Using melanogenesis to study anti-oxidants

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Abstract Building upon our research on the non-enzymatic synthesis of melanins, we explored the possibility that the reactions involved in the synthesis of melanins could serve as the basis for the study of and search for compounds with anti-oxidant properties. The synthesis of melanins can be achieved through air-mediated oxidation or through reactive-oxygen-species-mediated oxidation and can readily be monitored by the changes in color. In addition, a broad variety of different precursors can be used to generate melanins. Thus, using melanogenesis as the foundation for an anti-oxidant assay, one can evaluate the effects of potential anti-oxidants against air- or reactive-oxygen-species mediated oxidations and evaluate the consistency of the anti-oxidant effects when different precursors for melanogenesis are used.

Keywords: Melanin, anti-oxidant, L-ascorbic acid, glutathione

1. Introduction
Melanins (MNs) are darkly colored pigments ubiquitously found in nature.1,2 The precise chemical structure of the MNs is not firmly established, but they are derived through the oxidation and subsequent polymerization of diphenolic or indolic precursors.

We have explored several approaches to the non-enzymatic synthesis of MNs from a wide variety of precursors using 1) air-mediated oxidation in an alkaline environment, 2) reactive-oxygen-species (ROS)-mediated oxidation using a combination of Fe2+ and H2O2 and 3) tyrosinate-mediated oxidation.3,4,5,6,7 The formation of MNs from their precursors is easy to monitor as the reaction mixtures rapidly change from colorless to a darker color.

We have begun to explore the possibility that the oxidation chemistry leading to the formation of MNs can serve as the basis for the study of and the search for potential anti-oxidants. The advantage of our approach is that both air- and ROS-mediated oxidation can be studied using the same type of chemical reaction. We present our preliminary results monitoring the MN synthesis from pyrogallol ((1) in Figure 1) or dopamine ((2) in Figure 1) and the effects of L-ascorbic acid ((3) in Figure 1) or glutathione ((4) in Figure 1) on this process. As many potential anti-oxidants are poorly soluble in water and require some amounts of organic solvent as a co-solvent for their study, we briefly explored the effects of the presence of methanol on the different melanogenesis reactions.

Figure 1: Chemical structures of pyrogallol (1), dopamine (2), L-ascorbic acid (3) and glutathione (4)

2 Materials and methods
2.1 Materials
Pyrogallol, dopamine.HCl, glutathione and L-tyrosinate disodium salt were obtained from Sigma-Aldrich (St Louis, MO). H2O2 solution at 3% (v/v) was obtained from Kroger Co (Cincinnati, OH) and used within one month of its purchase. L-ascorbic acid and FeCl2.2H2O and all other chemicals were obtained from Fisher Scientific (Waltham, MA).
2.2 Kinetic anti-oxidant assays

All reaction mixtures were prepared in water and contained either 5mM (1) or (2) as the substrate for MN synthesis. Compounds (3) or (4) were tested as anti-oxidants and were added in a final concentration of 0.16 or 0.33mM. The MN synthesis was initiated by the addition of: 1) NaOH to a final concentration of 1.5mM, 2) L-tyrosinate to a final concentration of 5mM and 3) the addition of Fe²⁺ at a final concentration of 0.1mM followed by the addition of H₂O₂ to final concentration of 0.01% (v/v). Immediately after the start of the reaction, a 200µL aliquot was transferred into a well of a microplate and the absorbance at 400nm was monitored as a function of reaction time at room temperature. Unless stated otherwise, all experiments were performed in triplicate and the results are presented as the average ± standard deviation.

2.3 UV-Vis spectroscopy

UV/Vis spectroscopic measurements were made in wells of a 96-well microplate using the SynergyHT microplate reader from Biotek (Winooski, VT).

3. Results

3.1 Experiments involving NaOH-mediated MN synthesis

Figure 2, panels A through D, show the kinetic profiles obtained for the anti-oxidant assays involving NaOH-mediated MN synthesis.

