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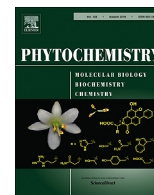

Structural characterization of allomelanin from black oat

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The brown to black coloration found in plants is due to the melanins, which have been relatively poorly investigated among the plant pigments. The aim of this work was to study the dark pigment extracted from the black oat hull with respect to composition and structure. Ultraviolet–visible (UV–Vis) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and Fourier transform infrared (FT-IR) spectroscopy were applied for the characterization of the pigment. UV–Vis spectroscopy revealed that the extracted material displays a broadband, structureless absorption profile a common feature of melanins. MALDI-TOF MS measurements demonstrated that oat melanin is a homopolymer built up from *p*-coumaric acid and consists mainly of low molecular weight (527–1499 Da) oligomers of 3–9 monomer units. The tetramer oligomer proved to be dominant. The results of the FT-IR analysis indicated that oat melanin is a fully conjugated aromatic system containing tetrasubstituted aromatic rings linked by C–C coupling. The *in vitro* preparation of melanin from *p*-coumaric acid by horseradish peroxidase was performed for comparison. The resulting polymer consisted of oligomers of 4–9 monomer units similarly to those in oat melanin. However, the building blocks proved to be connected to each other via C–O–C linkages in contrast with the C–C linkages in oat melanin.

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1. Introduction

Among the plant pigments the chemistry, biochemistry and biosynthetic pathways of the flavonoids responsible for the pale-yellow to blue coloration and the yellow to red carotenoids and betalains have been thoroughly investigated (Tanaka et al., 2008; Grotewold, 2006). In contrast the origin and nature of the black coloration in plants have been poorly studied (Nicolaus, 1968).

The brown to black pigments found in animals, microorganisms and plants are melanins, pigments that are not essential for development, but rather have a defensive role in all organisms. In man and other vertebrates, melanins function in camouflage (Morison, 1985) and photoprotection (Ortonne, 2002). Melanins protect fungi against microbial and environmental stresses such as UV irradiation or desiccation. (Bell and Wheeler, 1986). In the plant kingdom, the intensity of melanin formation is often correlated with resistance to microbial and viral infections and unfavorable climatic conditions (Bell, 1981). The response of plants to bruising or cutting likewise includes the production of melanins (Marshall et al., 2000).

Melanins are biopolymers formed from phenolic compounds by polymerization via quinones. The production of quinones is catalyzed by the phenoloxidases.

Melanins can be classified on the basis of their precursor molecule. In animals, the black eumelanins and the reddish-brown pheomelanins are derived from tyrosine. Melanins in fungi and other microorganisms are derived from tyrosine via 3, 4-dihydroxyphenylalanine (DOPA), γ-glutaminyl-3, 4-dihydroxybenzene (GDHB) or catechol, and 1, 8-

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dihydroxynaphthalene (DHN) (Bell and Wheeler, 1986). It is believed that the precursor in plants is catechol, caffeic, chlorogenic, protocatechuic, or gallic acid (Solano, 2014). However, there has not yet been sufficient evidence to support this view. The structures of melanins have rarely been studied due to the difficulties in the isolation of melanins from natural sources and the poor solubility of the pigments. Melanins are insoluble in water and common organic solvents (such as hexane, chloroform, ethyl acetate, ethanol, methanol or acetone) (Sava et al., 2001a; Hsieh and Lien, 2012; Wang et al., 2006) and can be dissolved only in alkaline solutions. The range of methods available for structural investigations is therefore limited. Knowledge of the structure has been derived largely from chemical degradation studies and the analysis of pigments synthesized *in vitro* from the presumed precursor monomers (Napolitano et al., 1996a, 1996b). To date, detailed accounts of the structural elucidation of melanins have been reported only in the case of the nitrogen-fixing bacteria *Azotobacter chroococcum* (Banerjee et al., 2014) and *Sepia officinalis* (Pezzella et al., 1997). Both pigments proved to be eumelanins built up from 5, 6-dihydroxyindole and/or 5, 6-dihydroxyindole-2-carboxylic acid monomer units. Analytical methods have been established for the quantitative determination of eumelanins and pheomelanins, in view of their important role in skin and hair pigmentation. The methods are based on oxidative degradation of the biological samples followed by high performance liquid chromatographic quantification of specific degradation products (Ito and Fujita, 1985; Napolitano et al., 2000). Despite their frequent occurrence, plant melanins have been poorly investigated. Studies are generally limited to their isolation and characterization of their physical and chemical properties (e.g. solubility, stability and oxidized bleaching) (Claussen and Pepper, 1968; Sava et al., 2001a). Little structural information is available, namely the pigment is devoid of nitrogen and the C/H ratio of 1.11 indicating an aromatic nature. However, enzymatic transformations of the presumed monomers (benzoic and cinnamic acids) by oxidoreductases have been thoroughly studied (Aktas et al., 2003; Hollmann and Arends, 2012; Xu et al., 2005). The *in vitro* oxidation of phenolic acids by laccase, tyrosinase and peroxidase originating from different sources is often performed to eliminate phenolic pollutants, producing novel, bioactive compounds and polymer resins. During *in vitro* enzymatic transformation of *p*-coumaric acid (El Aghaa and Makrisb, 2012; Liu et al., 2007) the structure of the dimers and oligomers (up to trimers) has been elucidated.

