NATURAL PRODUCT RESEARCH, 2015
http://dx.doi.org/10.1080/14786419.2015.1079185

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SHORT COMMUNICATION

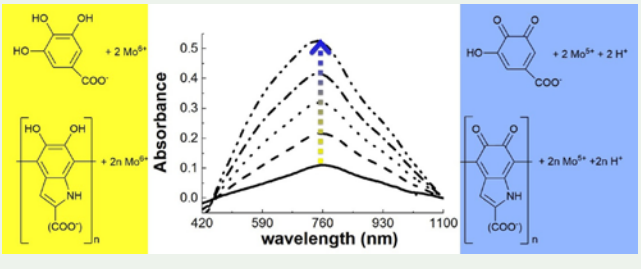
Redox activity of melanin from the ink sac of *Sepia officinalis* by means of colorimetric oxidative assay

Pathomthat Srisuk[abc], Vitor M. Correlo[ab], Isabel B. Leonor[ab], Pasquale Palladino[ab] and Rui L. Reis[ab]

[a]3B's Research Group – Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, University of Minho, Guimarães, Portugal; [b]PT Government Associate Laboratory, ICVS/3B's, Guimarães, Portugal; [c]Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand

ABSTRACT

The redox properties of natural extract from cuttlefish ink sac (*Sepia officinalis*) and synthetic melanin used as a biomimetic in melanin structural investigation were determined by comparison of this phenol-based heterogeneous pigment with gallic acid used as a standard in Folin–Ciocalteu colorimetric assay widely employed for characterisation of oxidative properties of biomaterials. Reactivity of sepia melanin reported here is much higher than previously indicated and this protocol should allow the redox characterisation of all melanins irrespective of their origin and composition.



ARTICLE HISTORY
Received 1 March 2015
Accepted 18 July 2015

KEYWORDS
Sepia officinalis; cuttlefish ink sac; sepia melanin; synthetic melanin; gallic acid; Folin–Ciocalteu phenol reagent; redox activity

1. Introduction

Melanins are ubiquitous phenol-based heterogeneous pigments found in most living kingdoms acting as a radical scavenger and antioxidant, and possessing peculiar physicochemical properties (Meredith & Sarna 2006) with the potential for widespread applications including cuisine, art, tissue engineering and bioelectronics (Mostert et al. 2012; Derby 2014). Animal melanins, like eumelanin and pheomelanin, are produced by tyrosinase enzyme action on tyrosine (Slominski et al. 2004). Eumelanin is a heteropolymer, dark and almost insoluble in water, derived from 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units. Several evidences suggest the presence of four to eight 5,6-dihydroxyindole

CONTACT Pasquale Palladino pasquale.palladino@unina.it; Rui L. Reis rgreis@dep.uminho.pt
Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/14786419.2015.1079185.

units per macromolecule forming sheets that aggregate up to nanoparticles (Cheng et al. 1994; Meredith & Sarna 2006). We used here the ability of melanin to undergo electron transfer reactions through the equilibrium between oxidised and reduced forms of 5,6-dihydroxyindole subunits to define its redox properties by comparison of reactivity of the natural extract, from cuttlefish ink sac (*Sepia officinalis*), and synthetic melanin, used as a biomimetic in melanin structural investigation (Perna & Capozzi 2012), with 3,4,5-trihydroxybenzoic acid (gallic acid) monohydrate, used as a standard in a widely employed assay in studying phenolic antioxidants, namely Folin–Ciocalteu colorimetric assay (Folin & Ciocalteu 1927; Huang et al. 2005; Dias et al. 2013), extending the protocols in the field of melanins to optical characterisation of oxidative properties (d'Ischia et al. 2013).

