

MELANIN IN BARLEY: FROM ISOLATION TO A POSSIBILITY TO INFLUENCE THE ACTIVITY OF BIOTRANSFORMATION ENZYMES

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ABSTRACT

This study describes the isolation and characterization of melanin from plant matrices using a modified version of the alkaline extraction method originally described by Sava et al. (2001). Melanin was isolated from barley (variety Nudimelanocriton) and purified through a series of organic solvent treatments, acidic hydrolysis, and repeated precipitations, yielding approximately 5 mg of pure melanin from 12 g of barley grain. Isolated melanin exhibited its characteristic properties

as insolubility in water, acids, and organic solvents, while being soluble in alkaline media and precipitable below pH 3.

Consistent with findings from Caldas et al. (2020) and Glagoleva et Shoeva (2020), melanin samples exhibited in alkaline media a broad-band almost monotonous decrease of UV/VIS absorption from initially 200 nm, with unresolved absorption band at about 270 nm, indicative of complex conjugated structures of aromatic character.

Additionally, effect of melanin on cytochrome P450 1A1/2 enzyme activity was assessed in HepG2 cells using 7-ethoxyresorufin O-dealkylation and high-performance liquid chromatography. Melanin at three concentrations (10 µg/mL, 1 µg/mL and 0.1 µg/mL) did not significantly induce cytochrome P450 1A1/2 enzyme activity (in contrast to CYP1A1/2 potent inducer, 2,3,7,8-tetrachlordibenzodioxin resulting in a nearly sixtyfold increase). These findings contribute to understanding the physicochemical properties of barley-derived melanin and its interaction with hepatic enzymes of xenobiotic biotransformation (as CYP1A1/2).

Keywords: melanin; allomelanin; barley; cytochrome P450; enzyme activity; cell culture; HepG2; UV-visible spectrophotometry

INTRODUCTION

Plant pigments are vital compounds that create the vibrant colours observed throughout the plant kingdom. These pigments not only provide plants with their varied hues but also play crucial roles in important biological processes. While pigments like anthocyanins and carotenoids are well-known, melanins are among the least studied. Though not essential for growth and development, melanin pigments

enhance the survival and competitiveness of species in specific environments (Solano et al., 2014). Melanin, a negatively charged brown or black biomacromolecule found in plants and other living organisms, has shown promising applications in various research fields, including biomedicine, dermocosmetics, nanotechnology, and bioengineering (Hou et al., 2019; Caldas et al., 2020).

In plants, melanin compounds play a crucial role in various functions essential for survival and adaptation. These pigments – in living systems as plants, animals, as well as in humans – primarily protect against environmental stressors such as ultraviolet (UV) radiation, extreme temperatures, microbial attacks, and oxidative damage, as well as chelating metals and, to some extent, involvement in nervous systems. These functions are determined by melanin's chemical and physical properties, including its molecular, supramolecular, and aggregate-level structures (Caldas et al., 2020; Pralea et al., 2019).

The high heterogeneity of melanins makes their analytical characterization quite challenging. Studies on melanin structures has been rare due to difficulties in isolating melanins from natural sources and their poor solubility. Melanins are insoluble in water and common organic solvents like hexane, chloroform, ethyl acetate, ethanol, methanol, or acetone, and can only be dissolved in alkaline solutions (Wang et al., 2006). However, recent advancements have been made in determining the structure of plant melanins using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). This technique successfully resolved the structure of oat melanin, revealing it as a homopolymer composed of p-coumaric acid, primarily

consisting of low molecular weight oligomers with 3-9 monomer units (Varga et al., 2016).

Melanins can be organized based on their precursor molecules in five categories: eumelanin, pheomelanin, neuromelanin, pyomelanin, and allomelanin (Singla et al., 2021; Peralta et al., 2023). Eumelanin is found in animals, microorganisms, and some fungi. It is derived from tyrosine and is black or brown in colour. Pheomelanin is endemic to higher animals, mammals, or birds. It is also a tyrosine derivative and is red or yellow in colour (Wakamatsu et al., 2002; Bell et Wheeler, 1986). Neuromelanin is predominantly found in the brain, particularly in regions like the substantia nigra and locus coeruleus. It is also derived from tyrosine (Zucca et al., 2014) and its biological function is still under investigation. Plant and fungal allomelanin is often nitrogen-deficient synthesized from phenolic compounds like caffeic, chlorogenic or gallic acid (Guo et al., 2023; Solano et al., 2014).

