

Study of Melanin Complex from Medicinal Mushroom *Phellinus robustus* (P. Karst.) Bourd. et Galz. (Aphyllphoromycetideae)

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ABSTRACT: The production of melanin complex of *Phellinus robustus* in submerged conditions was studied. It was estimated that exo- and endomelanins consist of carbon (C), 37.2%–36.9%; nitrogen (N), 5.08%–5.30%; and hydrogen (H), 4.9%–5.2%. The content of carbonyl groups in exo- and endomelanins was 3.8%–1.58%; carboxyl, 0.66%–1.28%; total OH, 15.23%–15.09%, including aliphatic OH, 13.35%–12.5%; and phenolic OH, 1.88%–2.58%. Our results demonstrated that melanins from *Ph. robustus* have high antioxidant and genoprotective properties.

KEY WORDS: melanin complex, production, spectra, properties, cultural liquid, mycelium, *Phellinus robustus*

INTRODUCTION

Fruiting bodies of *Phellinus linteus* (Berk. et Curt.) Teng and *Ph. robustus* (P. Karst.) Bourd. et Galz. have been used in traditional medicine for treatment of stomach and arthritis. The modern investigations of *Phellinus* spp. demonstrated the antioxidant activity, antitumor action, hypoglycemic effects, and other medicinal effects (Ikekawa et al., 1968; Kim et al., 2001; Nakamura et al., 2003)

Phenolic compounds are a large and most interesting group of natural antioxidants, which can undergo reversible oxidation and reduction and display the properties of donors or acceptors of electrons and

protons. Animal and plant tocopherols, naphthoquinones, ubiquinones, flavonoids, and plant pigments are phenolic compounds (Bisko et al., 2005). However, melanin pigments produced by nearly all living organisms have received little attention. Melanins are heteropolymers formed by oxidative polymerization of tyrosine, dihydroxyphenylalanine, and catecholamines (stable free radicals) (Kurchenko et al., 2004; Ikonnikova et al., 2004). Melanins readily interact with free radicals and other reactive species as a result of the presence of unpaired electrons in their molecules.

Some xylotrophic higher Basidiomycetes are able to produce melanin pigments in high quantities while

ABBREVIATIONS

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

growing. According to the literature, these melanins have photo- and radioprotective properties as well as antioxidant and genoprotective action (Babitskaya et al., 2002; Kurchenko et al., 2004; Ikonnikova et al., 2004, 2006; Bisko et al., 2005). The study of melanin complex produced by xylophilic basidiomycete *Ph. robustus* in submerged conditions and an estimation of its physical and chemical properties, structure, and genoprotective and antioxidant activities was the aim of our work.

MATERIALS AND METHODS

Strain

The studied strain of *Ph. robustus* F-320 was obtained from the culture collection of the Institute of Microbiology of the National Academy of Sciences of Belarus, Minsk. It was isolated from fruiting bodies, which were grown on *Quercus robur* (Minsk region, Belarus).

Cultivation

Mycelium of this strain was grown in submerged culture on glucose-peptone medium, (g L⁻¹): glucose, 10; peptone, 3; K₂HPO₄, 1; KH₂PO₄, 1; MgSO₄ · 7H₂O, 0.25; corn extract, 20 mL; deionized water, 1 L; pH 5.5. Mycelium was grown in 250 mL flasks using the submerged cultivation technique at 24°C–26°C for 7 days. Mycelium was separated from the cultural liquid medium by filtration and washed with distilled water.

Methods of Extraction and Identification of Melanins

Endomelanin 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. Exomelanin was separated from cultural medium by acidation to pH

2.0 with concentrated HCl. The coagulable pigment was separated from the liquid by centrifugation at 6000 g for 15 minutes. 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.

