Journal of Chromatography B 1061–1062 (2017) 11–16

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Using size exclusion chromatography to monitor the synthesis of melanins from catecholamines

Koen P. Vercruysse*, Astiney M. Clark, Paola A.F. Bello, Majidah Alhumaidi

Department of Chemistry, Tennessee State University, 3500 John A. Merritt Blvd., Nashville, TN 37209, United States

ARTICLE INFO

Keywords:
Size exclusion chromatography
Catecholamines
Melanin
Polysaccharides

ABSTRACT

We have employed size exclusion chromatography (SEC) to the study of the auto- and Cu^{2+}-mediated oxidation of the catecholamines, dopamine, epinephrine and norepinephrine, into melanins. We observed that, due to non-size exclusion-mediated effects, the catecholamines and some of the low molecular mass intermediates generated during the oxidation reactions, could be resolved from each other and from the high molecular mass pigment generated. Thus, SEC allowed us to monitor the disappearance of the catecholamine starting compounds, the appearance and subsequent disappearance of the low molecular mass chromophores generated in the initial phase of the reactions and the appearance of the high molecular mass melanins. In the process of this research, we observed that many, mostly anionic polysaccharides (PS), enhanced both the auto- and Cu^{2+}-mediated oxidation of all three catecholamines. SEC analyses of reaction mixtures involving PS suggested that very high molecular mass aggregates between PS and melanins can be generated. In addition, SEC analysis allowed us to verify the efficiency of the dialysis purification process employed to obtain pure and dried melanin materials for cell-biological studies.

1. Introduction

The pigments generated in our experiments belong to the category of the melanins (MN) and related biomolecules. MN are ubiquitously found in nature and excellent reviews regarding their biosynthesis, chemistry, classification and functions have been written [1–3]. The MN found in animals are built from L-DOPA which is derived from L-tyrosine. These types of MN are broadly divided into eumelanins and pheomelanins. The pheomelanins differ from the eumelanins that the amino acid L-cysteine is built into its structure. The pheomelanins have not yet been the subject of our experiments. The biosynthesis of MN involves a sequence of oxidation, cyclization and polymerization reactions most commonly described by the Raper-Mason scheme and illustrated in Fig. 1 [1]. In a first phase of this reaction scheme, the catechol portion of the precursor is converted into the *o*-quinone oxidation product and such *o*-quinones often exhibit absorbance in the visible region of the electromagnetic spectrum. Catecholamines (CAs) like epinephrine (EPI), norepinephrine (NE) or dopamine (DA) are biomolecules derived from L-DOPA and can undergo a similar sequence of reactions leading to neuromelanins or other melanins, similar as for L-DOPA leading to eumelanins (see Fig. 1). CAs like DA or NE are considered to be the precursors of the neuromelanins found in select areas of the brain [4,5]. MN types of pigments are also produced by plants, fungi and bacteria, but the precursors of some of these pigments are often nitrogen-free phenols like catechol, caffeic acid, homogentisic acid or others [3].

Given the fact that MN constitute a broad range of biomolecules derived from a broad range of potential precursors, the qualitative and quantitative analysis and characterization is challenging, primarily due to the fact that the naturally-occurring MN are often insoluble [6]. Chromatographic approaches to the analysis of MN often involve the degradation of the pigment and the subsequent analysis of the degradation products [6,7]. In this report we demonstrate the versatility of size exclusion chromatography (SEC) for the study of the formation of synthetic MN from CA precursors. In addition, we applied SEC to study the synthesis of MN from CAs in the presence of polysaccharides (PS). We observed that many PS promoted the oxidation reaction and that water-soluble, high molecular mass pigment/PS are generated in the process. Finally, we employed SEC to verify the dialysis purification process of the MN generated in our experiments.

2. Materials and methods

2.1. Materials

Chondroitin sulfate type A (sodium salt from bovine trachea; 70%

* Corresponding author.
E-mail address: kvercruysse@tnstate.edu (K.P. Vercruysse).

http://dx.doi.org/10.1016/j.jchromb.2017.04.005
Received 25 January 2017; Received in revised form 22 March 2017; Accepted 1 April 2017
Available online 03 July 2017
1570-0232/