Oat a cereal crop is primarily used as animal fodder, but an increasing amount is now being grown for human consumption, following the recent recognition of its nutritional benefits. There are several oat subspecies. *Avena sativa* (Poaceae) and *Avena byzantina* are the most commonly cultivated in the world. The oat grain is comprised of the hull and kernel (groat). The whole oat usually consists of 25–35% hull, depending on environmental and genetic factors, but there are naked oat cultivars too (Coffman, 1961). The hull varies in color in the different varieties, the most common colors being white, yellow, reddish-brown and black. It mainly consists of hemicelluloses, cellulose and lignin (Rasper, 1979). In addition, the hull contains proteins (Welch et al., 1983) and phenolic compounds in low quantities (Chen et al., 1982). No data are available as concerns the pigments present in the hull.

The objective of the present study is to characterize the dark pigment extracted from the black oat hull with respect to its composition and structure. The analytical techniques applied for the characterization of the pigment included ultraviolet–visible (UV–Vis) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and Fourier transform infrared (FT-IR) spectroscopy. The biopolymer synthetized *in vitro* from the presumed precursor monomer by peroxidase-initiated polymerization was studied for comparison.

2. Results and discussion

2.1. Characterization of the dark pigment obtained from oat hull

The black pigment of the oat hull was isolated from the plant matrix by alkaline extraction. Purification was performed according to Sava et al. (2001b). The multistep process started with acidic hydrolysis to remove carbohydrates and proteins, which was followed by washing with organic solvents to remove lipids. The final step was repeated precipitations to eliminate phenolic compounds. The extraction and purification processes afforded 5 mg pigment from 1 g oat hull.

2.1.1. UV–Vis and EPR spectroscopy

UV–Vis spectroscopy and electron paramagnetic resonance (EPR) spectroscopy were applied to identify obtained melanin.

The UV–Vis spectrum of the extracted and purified pigment is shown in Suppl. Fig. S1. The absorbance increases progressively towards shorter wavelengths. This broadband, structureless absorption profile with monotonously increasing function of energy is a common feature of the melanins (Crippa et al., 1978). It has been suggested, that this absorption profile may be due to the superposition of the absorption of the individual, chemically distinct constituents of the melanins (Tran et al., 2006).

Melanins are paramagnetic biopolymers due to the presence of stable free radicals. Free radicals can be easily observed by electron paramagnetic resonance spectroscopy. The EPR signal (Suppl. Fig. S2) of the dark pigment extracted from oat hull appears as a singlet, without any fine structure similarly to the EPR spectra of other synthetic and natural melanins of different origin (Mason et al., 1960; El-Obeid et al., 2006). The g value of the signal was calculated to be 2.0051 typical to carbon-centered organic free radicals with a conjugated structure bearing oxygen containing functional groups (Atherton et al., 1993).

2.1.2. MALDI-TOF mass spectrometry

MALDI-TOF MS is a powerful method to obtain structural information on polymers such as the degree of polymerization as well as molecular masses of the repeating units, and the oligomers (Nielen, 1999).

The MALDI-TOF mass spectrum of oat melanin (Fig. 1) resembles those of synthetic homopolymers. It is characterized by a series of peaks representing oligomers with different degrees of polymerization. The spectrum reflects the oligomers in their potassium adduct form. Because of the high cation affinity of the polymers, their ionization often takes place by the formation of metal ion adducts (Knochenmuss et al., 1998). Potassium impurity may originate from the matrix, solvents and the surface of the sample carrier. It has been also demonstrated that attachment of free gas-phase cations, rather than cation transfer from the cationized matrix, is the predominant process (Zhang and Zenobi, 2004). The peak-to-peak distance which is the molecular weight of the repeating unit is 162 Da. If the monomers are linked to each other via C–C linkages, the molecular mass of the monomer can be calculated (162 + 2 Da). The allomelanins are known to be formed from phenolic compounds by phenoloxidase enzymes. The two groups of phenolic acids in plants are hydroxylated derivatives of benzoic and cinnamic acid (Fig. 2). The hydroxybenzoic acids include *p*-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acids featuring a C6–C1 structure. The hydroxycinnamic acids, on the other hand, contain a three-carbon side-chain (C6–C3) structure, with *p*-coumaric, caffeic, ferulic and sinapic

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