The sediment exo- and endomelanins were dissolved in 2% NaOH. The alkali-soluble pigments were purified by gel filtration on Toyopearl HW-65 (Japan) in 0.01 N NaOH and lyophilized. Gel chromatography was performed in a Sephadex G-75 column (Pharmacia, Sweden) (Determan, 1967; Smychnik and Bambalov, 1980). Melanin pigments were identified by qualitative reaction 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 the 0.001%–0.1% solutions of pigment was estimated on spectrophotometer Specord M-40 (VEB Carl Zeiss Jena, Germany). IR spectra of melanins in tablet form with KBr was estimated on spectrophotometer Specord M-80. The quantity of paramagnetic centers (PMC) was estimated on radiospectrometer Varian E-112 (Varian, USA) with Mn⁺⁺ in powder MnO as standard.

Antioxidant Activity and Genoprotective Properties

The peroxidase-catalyzed oxidation of aminobiphenyls 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 H₂O₂. The reaction mixture was incubated at 30°C for 4 minutes. The reaction was started by adding H₂O₂ and was then monitored by the accumulation of colored products. Complete oxidation of *o*-dianisidine (DA) in 4 minutes was taken as 100% (Kukulyanskaya and Kurchenko, 1997).

Damage to bacteriophage-λ DNA by the products of peroxidase-mediated oxidation of DA in 0.1 M citrate–acetate buffer (pH 5.5) at 30°C for 30 minutes

was determined from changes in the electrophoretic mobility of cross-linked molecules of nucleic acids. Bacteriophage- λ DNA at a concentration of $0.2 \mu\text{g mL}^{-1}$ was added to the reaction mixture, which consisted of 10^{-5} M DA, 2×10^{-3} M HP, and 4×10^{-3} M H_2O_2 . Electrophoresis was performed in 0.9% agar gel in 0.1 M Tris-phosphate buffer (pH 8.0).

Fungal melanin was added to the reaction mixture at a concentration of $2\text{--}400 \mu\text{g mL}^{-1}$ to study its antioxidant and genoprotective effects.

RESULTS AND DISCUSSION

The qualitative reactions of the *Ph. robustus* pigment with KMnO_4 , H_2O_2 , and FeCl_3 is the basis of identification of this pigment as melanin.

Figure 1 demonstrates UV and visible spectrum of the absorption of 0.001% solution of melanin from *Ph. robustus*. This spectrum does not have the top that is characteristic for fungal melanins (Lyakh, 1981). However, the melanin from *Ph. robustus*, similar to the melanin from *Inonotus obliquus* (Pers.: Fr.) Pilat, displayed a lower absorption in visible light

than did melanins isolated from micromycetes (Malama et al., 1996; Babitskaya et al., 2002).

During submerged cultivation, *Ph. robustus* produced melanin in both mycelium and cultural liquid. The yield of exomelanin (in cultural liquid) was 2.4 g/L; the content of endomelanin in biomass was 1.8% of absolute dry biomass (a.d.m).

The molecular weight of exo- and endomelanines from *Ph. robustus* was 40–60 kDa. The obtained data indicate that the alkali fraction of exo- and endomelanins of *Ph. robustus* had molecular weight of 65–70 kDa and alcohol fraction of 40–45 kDa. The melanin of *Ph. robustus* also contained a small quantity of substances with molecular weight of 100–120 kDa. The analogous data were obtained for the melanin complex from *I. obliquus* (Babitskaya et al., 2002).

The investigation of the element composition of the pigments shows that exo- and endomelanin of *Ph. robustus* differed slightly (Table 1).

Analysis of the functional groups of endo- and exomelanins shows that the quantity of OCH_3 , aliphatic OH, and total hydroxyl groups was almost equal (Table 1). It has been demonstrated that the