While the primary structure of melanin, composed of DHI (5,6-dihydroxyindole) and DHICA (5,6-dihydroxyindole-2-carboxylic acid), is widely accepted in the scientific community, its final macromolecular structure remains unclear. Depending on the coupling site of the monomers, melanin can form either a large heteropolymer or, as some researchers suggest, a stacked oligomer. Nonetheless, both structural forms – DHI and DHICA result in melanin particles with identical chemical properties (Caldas et al., 2020).

Given that varieties of products enriched with melanin content are beginning to be developed, it is highly necessary to study its possible influence on the human organism, specifically on the metabolism of foreign substances mediated by cytochromes P450 (CYPs). All of the

recent findings may be a key helping to uncover the potential interaction between melanin pigments and the main enzymes of metabolism of xenobiotics incl. drugs. Melanin may modulate the activity of CYPs through physical binding or sequestration, altering their substrate accessibility and catalytic efficiency. Among human drug-metabolizing enzymes, forms of cytochrome P450 labeled as CYP1A1 and 1A2 are important for metabolism of aromatic structures and for their activation in chemical carcinogenesis. These two enzymes are often collectively named as CYP1A1/2 as they share high amino acid sequence similarity and share also most of substrates incl. the 7-ethoxyresorufin, used also here to evaluate their possible induction and hence an increase of enzyme activity (Anzenbacher et Anzenbacherová, 2001). Revelation of potential crosstalk between melanin and CYP may be crucial for evaluation of safety of future melanin-cereal products.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO), penicillin, streptomycin, ethanol, chloroform, methanol, acetonitrile, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin, sodium pyruvate, insulin, glucagon, NADP⁺, and Eagle's Minimal Essential Medium (EMEM 4655) were obtained from Merck (Darmstadt, Germany). Minimum Essential Medium (MEM 31095), foetal bovine serum, glutamin, and non-essential amino acids were obtained from Gibco (Billings, MT, USA). 7-ethoxyresorufin (ETRR) was obtained from Lipomed (Arlesheim, Switzerland). All chemicals were of the highest purity available.

Isolation and purification

The pigment was isolated from the plant matrix via alkaline extraction as described by Sava et al. (2001) with modifications. The grounded barley (2 g) was suspended in 0.5M NaOH (24 mL) in a glass flask. The sample was heated on a heater (IKA Labortechnik) at 100 °C for 1 h with mixing. Supernatant was filtered through gauze and acidified with 7M HCl to pH 2. Mixture was stored at room temperature for 24 h.

After centrifugation at 10000 rpm for 10 min, purification was conducted involving acidic hydrolysis 7 M HCl (12 mL) of the residue at 100 °C for 2 h, to remove carbohydrates and proteins. The resulting suspension was centrifuged at 10000 rpm for 10 min and the residue was washed with water until the supernatant was neutral. The brown-black solid material was washed in turn with chloroform, ethyl acetate and ethanol to eliminate lipids and repeatedly precipitated to extract phenolic compounds. The precipitate was filtered off and washed with water.

The extracted pigment material appears as a dark, glossy powder. It is insoluble in water and most organic solvents, partially soluble in concentrated sulfuric and nitric acids, and completely soluble in sodium hydroxide (Glagoleva et al., 2020).

UV-Vis spectrophotometry

Regarding UV and visible absorption spectrum, melanins of different origin evidence a broad-band monotonic absorption in visible and ultraviolet spectrum with the maximum at 196–300 nm (Caldas et al., 2020, Glagoleva et al., 2020). Melanin was dissolved in 20 mM potassium phosphate buffer (KH₂PO₄/K₂HPO₄) with different pH at

final concentration of 50 µg/mL with corresponding solution as a reference. The UV–visible absorption spectrum of melanin was scanned in the wavelength range of 600–200 nm with a UV–visible spectrophotometer (UV-2700i, Shimadzu).

Cell culture

HepG2 cells (ATCC) were cultured in Dulbecco's Modified Eagles medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO₂ at 37 °C and medium was refreshed every three or four days with subculturing.

Preparation of melanin solutions

Melanin was dissolved in pure DMSO. A series of 10-fold dilutions were then made from the stock solution using DMSO. These DMSO solutions were subsequently added to the culture medium, resulting in a final DMSO concentration of 0.1%.