2. Results and discussion

Although melanin is only slightly soluble and may only be finely suspended in water, we confidently assume that all the DHI/DHICA units are promptly available for redox reactions because it was demonstrated that melanin is an efficient electron transfer medium (Menter & Willis 1997), and large amount of metal ions can move easily within the channels of melanin granules (Liu & Simon 2005). A previous attempt to characterise sepia melanin, in comparison with DHI and DHICA molecules, by the analysis of the rate of ferricyanide consumption surprisingly indicated this pigment as redox inactive and irreversibly degraded as carboxylated pyrroles (Pezzella, d'Ischia, et al. 1997), usually formed when harsh purification conditions of natural extract are used (Ito et al. 2013). However, it is known that melanin may either retard or accelerate ferricyanide reduction (Menter & Willis 1997) and, more in general, the ferric reduction/oxidising power can vary strongly depending on the reductant (Prior et al. 2005). Based on this literature survey, we tentatively suggest that the limited reaction time of 1 min for ferricyanide reduction in presence of sepia melanin could be responsible for the very low redox activity of sepia melanin previously described (Pezzella, d'Ischia, et al. 1997; Pezzella, Napolitano, et al. 1997). The FCR used here consists of a mixture of the heteropoly acids of molybdenum and tungsten (Folin & Ciocalteu 1927). These metals act as redox probes changing the colour of solution from yellow to blue in presence of reducing agents, possibly by the formation of $(PMoW_{11}O_{40})^{4-}$ anion (Huang et al. 2005). We assume the same redox reaction of aromatic *ortho*-diols of gallic acid and melanin, synthetic or natural, oxidised to *ortho*-quinones by FCR solution as depicted in Figure 1. It is always necessary to use a standard molecule like gallic acid and refer to a calibration curve for ensuring the reliability of the FCR assay because molar absorptivity of molybdenum blue depends on temperature, pH, composition and ageing of FCR solution employed (Berenblum & Chain 1938). However, FCR assay is simple and reproducible even for complex matrix samples (Huang et al. 2005; Prior et al. 2005). From the analysis of visible spectra relative to the so-called molybdenum blue formation in presence of reductants (see Supplementary material), we have observed that gallic acid (Figure S1A), synthetic melanin (Figure S1B) and sepia melanin (Figure S1C) reacted strongly with the FCR solution, giving a value of 50.5 ± 0.5 g^{-1} L (R^2 = 0.999), 16.9 ± 0.2 g^{-1} L (R^2 = 0.999) and 6.7 ± 0.4 g^{-1} L (R^2 = 0.987), respectively, for the slope of the concentration-dependent absorbance at 760 nm reported in Figure 2. Considering the presence of at least four DHI/DHICA units per sepia melanin macromolecule (Cheng et al. 1994; Meredith & Sarna 2006), the reducing capacity per unit of DHI/DHICA is around 40% of that of gallic acid, i.e. the 5,6-dihydroxyindole units are preserved to a much higher extent

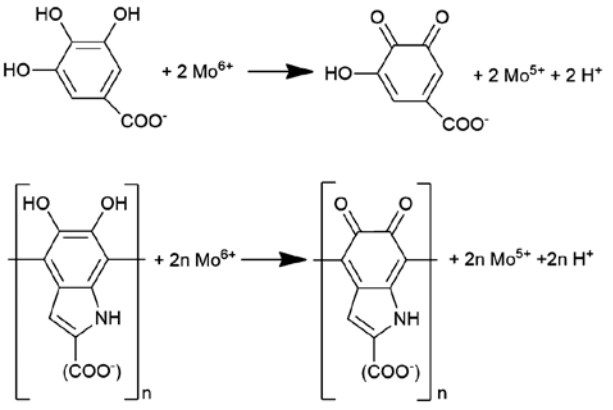


Figure 1. Simplified view of gallic acid and eumelanin aromatic *ortho*-diols oxidation to *ortho*-quinones in presence of Folin–Ciocalteu reagent.

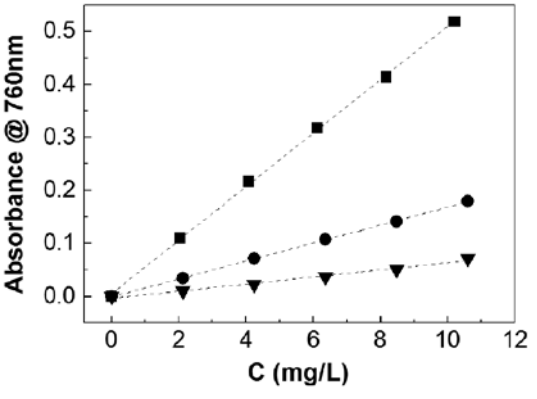


Figure 2. Plot of absorbance at 760 nm vs. concentration of gallic acid monohydrate (square), synthetic melanin (circle) and sepia melanin (triangle).

compared to previous estimate of less than 5% of intact units for this pigment (Pezzella, d'Ischia, et al. 1997). In first approximation, the ratio between the slope of gallic acid and the one from melanins plot corresponds to the molecular weight ratio between eumelanin macromolecule, synthetic or natural, and gallic acid monohydrate. Rigorous estimation of the amount of residual interacting melanosomal protein(s), elsewhere valued at 6–8% in weight of water-purified sepia melanin extract (Liu & Simon 2005), and taking into account the simultaneous presence in eumelanin of oxidised, reduced and semi-reduced subunits, i.e. the comproportionation equilibrium (Mostert et al. 2012), could give a further structural insight of eumelanin. However, molecular structure determination is out of the scope of this paper.

3. Conclusion

In this study, it has been found that synthetic melanin, prepared by oxidation of tyrosine with hydrogen peroxide, and natural melanins, isolated from cuttlefish ink sac, are promptly

oxidised by Folin–Ciocalteu phenol reagent. Sepia melanin is redox active and not degraded as pyrrolecarboxylic acids. We believe that the results presented here should provide a useful method and protocol to determine the redox properties of all the melanins irrespective of their origin and composition.

Supplementary material

Visible spectra of molybdenum blue formation in presence of reductants and experimental details relating to this paper are available online.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the European Union's Seventh Framework Programme (FP7/2007-2013) [grant agreement number REGPOT-CT2012-316331-POLARIS]; Khon Kaen University grant to PS.

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