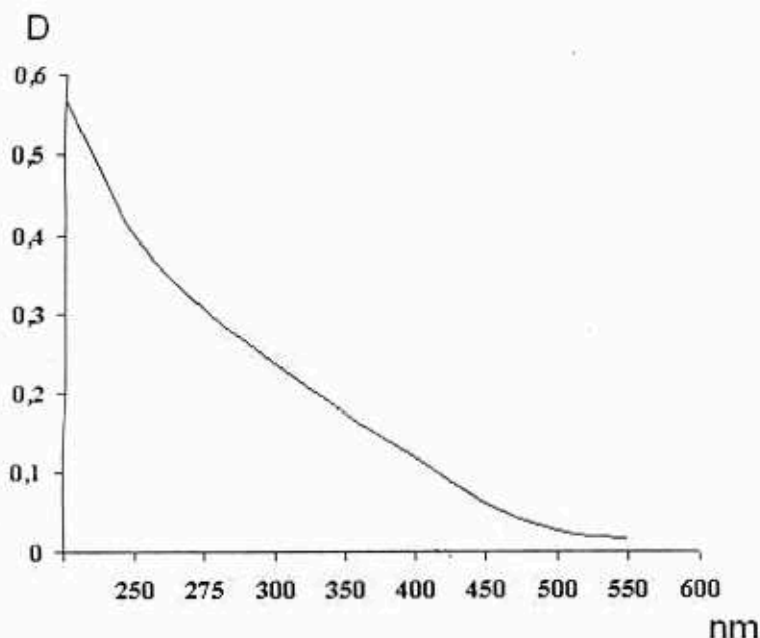


FIGURE 1. UV and visible spectra of melanin from *Phellinus robustus*. Horizontal axis, length of wave; vertical axis, density of absorption.

Table 1. Elemental and Functional Group Composition of Exo- and Endomelanins of *Phellinus robustus*, % a.d.m.

Element	Exomelanin	Endomelanin
C	37.20	36.90
N	5.08	5.30
H	4.90	5.20

Functional group	Exomelanin	Endomelanin
-OCH ₃	Trace	Trace
Aliphatic OH	13.35	12.50
Phenolic OH	1.88	2.58
Total OH	15.23	15.09
Carbonyl	3.80	1.58
Carboxyl	0.66	1.28

Note: a.d.m. = absolute dry biomass; C = carbon, N = nitrogen, H = hydrogen.

content of carboxyl groups in endomelanins was 2 times higher and phenolic OH was 27% higher than in exomelanins (Table 1). However, the content of carbonyl groups in exomelanins was 2 times higher than in endomelanins.

IR spectra of exo- and endomelanins from *Ph. robustus* were similar and had several bands typical for fungal melanin. The broad band in the region 3400 cm⁻¹ indicates the presence of ≡N-H groups (Fig. 2). The most intensive top, in the region 1660–1650 cm⁻¹, was typical for melanins and caused the valence vibration of the =C=O groups (Fig. 2).

IR spectra of the alkali-soluble and alkali-insoluble pigments have several general characteristics for both fractions bands: an intensive broad band in the regions 3400 to 3350 cm⁻¹ and a middle band in the regions 1680 to 1660 cm⁻¹ (Fig. 3). However, the spectrum of alkali-soluble pigment has a sharp intensive top of 1100 cm⁻¹, which probably is connected with the presence of carbohydrates—for example,

