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Stable free radicals in insect cuticles: Electron spin resonance spectroscopy reveals differences between melanization and sclerotization

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Abstract

Insect cuticles (exuviae; cast skins) were examined for the first time by ESR spectroscopy for the presence of stable free radicals, as found in melanins. All cuticles, except those from a locust albino strain, irrespective of the presence of melanin, provided single-line signals of varied *g*-values and linewidths. The ESR signals of melanins, isolated or in cuticles, were characterized by *g*-values <2.004 and small linewidths in the range of 4–6 G, while sclerotized cuticles, lacking melanin, showed *g*-values >2.004 and broad linewidths of 5–11 G. The melanin spectra were comparable to those reported for eumelanins with indol-based monomers. Minor signals ascribed to pheomelanins were found in several probes. The 'sclerotin' spectra were broader and displayed unresolved hyperfine structure in some cases. As for melanins, the location and environment of the radicals in cuticles giving rise to the two types of ESR spectra could not be assigned. Changes in the radical environment due to insecticide or solvent treatment can be detected by ESR spectroscopy.

Keywords: Electron spin resonance spectroscopy; Stable free radicals; Melanin; Cuticle; Scleotization; Insects

Melanins are amorphous, irregular, polymeric pigments widely occurring in living organisms [1]. While melanins are commonly known to be associated with skin and hair color in humans and other mammalians, various types of melanins are also present in the inner ear, eyes, brain, bird feathers, hoof horn, plants, fungi, and the ink sac of cuttlefish [2–4]. Melanin pigments are classified into two groups: eumelanins, the indole-type arising from enzymatic oxidation of tyrosine through 3,4-dihydroxyphenylalanine (dopa)[1], and pheomelanins formed by enzymatic oxidation of tyrosine in the presence of cysteine with cysteinyldopa as intermediate (or related sulfhydryl compounds) [5,6]. Formed from different precursors and hence resulting in different polymers the colors of melanins vary from the pitch black to brown and even red.

Research on melanins has revealed an unusual variety of properties. Formed by oxidative polymerization of various phenolic compounds, melanins can act as both oxidants and reductants, showing a high activity in binding drugs and metal ions [3,4,7–10]. The chemical properties of melanins such as high molecular weight, insolubility in organic solvents and water render them difficult to usual physicochemical or histochemical analysis. Because these pigments uniquely contain a stable population of organic free radicals of *o*-semiquinone type, ESR spectroscopy is a suitable method for studying them [11,12]. Thus a set of qualitative ESR criteria has been established for the identification of these types of free radicals [13]. ESR spectra of eumelanins and pheomelanins differ in lineshape. ESR spectra of eumelanins are single lines, whereas the spectra of pheomelanins reveal also hyperfine splitting [6]. Therefore, the analysis of the ESR lineshape permits to determine the type of melanin in the biological samples under study [14].

Melanins have also been reported from insects, where they are typically located in cuticles and hence contribute to

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[1] *Abbreviations used:* dopa, 3,4-dihydroxyphenylalanine; *g*-factor, gyromagnetic factor.

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the insects' coloration. However, since appropriate insect material is commonly not available in necessary amount, these pigments have been chemically characterized in only few species, and hence the terms 'melanins' or 'melanization' are commonly used to describe just black pigmentation [15]. In vertebrate cells, melanins have been well described as osmiophilic granules at the ultrastructural level. Similar results have been obtained also from the integument of some insects, while in other cases of visual black pigmentation, no comparable granular material was found [16].

The present study examines the use of ESR spectroscopy to identify and characterize melanin-based pigmentation of insect cuticles (exuviae as cast at molting) from various orders. The ESR spectra of melanins prepared from human hair as well as from insect cuticles by acid hydrolysis were compared with those obtained from intact cuticles that displayed black pigmentation. Unexpectedly, also cuticles without any visible 'melanin' pigments, initially expected to provide melanin-free controls, also exhibited ESR signals of comparable intensity. However, these ESR signals, differed significantly from those of isolated melanins and melanin-containing cuticles. Thus, ESR spectroscopy not only proved to be a useful tool to characterize melanin-type pigmentation of intact insect cuticles, but also allowed to discriminate between true melanins and melanin-like polymers in non-melanized sclerotized cuticles, based on the gyromagnetic factor and linewidth values of their ESR spectra. Apart of some early and tentative experiments by Mason and colleagues [17], the present study is the first comprehensive one applying ESR spectroscopy to characterize cuticles from a broad range of insects.

