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Purification, characterisation and analysis of melanin extracted from *Tuber melanosporum* Vitt.

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A new method for the purification of melanin extracted from *Tuber melanosporum* Vitt. with KOH under nitrogen was developed. The physical and chemical properties of the resulting dark pigment were determined, including its elemental composition and ultraviolet (UV) and infrared (IR) spectra. The results of this study were calibrated using data obtained with synthetic dihydroxyphenylalanine (DOPA) melanin and indicate that (1) purification with chloroform gave the best results, as shown by elemental and infrared analysis; and (2) the synthetic and natural melanins have the same physical and chemical properties, with the same percentage of nitrogen, the same functional groups and the same degree of polymerisation. However, they showed large differences in: (1) the time necessary to become decolorised by oxidizing agents (NaOCl and H_2O_2); (2) the slope of log absorbance vs wavelength. These results and data found in the literature lead to a proposal that melanin of *T. melanosporum* is derived from a new nitrogen precursor. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Physical and chemical properties have been used to characterize melanins: high molecular weight; insolubility in water, aqueous acid, and common organic solvents; decolorisation by oxidizing agents (NaOCl and H_2O_2); and a positive reaction for polyphenols. The absorption spectra of their alkaline solutions show no maxima or minima in the ultraviolet or visible ranges and the plots of log absorbance vs wavelength give essentially straight lines with negative gradients.

Black pigments have been extracted from animal sources (Piattelli *et al.*, 1963; Nicolaus *et al.*, 1964; Chet *et al.*, 1967; Pawelek & Korner, 1982) and fungal sources (Schaeffer, 1953; Lingappa *et al.*, 1963; Sussman *et al.*, 1963; Bartnicki-Garcia & Reyes, 1964; Bainbridge *et al.*, 1971; Ellis & Griffiths, 1974; Bell *et al.*, 1976; Ramberg & McLaughlin, 1980; Stussi & Rast, 1981). They were found to conform with the physical and chemical properties characteristic of melanins. According to these investigations, melanins from plants and fungi are derived from GHB (γ-glutaminyl-4-hydroxybenzene), DHN (1,8-dihydroxy-naphthalene) or catechol precursors, whereas animal melanins come mostly from indoles (or sulfur-containing products).

Species of the genus *Tuber* are mycorrizal ascomycetous fungi with subterranean fruiting bodies. The presence of melanin in the ripe ascocarps of *T. melanosporum* has been recognized (Fonvieille *et al.*, 1990; Ragnelli *et al.*, 1992), but no studies have been carried out with regard to the precise determination of its origin and nature.

The aim of this work was: (1) to extract melanin from *T. melanosporum* and purify it; (2) to characterize it through its physical and chemical properties including its elemental composition and ultraviolet and infrared spectra; and (3) to compare the results obtained with those from synthetic DOPA melanin and data found in the literature relating to fungal melanins.

MATERIALS AND METHODS

Truffles

500 g of *T. melanosporum* were collected near Cahors (France) during March 1991. They were freeze-dried and stored in a freezer at −25°C.

Fungal melanin

Melanin was extracted from the gleba of *T. melanosporum* using a method similar to that reported for the extraction of melanin from walls isolated from different fungal species (Ellis & Griffiths, 1974). Briefly, 5 g of gleba were homogenised in 50 ml of 1 M KOH using an Ultraturax Rototransfo (8000 r.p.m. for 10 min) and then sonicated with an Ultrasonic disintegrator (12 Kc/s for 5 min). The melanin was extracted from the cell debris by treatment with hot alkali (1 M KOH at 100°C for 5 h) under reflux in an atmosphere of nitrogen. After filtration, the dark brown filtrate was acidified to pH 2. The resulting black precipitate was collected by centrifugation (10,000 r.p.m. for 10 min) and washed with distilled water. The precipitate of crude melanin was dried in a desiccator and kept under nitrogen.

Purification of melanin involved the elimination, by hydrolysis, of the carbohydrates, proteins and lipids associated with the crude residue.

Acid hydrolysis

The crude residue was hydrolysed with 10 ml of 7 M HCl for 2 h at 100°C. The non-hydrolysable residue was collected by centrifugation (1000 r.p.m. for 10 min) and firstly washed in 0.01 M HCl solution and then in distilled water. The precipitate, the non-hydrolysable melanin, was dried in a desiccator and kept under nitrogen.