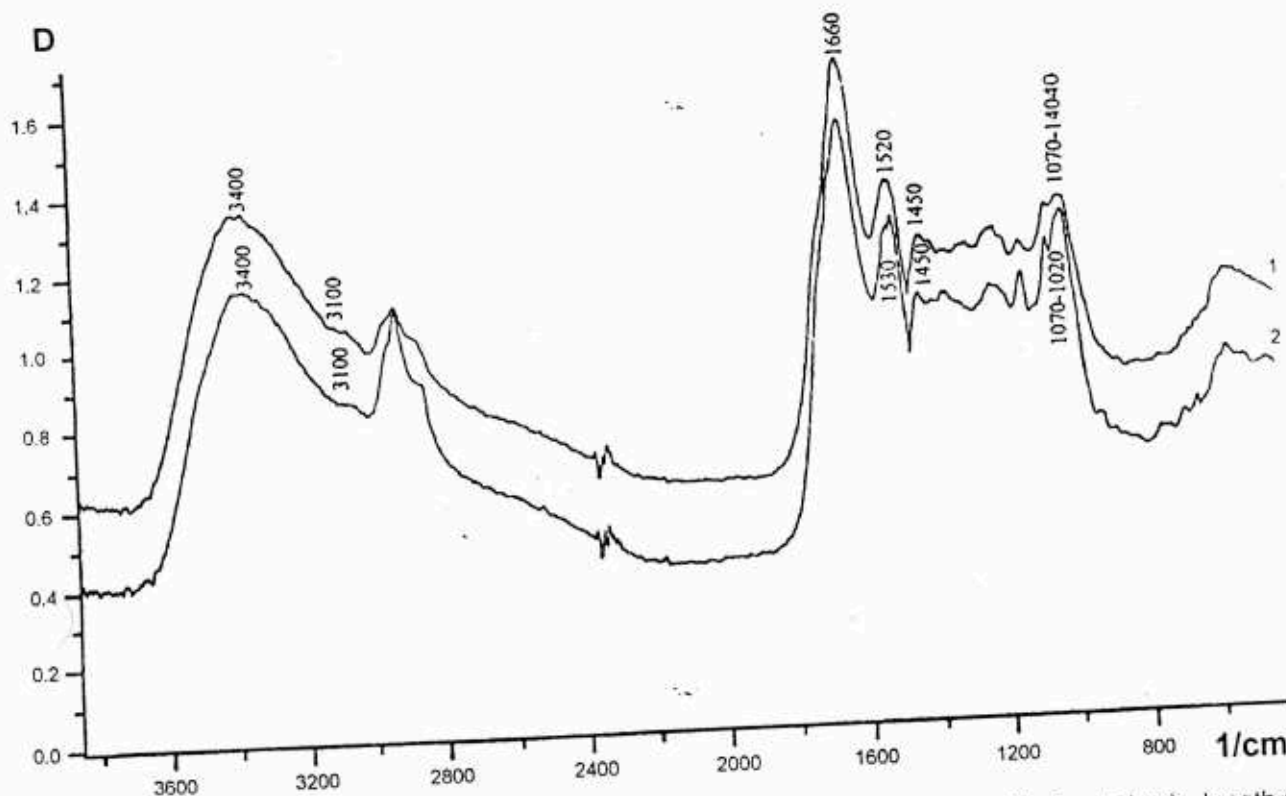


FIGURE 2. IR spectra of exo- (1) and endomelanins (2) from *Phellinus robustus*. Horizontal axis, length of wave; vertical axis, density of absorption.

