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Characterization of Molecular Composition of Bacterial Melanin Isolated from *Streptomyces glaucescens* Using Ultra-High-Resolution FT-ICR Mass Spectrometry

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Abstract : In this study, the chemical composition of bacterial melanin isolated from the *Streptomyces glaucescens* strain was elucidated by ultra-high-resolution Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry. Ultra-high-resolution mass profiles of the microbial melanin product were acquired using a 15 Tesla FT-ICR mass spectrometer in positive and negative ion modes *via* electrospray ionization to obtain more complete descriptions of the molecular compositions of melanin-derived organic constituents. A mass resolving power of 500,000 (at *m/z* 400) was achieved for all spectra while collecting 400 scans per sample with a 4 M transient. The results of this analysis revealed that the melanin pigment isolated from *S. glaucescens* predominantly exhibits CHON and CHO species, which belong to the proteins class of compounds, with the mean C/O and C/N ratios of 4.3 and 13.1, thus suggesting that the melanin could be eumelanin. This analytical approach could be utilized to investigate the molecular compositions of a variety of natural or synthetic melanins. The compositional features of melanins are important for understanding their formation mechanisms and physico-chemical properties.

Key words: Melanin, *Streptomyces glaucescens*, FT-ICR MS, Chemical composition, Eumelanin

Introduction

Melanins, a group of complex natural pigments found in most organisms, are synthesized *via* polymerization of indolic and phenolic compounds and classified into three major types (*i.e.*, eumelanins, pheomelanins and allomelanins) based on color and structural classes.[1,2] Typically, biosynthesis of both eumelanins and pheomelanins is initiated from L-tyrosine to L-3,4-dihydroxyphenyl alanine (L-DOPA), and then undergoes conversion to dopachrome to form eumelanins or cysteinylation to generate pheomelanins (see **Figure 1**).[2] Unlike eumelanins and pheomelanins, allomelanins are the most heterogeneous group of melanin polymers, deficient of nitrogen and catalyzed by catechol precursors.[3]

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Melanins have often been considered important functional bio-polymers, owing to their diverse biological functions, such as thermoregulation, photo protection, acting as free radical sinks, acting as cation chelators, and antibiotic resistance.[4-7] Moreover, melanins can be utilized as pigment additives for food, cosmetics, and textiles. Melanin is known to exhibit high structural diversity and complexity of repeating units and unit linkages due to the complicated biosynthetic pathways. Therefore, the elucidation of the physiochemical properties of natural melanins remains challenging. Recently, El-Naggar and colleagues reported the efficient production of melanin pigments in the *Streptomyces glaucescens* NEAE-H strain and its anticancer and antioxidant activities.[8] They characterized the melanin product using various analytical techniques such as UV-vis, Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR) and scanning electron microscopy (SEM) to show the physico-chemical properties of the melanin are similar to those of other species. Prados-Rosales and coworkers also investigated the structural characteristics of the melanin isolated from mushrooms by using SEM, transmission electron microscopy (TEM), electron paramagnetic resonance (EPR) and NMR spectroscopy.[9] However, detailed chemical information on natural melanin remains veiled due to the complexity of the structure and the lack of analytical methods for it.

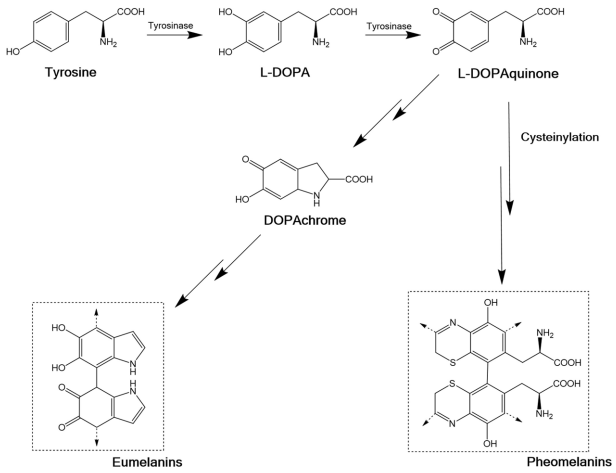

Figure 1. Simplified melanin biosynthesis pathway.