The results presented in Figure 2 suggest that (3) and (4) had no or minimal effects on the NaOH-
initiated synthesis of MN from (1). On the other hand, (3) and (4) completely blocked the NaOH-initiated MN synthesis from (2).

3.2 Experiments involving tyrosinate-mediated MN synthesis

Figure 3, panels A through D, show the kinetic profiles obtained for the antioxidant assays involving tyrosinate-mediated MN synthesis.

The results presented in Figure 3 suggest that (3) and (4) had no or minimal effects on the tyrosinate-initiated synthesis of MN from (1). On the other hand, (3) and (4) appeared to delay, but not slow down the tyrosinate-initiated MN synthesis from (2). Although in Figure 3, panel C, the addition of (3) at 0.33mM appeared to block the reaction completely, visual observations indicated that MN formation did occur, but much later after the collection of data was terminated.

3.3 Experiments involving Fe$^{2+}$/H$_2$O$_2$-mediated MN synthesis

Figure 4, panels A through D, show the kinetic profiles obtained for the antioxidant assays involving Fe$^{2+}$/H$_2$O$_2$-mediated MN synthesis.
Figure 4: Kinetic profiles of the Fe$^{2+}$/H$_2$O$_2$-initiated MN synthesis using (1) (panels A and B) or (2) (panels C and D) as the precursor in the presence of 0.00, 0.16 or 0.33mM (3) (panels A and C) or (4) (panels B and D). Experimental details are described in the Materials and Methods section.

The results presented in Figure 4 that, for both precursors used, the presence of (3) or (4) did not or only minimally affect the reaction kinetics of the Fe$^{2+}$/H$_2$O$_2$-mediated MN synthesis.

3.4 Experiments involving the effect of methanol on the MN synthesis

Using (1) as the precursor, we briefly investigated the effects of methanol on the NaOH-, tyrosinate- or Fe$^{2+}$/H$_2$O$_2$-initiated MN synthesis. Those experiments were not performed in triplicate, but the effect of methanol was evaluated at five different concentrations to observe any patterns that may emerge. Figure 5, panels A through C, show the kinetic profiles of these experiments.
Figure 5: Kinetic profiles of the NaOH- (panel A), tyrosinate- (panel B) or Fe²⁺/H₂O₂-initiated (panel C) MN synthesis using (1) as the precursor in the presence of varying concentrations of methanol (in % v/v). Experimental details are described in the Materials and Methods section.

The results presented in Figure 5 suggest that methanol had no effect on the NaOH- or tyrosinate-mediated oxidation of (1) into MN, but did have an inhibitory effect on the Fe²⁺/H₂O₂-initiated synthesis of MN from (1).

4. Discussion

Our preliminary results suggest that using melanogenesis to study or search for anti-oxidants is complicated. While exploring the anti-oxidant properties of (3) or (4), different patterns of results were obtained depending on the precursor and reaction conditions used to generate MN. Particularly when MN is generated using NaOH or tyrosinate the experimental results show striking differences whether (1) or (2) are used as precursor. However, in all cases studied, similar patterns of results were obtained whether (3) or (4) were evaluated as anti-oxidants. Thus, our observations regarding the effects of (3) or (4) or any other compound on the melanogenesis process could provide additional insights regarding the various steps involved in melanogenesis. Given that (1) is structurally much less complicated than (2) (e.g., no side chain or amine functionality), it is very likely that the melanogenesis process involving (1) is less complicated than the process involving (2). The qualitative and quantitative differences observed in our studies of the inhibition of the melanogenesis process using (1) or (2) could well be due to the underlying differences in the melanogenesis process involved.

In addition, the presence of methanol had a variable effect on the kinetics of the MN synthesis depending on the reaction conditions evaluated. More detailed evaluations, involving different analytical tools, will be needed to explain the qualitative differences observed in our results.

Apart of the study of anti-oxidants, our efforts could make contributions to the extensive efforts made in the search for inhibitors of melanogenesis. These efforts are focused on the search for inhibitors of tyrosinase or the melanogenesis signaling pathway. However, non-enzymatic factors could make important contributions to the melanogenesis process leading to the appearance of a dark color.

REFERENCES