Washing with chloroform

The non-hydrolysable melanin was redissolved in 5 ml of 1 M KOH and 2 ml of chloroform were added, followed by 0.2 ml of 1-butanol. The mixture was shaken for 30 min under nitrogen and centrifuged (6000 r.p.m. for 10 min). The chloroform phase was discarded and the operations were repeated twice on the alkaline melanin solution. Finally, the melanin was precipitated by acidification and washed with distilled water. The precipitate was dried in a desiccator and kept under nitrogen as 'pure' melanin.

Synthetic melanin. DOPA melanin was used to calibrate the results of the study; another fungal melanin would have been preferred, but DOPA melanin was the only one commercially available. It was purchased from Sigma (ref. M: 8631 batch N° 10 H 0292 8049–97–6). It did not receive any further treatment.

Analytical methods

The elemental composition of the melanin samples was determined by a C, H, N-elemental analyser, Carlo Erba Strumentazione Model 1106. Infrared (IR) spectra were recorded on an IR spectrometer Perkin Elmer Model 1600 FT. KBr pellets were obtained by pressing, under vacuum, uniformly prepared mixtures of 1 mg sample and 99 mg spectrometry grade KBr.

RESULTS AND DISCUSSION

Purification

Melanins are insoluble in acid solution. Therefore, the acid hydrolysis classically used to purify them is, probably, insufficient to remove all associated macromolecules. Further purification was achieved by washing the alkaline solution of melanin with chloroform. This treatment gave a better purification as illustrated by the infrared spectrum and the results of elemental analysis of the 'pure' melanin. Since this technique removes only small quantities of macromolecules, it is necessary to repeat it several times.

Characterization

The black pigment extracted from *T. melanosporum* presents all the physical and chemical properties common to the natural melanins previously identified. It was insoluble in both water and organic solvents: ethanol, hexane, acetone, benzene and chloroform. It was decolorised by oxidizing agents (NaOCl and H_2O_2) and gave a positive reaction for polyphenols by producing flocculent brown precipitates with $FeCl_3$. The results are summarized in Table 1, which also shows the properties of the DOPA melanin sample used for calibration. Both

Table 1. Diagnostic tests for *Tuber melanosporum* and synthetic DOPA melanins

Tests	'Pure' melanin of *Tuber melanosporum*	Synthetic DOPA melanin
Solubility in H_2O at 25°C	–	–
Solubility in organic solvents (ethanol, hexane, acetone,benzene and chloroform)	–	–
Solubility in 1 M KOH at100°C (2 mg/5 ml)	+	+
Reaction to oxidizing agent: NaOCl, 3.5% active Cl	+(15 min)	+(30 min) (10% ov V[a])
– H_2O_2 vol. 30 (10% of V[a])	+(20 min)	+(25 min)
Precipitation by 7 M HCl	+at pH 2	+at pH 2
Reaction for polyphenols with $FeCl_3$	+	+

+ Positive response.
–Negative response.
[a]Volume of alkaline solution of melanin (2 mg/5 ml of 1M KOH).

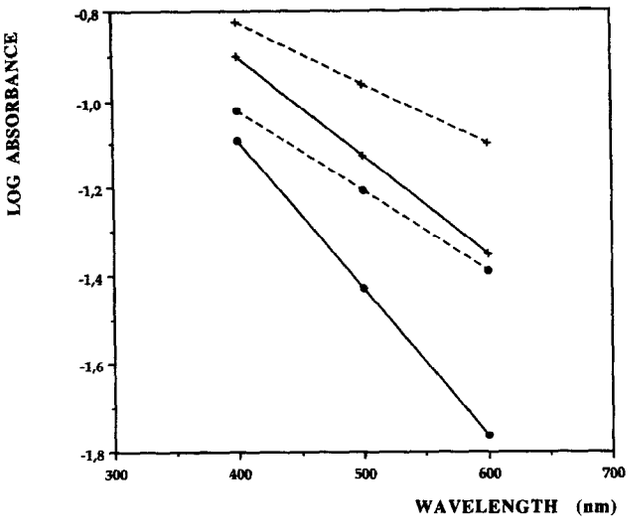

Fig. 1. Linear plots with negative slopes at t = 0 (dashed lines) and after 48 h (solid lines). Alkaline solution of melanin solution used 80 mg/ml. + *T. melanosporum* melanin. • Synthetic DOPA melanin.

melanins showed, in general, the same behaviour, except for the time required to bleach their alkaline solutions by the oxidizing agent used. This difference is important, because it depends on the structure and type of each melanin. Indeed, different reaction rates with these reagents are obtained with DOPA, catechol and DHN melanin (Zhdanova & Pokhodenko, 1970; Wheeler *et al.*, 1978).