Materials and methods

Preparation of cuticles and of melanins

All 'cuticles' examined were exuviae representing the distal part of the cuticles that are shed during molting of larvae and pupae, respectively. In the present work, the more commonly known term 'cuticles' is used throughout. Puparia are sclerotized exuviae from last instar fly larvae undergoing pupation within the puparium. Insects were obtained from in-house stocks, commercial breeders, Syngenta Crop Protection and Novartis Animal Health. For the insecticide study, the fly larvae were feed diet supplemented with 0.25 mg/L cyromazine and 0.015 mg/L dicyclanil, respectively.

The cuticles were mechanically cleaned from any contaminating material under a binocular microscope. In case of puparia, the pupal cuticles (after emergence of the flies) and the dead flies (after insecticide treatment), respectively, were completely removed. The cuticles (except of those from Hymenopterans) were then washed with usually three changes of either water or 0.1 N HCl or 0.1 N NaOH or acetone under magnetic stirring and suspension in an ultrasonic bath. Samples treated with HCl or NaOH were subsequently washed several times with water until neutrality. Next, the cuticles were air-dried and crushed in a mortar to obtain as small as possible particles. This coarse cuticle powder was filled into standard NMR tubes (4 mm diameter) at quantities of 40–90 mg/tube. Less material was available in only a few probes of melanins. All procedures were carried out at room temperature and under ambient light.

To prepare melanins, cuticles were subjected to a series of washing steps with 0.1 N NaOH, water, 0.1 N HCl, water, ethanol, and finally acetone. The washed cuticles were dried at 70 °C and crushed in a mortar. The cuticle material was next hydrolyzed in 6 N HCl at 110 °C under nitrogen to prevent autoxidation of released catechols that could give rise to melanin formation, as observed in initial experiments. The HCl was renewed four to six times every 12 h until the supernatants were fairly colorless. These extracts were discarded. The remaining dark material was washed with acetone, which remained colorless with most samples. When the acetone extract was brown (pupal cuticles from *Inachis io*; larval and pupal cuticles from *Pieris brassicae*), it was diluted with water and acidified with HCl to pH < 1 resulting in a dark precipitate that was collected by centrifugation and dried. The dark pigment thus obtained was soluble in 0.5 N NaOH. The remaining HCl-resistant dark material was dissolved in 0.5 N NaOH and dark pigment was precipitated upon acidification, as above. The yield of this dark pigment, called melanin here, varied between 24% in the black larval cuticles from *I. io* (nymphalid butterfly) and 1% in the brown puparia from *Calliphora vicina* (blowfly).

Electron spin resonance (ESR) spectroscopy

ESR measurements were performed using X-band spectrometers (Brucker ESP300 or Brucker ElexSys E500). The samples were measured without further preparation at room temperature and in contact with atmospheric oxygen. The ESR parameters gyromagnetic factor (*g*-factor) and linewidth ($\Delta\Gamma$) were obtained using DPPH (α,α-diphenyl-β-picryl hydrazyl) as internal standard and peak-to-peak distance of the spectrum, respectively. The value of the double integral of the ESR spectrum divided by the receiver gain value and the mass of the sample, J^* was used to compare the concentration of paramagnetic centers in the various samples. The area under the absorption curve was obtained by using double integration of the ESR spectrum. All samples were measured in the same experimental conditions: modulation amplitude (1 G), microwave power (2 mW), time constant (20 ms); only the receiver gain was varied for an optimum measurement of the ESR spectra.

Results and discussion

The examined samples comprised cuticles, as shed at each molt (i.e., exuviae), from larvae and pupae representing several insects orders, as well as melanins isolated from