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) has emerged as a powerful tool for the analysis of extremely complex mixtures (*e.g.*, crude oils, water and soil-derived organic substances), resulting in rapid and accurate interpretation of the elemental compositions of complicated samples.[10-15] From the highly accurate mass data acquired by using FT-ICR MS, the assignment of elemental compositions based on exact mass differences is made, and then the assigned formulas are sorted by chemical class, double bond equivalent and carbon number. Marshall et al. demonstrated that a mass accuracy of ~1 mDa at 500 Da (e.g., ~200 ppb), corresponding to the mass resolving power of ~400,000, is required to efficiently achieve unique elemental composition assignment;[16] therefore, the unsurpassed resolving power (full width at half maximum, FWHM: >400,000 at *m/z* 400) and mass accuracy (<1 ppm) of FT-ICR MS and the development of advanced data processing tools and graphical methods has made it possible to successfully identify the chemical compositions of extremely complicated samples, compared to other types of high-resolution mass analyzers such as Orbitrap and Q-TOF.[11,17,18] Recently, high-resolution FT-ICR MS has also been utilized to characterize chemically or enzymatically synthesized humic-like substances.[19,20]

In this study, we examined the chemical composition of natural melanin isolated from the *S. glaucescens* strain using an ultra-high-resolution 15 Tesla FT-ICR MS. The resulting data were interpreted to investigate the molecular characteristics of the microbial melanin, providing clues to the type of melanin. This approach has offered insights into melanin structure and physico-chemical properties.

Experimental

Preparation of bacterial melanin for MS analysis

The isolation of melanin pigment from the culture of *Streptomyces glaucescens* strain was done, as previously described by El-Naggar et al.[8] The isolated melanin was dissolved in 7% (v/v) aqueous ammonium hydroxide solution and filtered using a 0.45 μm PTFE (Teflon) syringe filter to remove insoluble materials. Inorganic constituents included in the resultant were removed by using a Bond Elut PPL cartridge (Agilent, Santa Clara, CA). The cartridge was first conditioned with 3 mL of methanol, followed by 3 mL of 0.1% formic acid. Then, the melanin sample was loaded to the cartridge, then washed with ultrapure water three times. The melanin-derived organic components, retained on the cartridge, were eluted with 1.5 mL of methanol containing 2% ammonium hydroxide solution. The resulting eluates were immediately dried under a gentle nitrogen stream and redissolved with 50% aqueous methanol containing 0.1% formic acid for FT-ICR MS analysis with positive ion mode. For negative ion mode analysis, it was reconstituted with 50% aqueous methanol containing 7% ammonium hydroxide solution.

FT-ICR MS analysis

Ultra-high-resolution mass spectrometric analysis was performed on a 15T FT-ICR mass spectrometer equipped with an electrospray ionization source (solariX XR^{TM} system, Bruker Daltonics, Billerica, MA), as demonstrated previously[21] with some modification. The melanin samples were directly infused into the 15T FT-ICR mass spectrometer using a chip-based nano-electrospray system (TriVersa NanoMate, Advion BioSciences, Ithaca, NY) at a flow rate of approximately 400 nL/min by a spray voltage of 1.4 kV and a gas pressure of 0.3 bar and analyzed in negative ion mode within the mass range of *m/z* 150-1200. The mass resolving power was set at 500,000 (at *m/z* 400) for all spectra, and 400 scans per sample were collected with a 4 M transient. The other MS parameters were as follows: a drying gas flow rate of 1.5 L/min, drying gas temperature of 180°C, ion accumulation time of 0.05 s and transient length of 1.39 s. All FT-ICR mass spectra were externally calibrated using a NaTFA solution (10 μg/mL in methanol). Data acquisition was controlled by ftmsControl 2.0 software (Bruker Daltonics).

Assignment of elemental compositions

Raw data obtained from 15T FT-ICR MS were processed using DataAnalysis (ver. 4.2, Bruker Daltonics) and Composer (Sierra Analytics, Modesto, CA) softwares. After 15T FT-ICR MS measurements, the raw spectra were imported to the DataAnalysis 4.2 for peak detection and recalibration. The Composer, a formula calculator, was employed for assignment of elemental compositions, as described previously,[22] with some modifications. Briefly, the empirical molecular formulae were calculated for the masses of singly charged ions in the range of *m/z* 150–1,000 by allowing up to the combinations of 200 ^{12}C, 400

Table 1. Chemical properties of the assigned molecular compositions of bacterial melanin isolated from *S. glaucescens*.