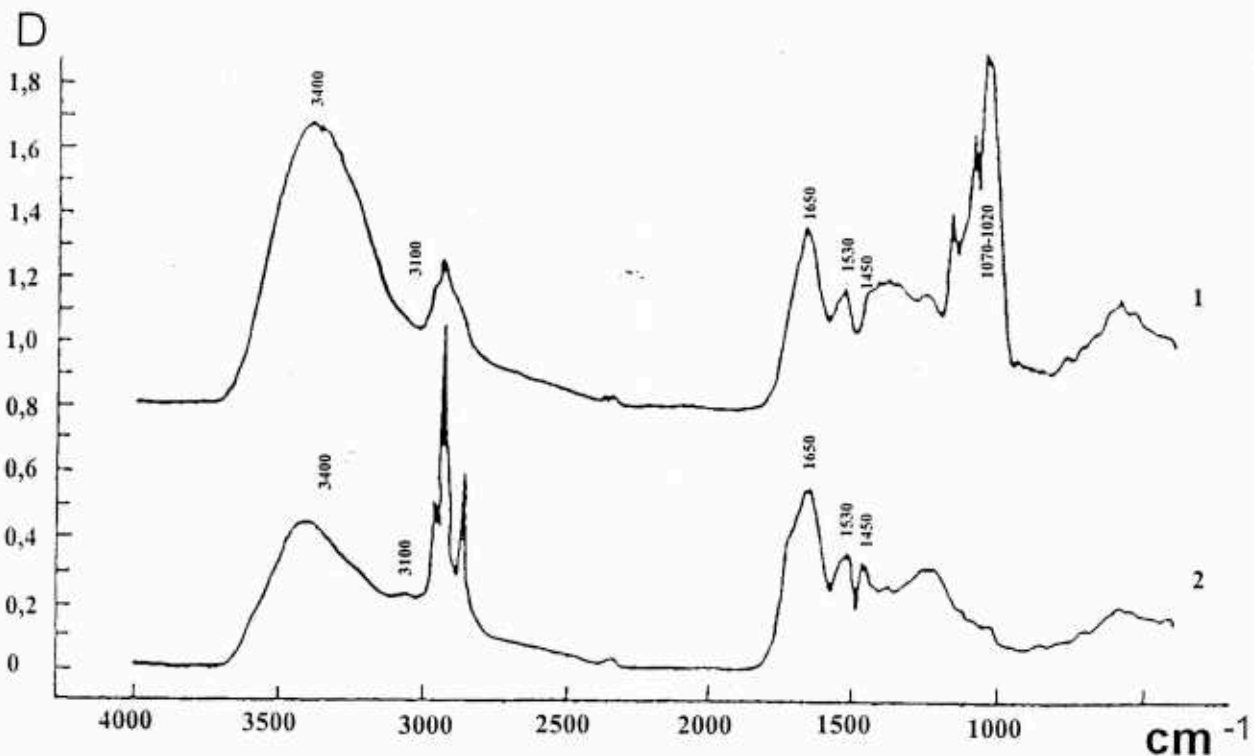


FIGURE 3. IR spectra of alkali-soluble (1) and alkali-insoluble (2) melanins from *Phellinus robustus*. Horizontal axis, length of wave; vertical axis, density of absorption.

amylase (Zbankov, 1964)—and the IR spectrum of alkali-insoluble pigment has a sharp intensive top in the region 2920 cm^{-1} ; this indicates the presence of CH_2 - and CH_3 - groups (Fig. 3). In the opinion of the investigators (Pridham and Woodhead, 1977), the absorption band in the region of 1600 cm^{-1} is connected with the guinoid structure of melanins.

We studied the antioxidant activity of melanin from *Ph. robustus* using benzidine (BD) and its methyl derivatives—3,3'-dimethylbenzidine (DMBD) and 3,3',5,5'-tetramethylbenzidine (TMBD)—which are indirect carcinogens. The conversion of these substances into high-reactive metabolites requires their metabolic activation. Peroxidase-mediated oxidation of aminobiphenyls results in the formation of electrophilic products that can react with nucleophilic groups of macromolecules (Scherba et al., 2000). Our data demonstrated that the inhibition efficiency of peroxidase-catalyzed oxidation of BD, DMBD, and TMBD by the melanin was different, in that it

decreases with an increase in the number of methyl substituents in the order BD, DMBD, and TMBD (Fig. 4). The melanin concentrations resulting in 50% inhibition of the oxidation rates of DMBD and TMBD were more than 2 and 6 times higher, respectively, than that of BD oxidation. Analogous data were obtained by using melanins from *I. obliquus* and several species of micromycetes (Kukulyanskaya and Kurchenko, 1997; Scherba et al., 2000).

Electrophilic products formed during metabolic activation of aminodiphenyls can interact with nucleophilic groups of biopolymers, in particular DNA, with the formation of DNA-DNA intermolecular links (Babitskaya et al., 2002). We used peroxidase-catalyzed DNA oxidation to study the influence of *Ph. robustus* melanin on this process. The main reaction product, the bifunctional reagent diimine, caused DNA cross-linking (Kukulyanskaya and Kurchenko, 1997): $\text{AH}_2 + \text{H}_2\text{O}_2 + 2\text{DNA} \xrightarrow{\text{HP}} \text{DNA-A-DNA} + 2\text{H}_2\text{O}$, where A is aminodiphenyl and HP is horseradish peroxidase.