Measurement of induction of CYP1A/2 enzyme activities in HepG2 cells

HepG2 cells were trypsinized, counted and resuspended in culture medium to a final concentration of 200 000 cells/ well for the CYP1A1/2 assay. 1 mL cell suspension was added to a well of a 12 well culture plate (Merc, Darmstadt, Germany). The 12 well plates were incubated for 24 h in a humidified atmosphere at 37 °C under 5% CO₂. The enzymatic activity of CYP1A1/2 was measured via the incubation of HepG2 cells with melanin (concentration range: 10 µg/mL–0,1 µg/mL), TCDD as an inductor and vehicle alone (0,1% DMSO) in a 12-well plate for 24 h. Thereafter, ETRR was added to a

final concentration of 2,6 μ M in a culture medium and incubated for 2 h. The supernatant was transferred into Eppendorf tubes, diluted with methanol (1:2), and centrifuged at 14000 RPM at 4 °C for 10 min. The activity of CYP 1A1/2 was measured as an amount of ETRR metabolite – resorufin, using HPLC with fluorescent detection (excitation 535 nm, emission 585 nm) according to methods described by Chang et Waxman (2006).

Cell viability was assessed using the MTT assay. This colorimetric test relies on the activity of oxidoreductase enzymes in living cells to convert the tetrazolium dye MTT into formazan crystals. After 24 hours of treatment, the cells were rinsed with PBS and incubated with a 5 mg/mL MTT solution diluted in serum-free medium (1:10) for one hour. The solution was then removed, and the formazan crystals were dissolved in a DMSO/0.1% NH₃ solution. Absorbance was measured spectrophotometrically at 540 nm.

RESULTS AND DISCUSSION

Isolation and purification

In this study, we isolated melanin from barley (var. Nudimelanocriton) (Fig. 1). After further purification with organic solvents, acidic hydrolysis and repeated precipitation, approx. mg of pure melanin was obtained from 12 g of barley grain (Fig. 2). It was insoluble in water, acids and organic solvents, soluble in alkaline media and could be precipitated by acidification below pH 3.



Figure 1. Barley (var. *Caesar*) without increased melanin content (left), barley (var. *Nudimelanocriton*) enriched with melanin content (right).



Figure 2. Melanin isolated from barley, var. *Nudimelanocriton*

UV-Vis spectrophotometry

The absorbance in alkaline aqueous media was higher than in neutral or acid ones (Fig. 3), indicating that melanin prepared was relatively stable under the alkaline pH. The absorbance gradually increased with the pH. The maximum absorption of melanin was reached at about 200 nm and decreased towards the visible region, which is a characteristic feature of melanin absorption spectra ascribed to apparently complex conjugated structures of the melanin molecules (Cockell et John, 1999). No distinct absorption peaks at 260 nm and 280 nm were found, indicating that the content of nucleic acid and

protein impurities was low (Hou et al., 2019). However, an unresolved absorption band at about 270 nm is probably indicative of complex conjugated structures of aromatic character (Fig. 3 and 4).

For melanin samples dissolved in potassium buffer with pH values from 8 to 10, no significant difference was observed between individual spectra (Fig. 4). This optical property of melanin samples in alkaline media may help in choosing an optimal wavelength for detection of melanin in HPLC separation.

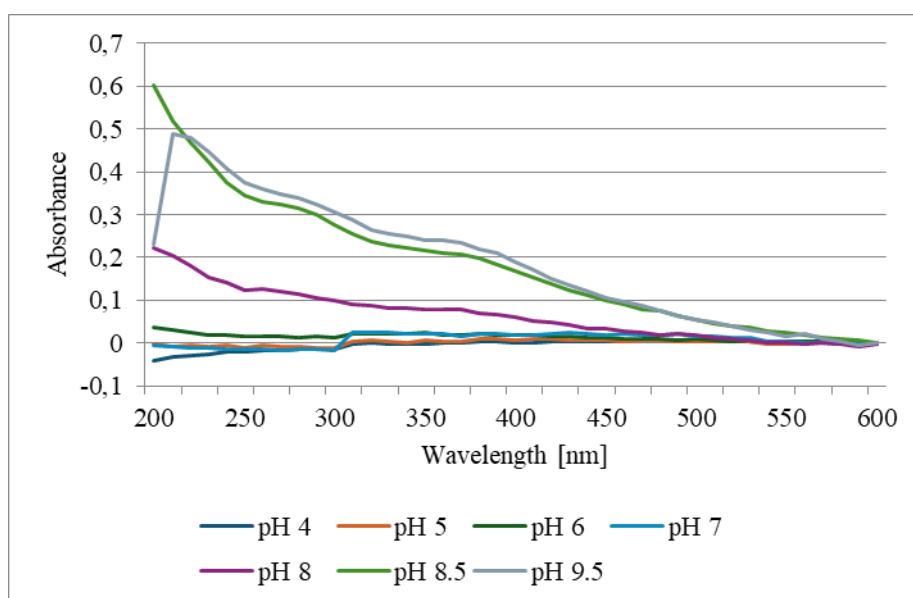


Figure 3. UV–VIS absorbance spectrum of melanin. 50 μg /mL of melanin dissolved in solutions of potassium buffer with different pH.