Mode	Assigned Peaks	Mean DBE*	Mean AI**	Mean H/C	Mean C/O	Mean C/N	Mean C/S
Positive	1546	5.73	0.09	1.68	4.52	12.09	174.37
Negative	851	5.74	0.10	1.66	4.15	14.20	156.86

*The double bond equivalent (DBE) value representing the sum of rings and double bonds in each molecule can be calculated from the number of atoms in chemical formulas by the following equation: *DBE=1+C-H/2+N/2*.
**Aromaticity index.

^{1}H and 60 ^{16}O atoms, followed by additional calculations of molecular formula including up to 5 ^{14}N and 2 ^{32}S atoms, as described by Koch et al.[23] The FTMS spectra were recalibrated iteratively using the exact masses of the assigned chemical formulae with assignment errors < 0.3 ppm and then the molecular formulas with assignment errors > 0.3 ppm were ruled out from the final list for further processing, as previously demonstrated.[21] The van Krevelen plot was used to visualize the assigned compositions of the samples based on their molar H/C and O/C ratios.[17]

Results and Discussion

Ultra-high-resolution FT-ICR MS analysis of melanin isolated from *S. glaucescens*

The melanin sample isolated from *S. glaucescens* was analyzed using an ultra-high-resolution 15T FT-ICR mass spectrometer equipped with an ESI source with positive and negative ion modes. As shown in **Figure 2a**, the FT-ICR MS spectra of the melanin extract appear to differ by ionization mode. After processing the raw data using Composer software, 1546 and 851 molecular formulas were assigned to the ESI (+) and (-) FT-ICR MS data, respectively. All chemical characteristics of the assigned elemental formulae that were calculated using the Composer software from the FTMS data of the bacterial melanin were listed in **Supplementary Tables 1** and **2**. 442 chemical formulas identified *via* negative ion mode were also analysed *via* positive ion mode. After excluding duplicate formulas, 1955 molecular compositions were identified from the bacterial melanin pigment. The overall chemical attributions of the melanin compounds are summarized in **Table 1**. Regardless of the ion detection mode, we observed that certain chemical properties [*i.e.*, double bond equivalent (DBE), aromaticity index (AI) and hydrogen-to-carbon (H/C) ratio] were consistent (see **Table 1**). In particular, relatively low carbon-to-oxygen (C/O) and carbon-to-nitrogen (C/N) ratios were observed in negative ion and positive ion modes, respectively, compared to the opposite modes. This is perhaps due to the intrinsic properties of analytes: organic compounds possessing hydroxyl and carboxyl functional groups are more readily detected by ESI in negative ion mode, whereas nitrogen-containing compounds are preferentially ionized on the ESI positive ion mode.

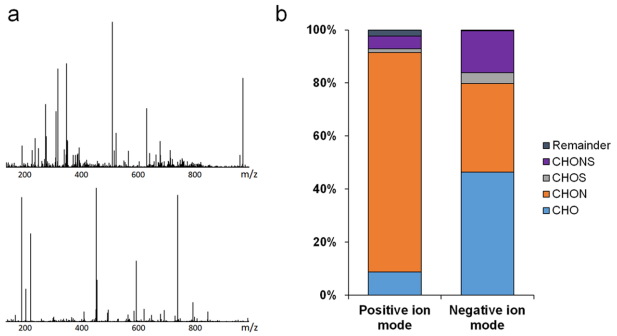

Figure 2. Reconstructed FT-ICR MS spectra of *S. glaucescens* melanin, analyzed *via* ESI-positive (upper panel in Figure 2a) and ESI-negative (lower panel in Figure 2a) ion modes. (b) Bar charts displaying the chemical class distribution of the microbial melanin, depending on the ion modes.

Molecular compositions and chemical class distributions of *S. glaucescens* melanin

ESI (+) FTMS analysis revealed that CHON class compounds were abundant in the melanin extract (82.8% of the total based on the relative ion intensity), whereas the CHON class species were 33.3% of the total in the ESI (-) FTMS analysis (**Figure 2b**). In contrast, CHO class compounds occupied 8.8% of the total in ESI (+) and made up a high proportion in ESI (-) (46.4% of the total). Sulfur-containing molecules were also detected in both ion modes in low proportion (6 to 20% of the total).