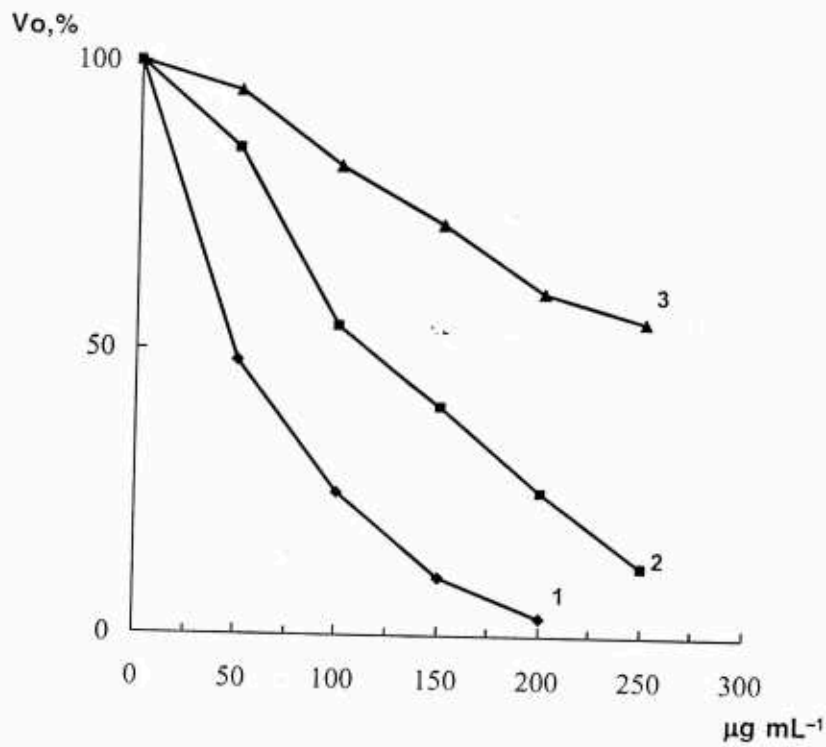


FIGURE 4. Effect of melanin concentration ($\mu\text{g mL}^{-1}$) on the rate of aminodiphenyl oxidation (V_o): 1, benzidine; 2, 3,3'-dimethylbenzidine; 3, 3,3',5,5'-tetramethylbenzidine. Complete oxidation of aminodiphenyls in 4 minutes was taken as 100%.

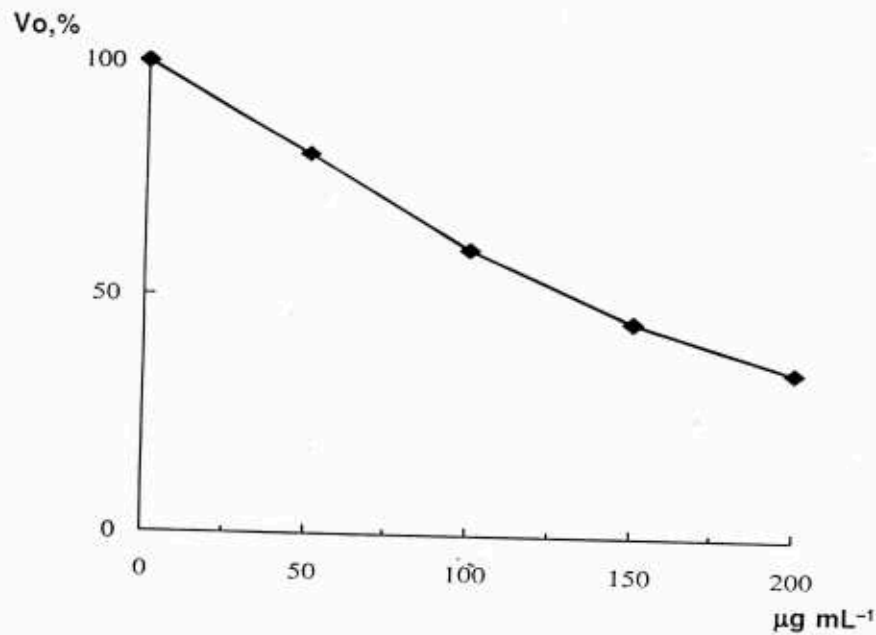


FIGURE 5. Effect of melanin concentration ($\mu\text{g mL}^{-1}$) on the rate of DA oxidation (V_o). Total oxidation of *o*-dianisidine (DA) in 4 minutes was taken as 100%.

