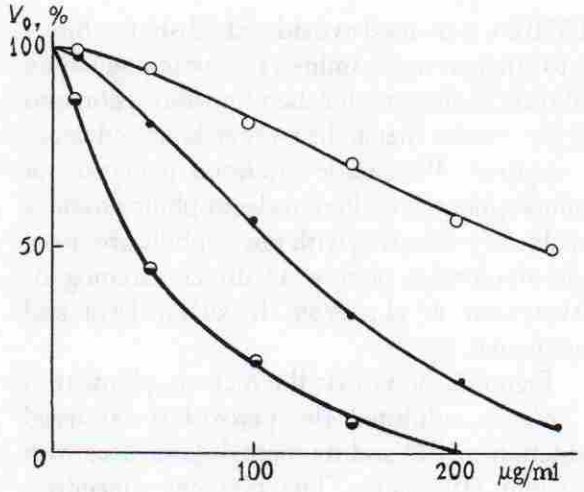

FIGURE 3. Effect of melanin concentration (μg/mL) on the rate of aminodiphenyl oxidation (V_0): (1) benzidine; (2) 3,3′–dimethylbenzidine; (3) 3,3′,5,5′–tetramethylbenzidine.

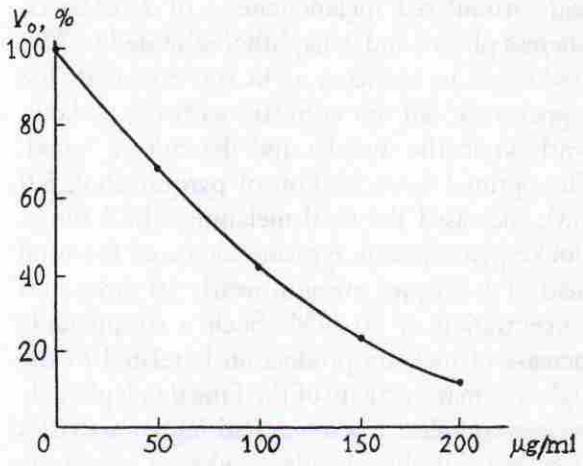

FIGURE 4. Effect of melanin concentration (μg/mL) on the rate of DA oxidation (V_0).

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 Kurchenko, 1997):

$$AH_2 + H_2O_2 + 2DNA \xrightarrow{HP} DNA\text{-}A\text{-}DNA + 2H_2O$$

where A is aminodiphenyl 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 aminodiphenyls 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-

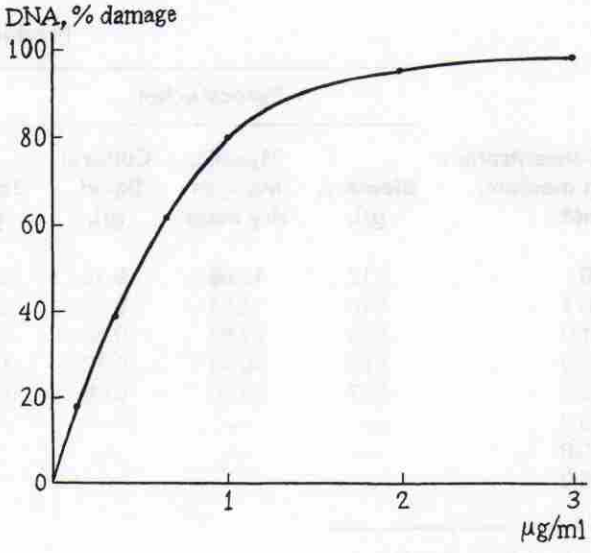

FIGURE 5. Effect of DA ($\times 10^{-5}$ μM) concentration on the damage of λ DNA phage cross-linking (%).

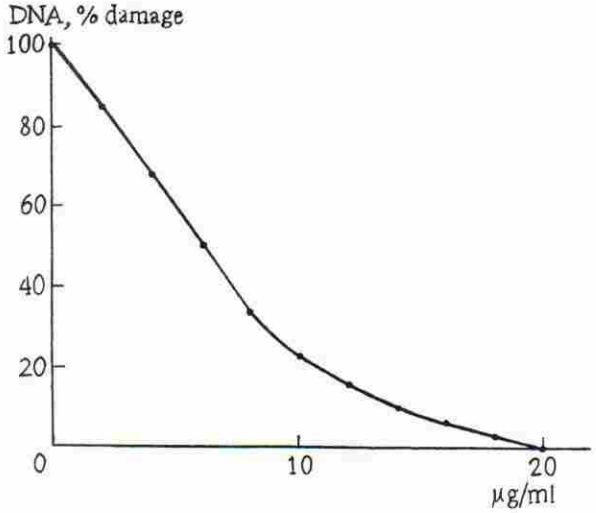

FIGURE 6. Effect of melanin concentration (μg/mL) on the damage of DNA (%) by products of peroxidase-catalyzed DA oxidation.

radical products of aminodiphenyl oxidation, preventing their transformation to diimines, the end products of this reaction. (4) Melanin protects DNA against free-radical damage through formation of DNA–melanin complexes (Kukulyanskaya and Kurchenko, 1997).

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

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