The assigned molecules were categorized by compound class using various chemical metrics (*i.e.*, lipids, proteins, unsaturated hydrocarbons, condensed aromatics, and lignins),[5,11] as displayed in the van Krevelen plots (**Figures 3a** and **b**). The van Krevelen diagrams show that the bacterial melanin-derived organic substances mostly belong to proteins, followed by lignins. The proteins class compounds were considered likely eumelanin-like molecules because of the nitrogen content in eumelanins, whereas the lignin-like species could be nitrogen-free allomelanins because lignins are primarily composed of carbohydrates with a low content of nitrogen. The analysis of the DBE versus the carbon number plot shows that most of the melanin constituents comprised compounds with DBE values of below 15 and 10-40 carbon atoms (**Figures 3c** and **d**). Taken together, the major contributors to the microbial melanin appear to be the CHON and CHO classes, and the

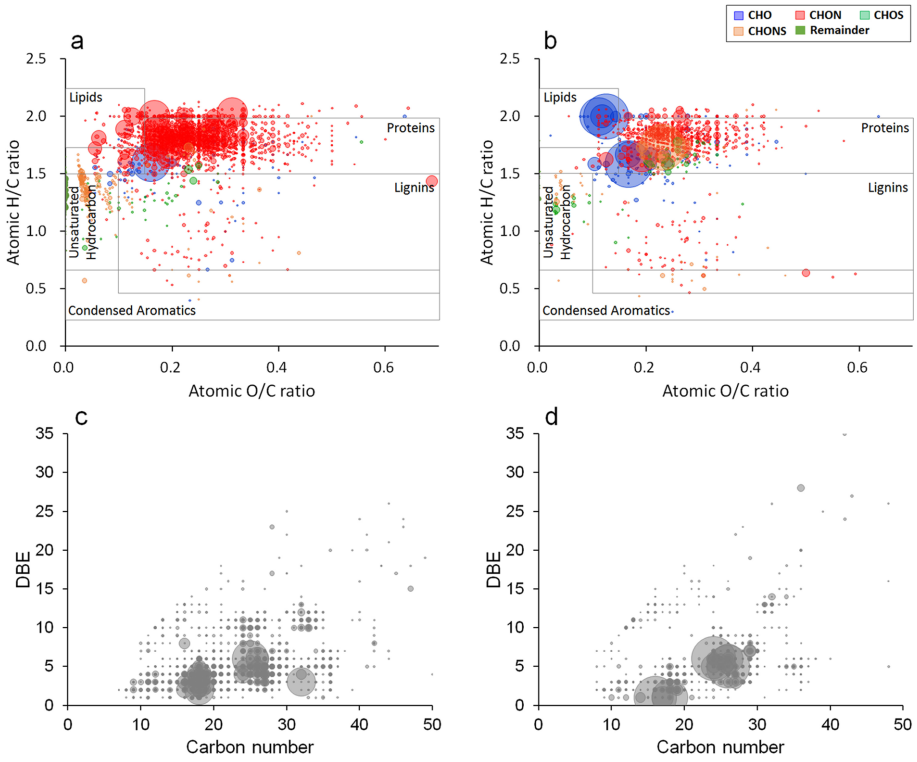

Figure 3. Van Krevelen diagrams showing the intensity-weighted distributions of chemical classes based on the molar H/C and O/C ratios of assigned elemental compositions from bacterial melanin, analyzed by ESI (+) and (-) FT-ICR MS (a and b) (color code: CH, red; CHO, blue; CHON, orange; CHOS, green; CHONS, purple; Remainder, dark green). The size of each circle is proportional to the intensity of the molecular formula. DBE and carbon number plots of the melanin, analyzed *via* ESI (+) and ESI (-) FT-ICR MS (c and d).

abundance of those compounds belonging to the proteins class is evidence supporting the classification of the bacterial melanin as eumelanin. Further investigation on the chemical characteristics of the melanin will be performed to better understand the formation mechanism.

Conclusions

In this study, the chemical composition of bacterial melanin was first investigated in detail using high-field FT-ICR mass spectrometry. The molecular composition and chemical distribution of the melanin pigment isolated from *S. glaucescens* unveiled the chemical characteristics of the microbial melanin. This analytical approach using ultra-high-resolution FT-ICR MS could be utilized to understand the molecular compositions of a variety of natural or synthetic melanins. The compositional features of melanins are important for understanding their formation mechanisms and physico-chemical properties.

Supporting Information

Supplementary information is available at https://drive.google.com/file/d/1Xu2iMfGN_0Hdbq_jlQaJq5eYNj3NBeQF/view?usp=sharing.

Acknowledgments

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