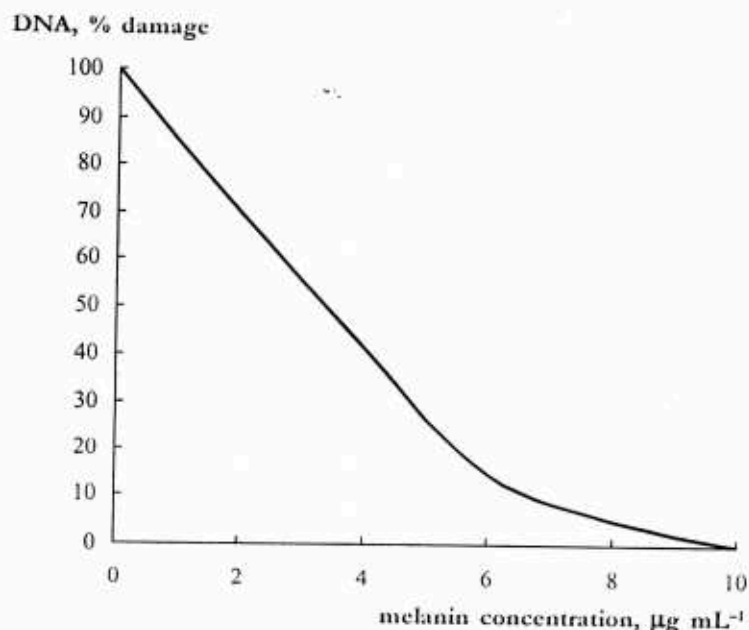


FIGURE 6. Effect of melanin concentration ($\mu\text{g mL}^{-1}$) on the damage of bacteriophage- λ DNA (%) by the products of peroxidase-catalyzed DA oxidation.

The antioxidant properties of melanin from *Ph. robustus* were studied in the reaction of peroxidase-catalyzed oxidation of DA. Figure 5 shows the dependence of the initial rate of DA oxidation on the melanin concentration in the reaction mixture. Melanin from *Ph. robustus*, as well as melanin from *I. obliquus*, inhibited this process at concentrations exceeding $20 \mu\text{g mL}^{-1}$ (Babitskaya et al., 2002). Melanin from *Ph. robustus* caused a 50% decrease of the rate of DA oxidation at a concentration of $135 \mu\text{g mL}^{-1}$ (Fig. 5). Some authors (Lyakh, 1981; Zhdanova and Vasilevskaya, 1998) reported that the ability of melanin pigments to interact with free radicals is due to the presence of PMC. Concentrations of PMC in melanins isolated from *Ph. robustus* are $2.3 \times 10^{17} \text{ spin g}^{-1}$. High concentrations of PMC in melanins provides their great electron-absorption capacity, which in turn fulfills the role of radio- and

photoprotector, to disactivate natural radicals that form in physical and chemical reactions.

The genoprotective properties of the studied melanin were investigated using the effect of the pigment on DNA I phase damage by the products of peroxidase-catalyzed DA oxidation. The rate of DNA damage depended on the initial DA concentration. It was demonstrated that at a concentration of 0.3×10^{-3} , DA caused 100% DNA damage (Babitskaya et al., 2002). The melanin from *Ph. robustus* at a concentration of $3.1 \mu\text{g mL}^{-1}$ prevented 50% of the DNA damage, and at $10 \mu\text{g mL}^{-1}$ the melanin completely prevented DNA damage (Fig. 6). Thus, the melanin from *Ph. robustus* has higher genoprotective properties than the melanin from *I. obliquus* (Babitskaya et al., 2002).

The data obtained in our study demonstrates the considerable antioxidant and genoprotective properties of melanin from *Ph. robustus in vitro*.

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