The absorption spectra of alkaline melanin solutions showed no maxima or minima in the visible ranges (400–600 nm), and the plots of log absorbance versus wavelength gave lines with negative slopes (Fig. 1). The gradients −0.0013 and −0.0018 for 'pure' melanin of *T. melanosporum* and DOPA melanin, respectively, are similar to those found for the melanin extracted (Ellis & Griffiths, 1974) from the aleuriospores of *Epicoccum nigrum* (−0.0015), from sclerotia of *Verticillium dahliae* (−0.0015) and *Colletotrichum coccodes* (−0.0026), and from hyphae of *Amorphotheca resinea* (−0.0025). These slopes have often been used to characterize and compare melanins (Schaeffer, 1953; Bartnicki-Garcia & Reyes, 1964; Chet *et al.*, 1967). However, the alkaline solutions of melanin, on account of oxidation, did not give the same slope after they had been allowed to stand for short periods of time at room temperature. So, the slopes of the two solutions of the melanins studied were, after 48 h, −0.0022 for the melanin of *T. melanosporum* and −0.0033 for DOPA melanin. Accordingly, all extractions made in the present investigation and all alkaline solutions of the melanin obtained were kept under an atmosphere of nitrogen so as to avoid oxidation of the pigment.

Elemental composition

Analytical data of the samples studied are listed in Table 2. Acid hydrolysis and washing with chloroform essentially led to a loss of aliphatic chains, hydrocarbons and proteins. The data as such cannot distinguish aliphatic chains from hydrocarbons but they were deduced by analysis of infrared spectra data.

The N%, determined after acid hydrolysis, for the different melanins previously studied were: (1) 8.8% for γ-glutaminyl-4-hydroxybenzene (GHB) melanin from basidiospores cell walls of *Agaricus bisporus* (Rast *et al.*, 1981); (2) 6.3% for indole melanin (DOPA melanin); (3) 2–4% for heterogeneous melanin (Rowley & Pirt, 1972; Senesi *et al.*, 1987); and (4) traces for DHN or catechol melanin (Piattelli *et al.*, 1963).

For the 'pure' melanin of *T. melanosporum*, we have found the N% and S content to be 6.16 and 9.23, respectively (Table 2); thus it must be derived from a nitrogen-sulfur precursor and therefore it differs from DHN and catechol melanins (phenolic type) (Bell *et al.*, 1976; Piattelli *et al.*, 1963).

Infrared spectra

Infrared spectrometric techniques gave information on the main functional groups in the melanin structure (Bull, 1970; Schnitzer *et al.*, 1973; Ellis & Griffiths, 1974; Senesi *et al.*, 1987; Paim *et al.*, 1990). A detailed comparative analysis of the infrared spectra of the melanins studied (Fig. 2A–2D) may supply valuable information on the effect of each treatment used to purify the melanin and the distinct functional groups prevailing in the

Table 2. Analytical data of samples studied and some fungal melanins

Samples	%					Atomic ratio		
	C	H	N	O	S[a]	C/H	C/N	O/C
Crude melanin	55.06	7.77	7.72	—	—	0.59	9	—
Non-hydrolysable melanin	68.35	9.20	2.87	—	—	0.61	33	—
'Pure' melanin	52.10	4.20	6.16	28.31	9.23	1.03	10	0.40
Synthetic DOPA melanin	47.40	3.60	6.29	39.43	3.28	1.09	9	0.62
GHB melanin[b]	57.9	3.8	8.8	—	—	—	—	—
Catechol melanin[c]	61.81	3.89	Traces	—	—	—	—	—

[a]Calculated by difference: % (S) = 100-%(C + H + N + O).
[b]As reported by Rast *et al.* (1981).
[c]As reported by Piattelli *et al.* (1963).