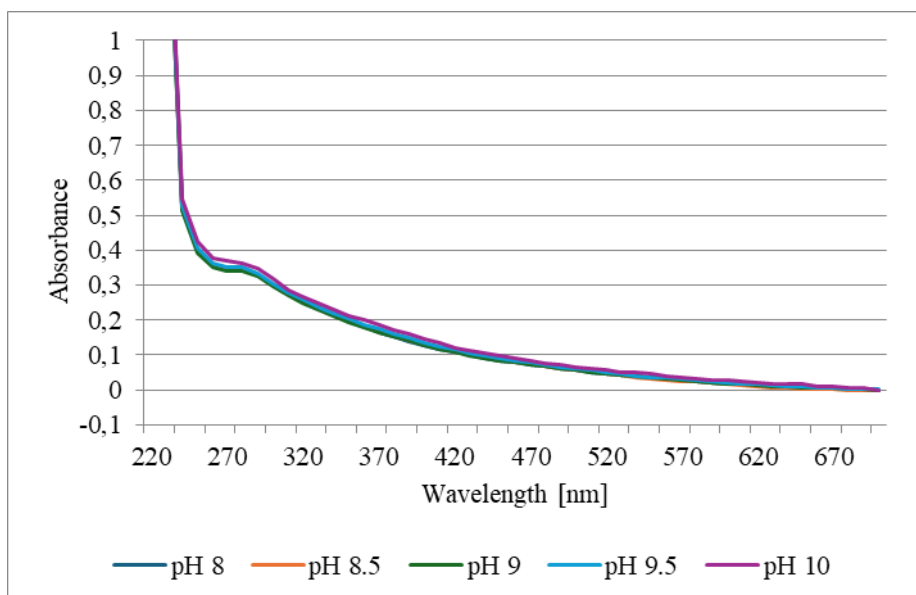


Figure 4. UV–VIS absorbance spectrum of melanin. 50 µg /mL of melanin dissolved in solutions of potassium buffer with different pH.

Induction of CYP1A1/2 in HepG2 cells

The activity of CYP1A1/2 was determined using O-dealkylation of prototypical CYP1A1/2 substrate, 7-ethoxyresorufin, as described by Pearce et al. 1996 and performing high performance liquid chromatography. Melanin tested in three different concentrations showed no significant induction above the basal CYP1A1/2 activity in HepG2 cells. TCDD was used as a control being a potent inducer, with a maximum activity almost sixty times greater than rest of the samples (Fig. 5). Melanin also showed no influence on viability of the HepG2 cell line (Fig. 6).