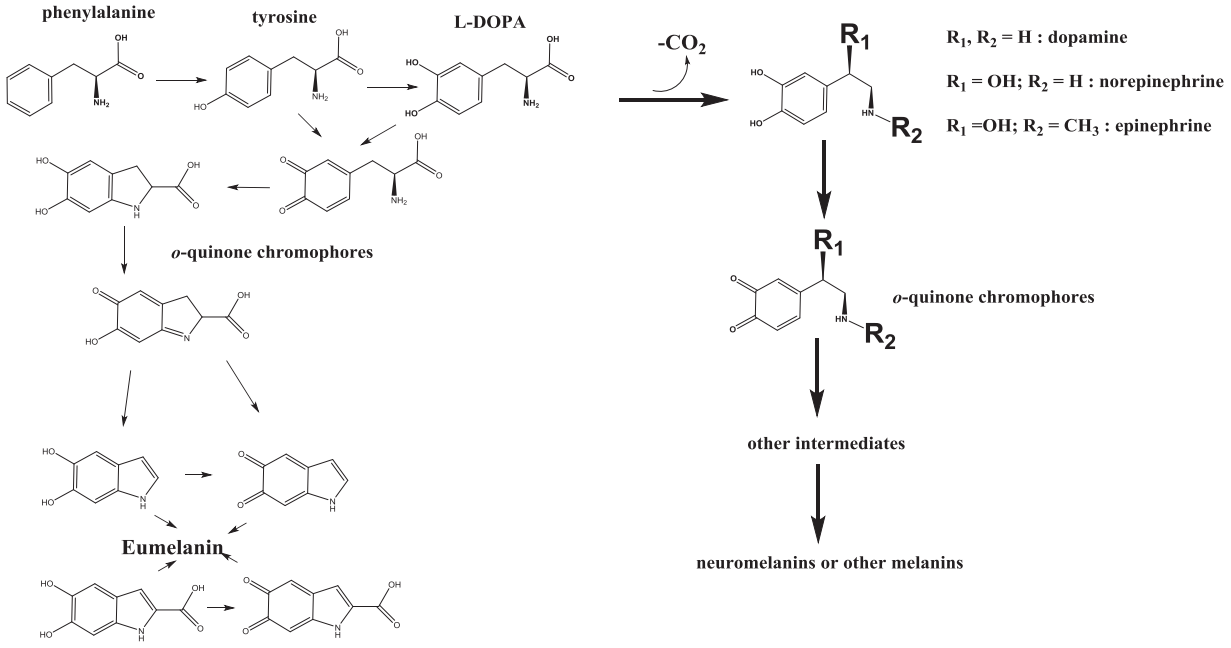

Fig. 1. Raper-Mason scheme for the synthesis of eumelanin from DOPA or catecholamines.

with counterbalance chondroitin sulfate type C), chondroitin sulfate type C (sodium salt from shark cartilage; 90% with counterbalance chondroitin sulfate type A), alginic acid sodium salt (Algin®, sodium alginate), ι-carrageenan (commercial grade, type II), carboxymethylcellulose (low viscosity grade; sodium salt), fucoidan (from *Fucus vesiculosus*, dopamine.HCl, epinephrine.HCl and norepinephrine.HCl, $CuCl_2{\cdot}2H_2O$ were obtained from Fisher Scientific (Suwanee, GA). All other reagents were of analytical grade.

2.2. UV/Vis spectroscopy

UV/Vis spectra of DA, NE and EPI were obtained using a DU 800 spectrophotometer from Beckman Coulter (Fullerton, CA) against water as the blank.

2.3. RP-HPLC analyses

RP-HPLC analyses were performed on a UFLC chromatography system equipped with dual LC-6AD solvent delivery pumps and SPD-M20A diode array detector from Shimadzu, USA (Columbia, MD). Analyses were performed on a BDS Hypersil C_8 column (125 × 4.6 mm) obtained from Fisher Scientific (Suwanee, GA). Analyses were performed in isocratic fashion using a mixture of water:methanol:acetic acid (90:10:0.05% v/v) as solvent. The sample volume was 20 µL. Samples were diluted to a CA concentration of approximately 0.1 mM.

2.4. Size exclusion chromatography (SEC)

SEC analyses were performed on a Breeze 2 HPLC system equipped with two 1500 series HPLC pumps and a model 2998 Photodiode array detector from Waters, Co (Milford, MA). Analyses were performed using an Ultrahydrogel 500 column (300 × 7.8 mm) obtained from Waters, Co (Milford, MA) in isocratic fashion using a mixture of 25 mM Na acetate:methanol:acetic acid (90:10:0.05% v/v) as solvent. Samples were diluted with SEC solvent, centrifuged and 20 µL was injected. Samples were diluted as indicated in the text or figures.

2.5. Dialysis and freeze drying

Select samples were dialyzed using Spectrum Spectra/Por RC dialysis membranes with molecular-weight-cut-off of 3.5 kDa obtained from Fisher Scientific (Suwanee, GA). Select dialyzed materials were frozen overnight and dried using a Labconco FreeZone Plus 4.5 L benchtop freeze-dry system obtained from Fisher Scientific (Suwanee, GA).

2.6. FT-IR spectral analysis

FT-IR spectroscopic scans were made using the NicoletiS10 instrument equipped with the SmartiTR Basic accessory from ThermoScientific (Waltham, MA). Scans were taken with a resolution of 4 cm^{-1} between 650 and 4000 cm^{-1} at room temperature using a KBr beam splitter and DTGS KBr detector. Each spectrum represents the accumulation of 24 scans.

3. Results

3.1. Preliminary observations

When Cu^{2+} was added to aqueous solutions containing EPI, DA or NE, a change in color, from colorless to pink, red or orange, appeared within hours of mixing depending on the concentrations of Cu^{2+} or CA involved. Fig. 2 illustrates the UV_Vis spectra of the chromophores thus generated. For all three compounds the UV_Vis spectrum exhibited a distinct absorbance maximum between 450 and 500 nm. It was presumed that these chromophores generated in the initial phase of the reaction corresponded to the *o*-quinone oxidation products of the CA precursors. When select PS were added to such reaction mixtures then within hours, e.g., fucoidan, or after overnight reaction, e.g., chondroitin sulfate (CS) A or C, the colors of the mixtures darkened to yellow-brown (EPI and NE) or grey-black (DA) (results not shown). These observations prompted us to speculate that the chromophore generated initially reacted further, generating other types of colored substances. The reaction of CAs in the presence of Cu^{2+}, with or without PS, was evaluated using RP-HPLC analyses. Fig. 3 shows a typical RP-HPLC profile of EPI after reaction with Cu^{2+} in the presence or absence of CS A. EPI had a retention time of about 6 min and following