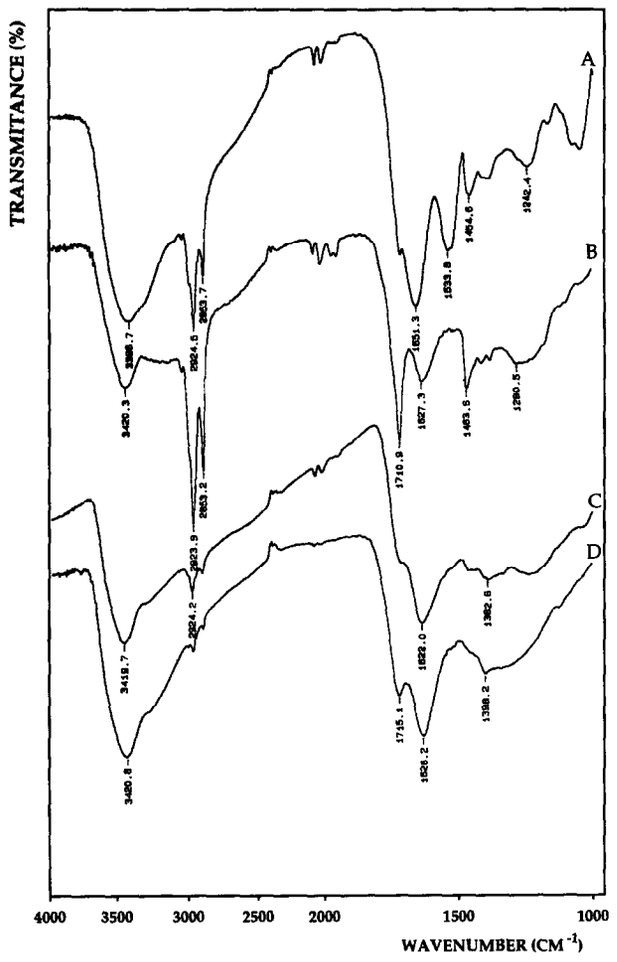

Fig. 2. Infrared spectra of melanins extracted from *Tuber melanosporum*: (A) crude melanin; (B) non-hydrolysable melanin; (C) 'pure' melanin; and (D) synthetic DOPA melanin.

various samples. The spectra of all the preparations display: (1) a strong, broad band at 3400 cm^{-1}, attributed to stretching vibrations of OH and NH_2 groups; (2) a strong band at 1650–1620 cm^{-1} due to the vibrations of aromatic C=C, of amide I C=O and/or of COO^- groups.

However, spectra (A) and (B) of the residues before and after acid hydrolysis showed bands that were strongly reduced or absent in the spectrum (C) of 'pure' melanin and DOPA melanin. These are (1) a strong band at 2930–2920 cm^{-1}; (2) a medium band at 2860–2850 cm^{-1} assigned to stretching vibration of aliphatic C–H; and (3) a band at 1460–1450 cm^{-1} attributed to bending vibration of aliphatic C–H.

The analysis of three spectra of melanins from *T. melanosporum* (A, B, and C) showed that (1) after acid hydrolysis of crude extract, the strong, broad band (3400 cm^{-1}), attributed to stretching vibrations of OH and NH_2 groups, and the band (1650–1620 cm^{-1}), due to C=O of amide I, aromatic C=C and/or COO^- groups, were weak (formation of H_2O and NH_3, and hydration of amide I to the corresponding acid, saturation of C=C and/or protonation of COO^- groups, respectively); (2) the bands characteristic of aliphatic C–H (2980–2920 cm^{-1}, 2860–2850 cm^{-1}, and 1460–1450 cm^{-1}) were absent or strongly reduced in the spectrum of the 'pure' melanin.

The spectrum of synthetic DOPA melanin shows exactly the same bands as *T. melanosporum* melanin, except for a weak band at 1720–1710 cm^{-1}, due to stretching vibrations of numerous C=O-containing groups (Filip *et al.*, 1974; Schnitzer & Neyroud, 1975; Russell *et al.*, 1983).

Strong absorption in this region (Spectrum B) is caused by free carboxyl groups which are practically absent from the 'pure' melanin. They are probably involved in the formation of hydrogen-bonding and partly ionized as carboxylate groups (Saiz Jimenez & Martin Martinez, 1972; Filip *et al.*, 1974).

From the results obtained in the present study: (1) acid hydrolysis followed by washing with chloroform gave the highest degree of melanin purification; (2) *T. melanosporum* melanin (N% evaluated at 6.16) is certainly different from DHN and catechol melanins (trace only of N). It is also different from GHB melanin because it does not display the strong band at 1100 cm^{-1} characteristic of this type of melanin (Rast *et al.*, 1981).

Tuber melanosporum melanin is derived from an unknown nitrogen precursor. Degradation of *T. melanosporum* melanin by potassium permanganate and identification of the decomposition products by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) are underway and should clarify the origin of this melanin.

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