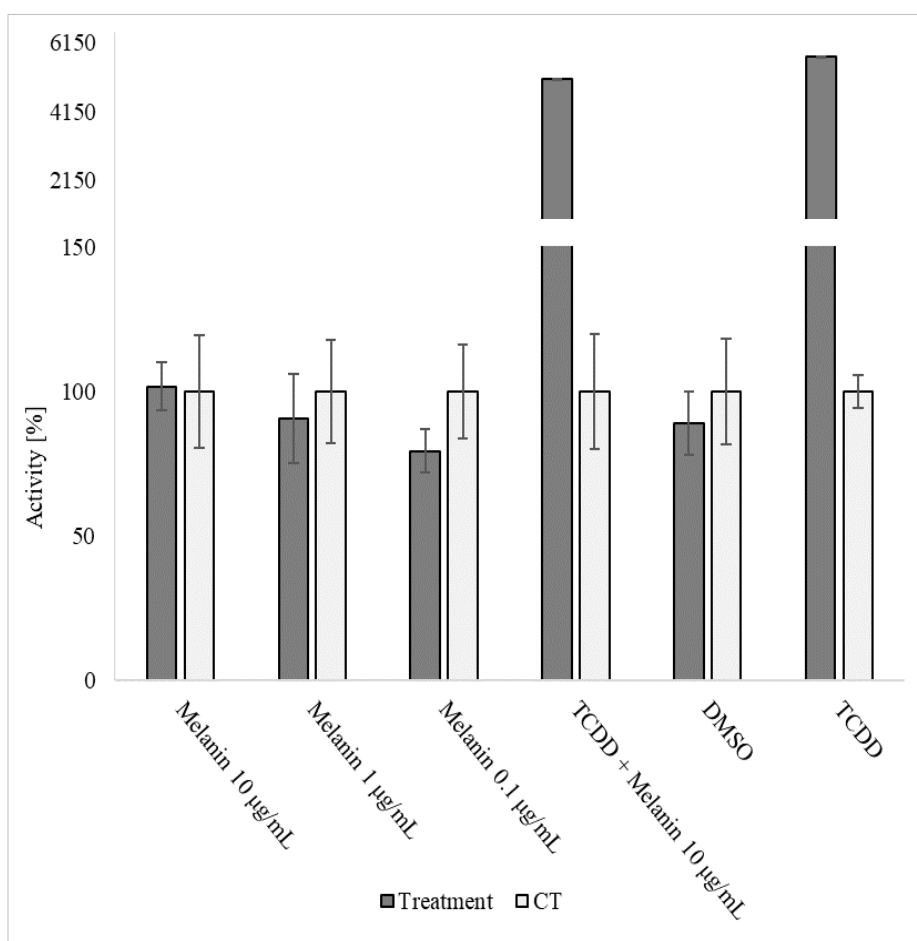


Figure 5. Effect of melanin on the activity of CYP1A1/2 cells after 24 h of treatment. 5 nM TCDD was used as a positive control. All experiments were performed in triplicate, all data were normalized to the control (CT), and each bar represents the mean $\pm$ SD of independent experiments.

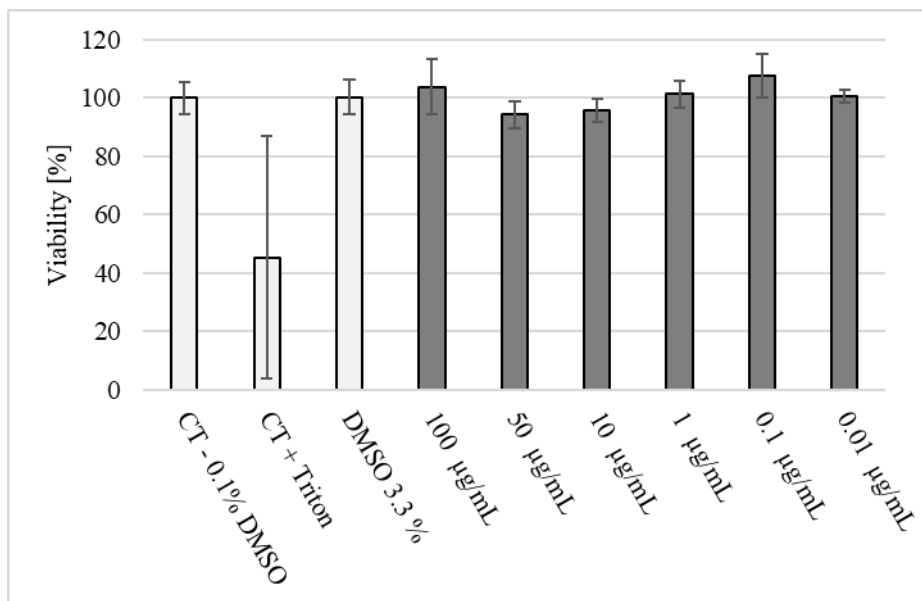


Figure 6: Effect of melanin on the viability of HepG2 cells after 24 h of treatment. All experiments were performed in triplicate, all data were normalized to the control (CT), and each bar represents the mean $\pm$ SD of independent experiments.

CONCLUSION

In conclusion, successful extraction and purification of melanin from barley (var. Nudimelanocriton) was realized yielding 5 mg of pure melanin from 12 g of barley grain. Melanin exhibited expected solubility properties, being insoluble in water, acids, and organic solvents, yet soluble in alkaline media and precipitable below pH 3. UV spectral analysis revealed characteristic absorption properties, with maximal absorption beyond 200 nm and stability under alkaline conditions. The absence of peaks at 260 nm and 280 nm confirmed minimal contamination by nucleic acids and proteins, however, an unresolved and broad band at about 270 nm of samples in alkaline

media indicates presence of an absorption due to aromatic structures. These spectral properties may help in HPLC detection of melanin in alkaline matrices. Furthermore, the study showed that barley-derived melanin did not significantly induce CYP1A1/2 activity in HepG2 cells, when compared with the potent inducer TCDD. These findings contribute to the understanding of the properties of barley-derived melanin, offering insights necessary for its potential applications.

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