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Isolation and Characterization of Melanin Pigment from Yesso Scallop *Patinopecten yessoensis*

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Abstract Melanin is one of the essential compounds in the pigments of molluscan shells. However, the effects of melanin on color variations in molluscs are largely unknown. Our previous study suggests that Yesso scallop *Patinopecten yessoensis* might contain melanin pigment in the dark brown shell. We therefore isolated melanin from the pigmented shells using hydrochloric acid method, and characterized the types of melanin pigments by spectrophotometry. The purified melanin, which was verified by spectrophotometry scanning and HPLC analysis, showed the typical characteristics of melanin absorption spectra and HPLC chromatograms. The contents of pheomelanin and eumelanin in pigmented shells, which were determined by the linear standard curve of melanin at 405 nm and 350 nm absorbance, were 48.23 ± 1.350 and 157.65 ± 5.905 mg, respectively. The present results indicate that the brown-pigmented shells of scallops comprise approximately 76.6% of eumelanin and 23.4% of pheomelanin, which supports the presence of eumelanin-rich pigment in scallop shells. Therefore, the combination of hydrochloric acid extraction and spectrophotometric quantification is a rapid and efficient method to isolate and quantify melanin in shells. This will facilitate the melanin studies related to shell color polymorphism and the selective breeding of bivalves with different shell colors.

Key words eumelanin; pheomelanin; hydrochloric acid method; spectrophotometry

1 Introduction

Melanin is a common colored substance produced by animals, plants, and micro-organisms (Bell and Wheeler, 1986; Prota, 1995; Wang *et al.*, 2006). For animals, the color of hair, skin, and eyes mainly depends on the quantity, quality, and distribution of melanin pigments. There are two chemically distinct types of melanin, including the insoluble, black to brown eumelanin and the alkaline-soluble, yellow to reddish-brown pheomelanin. The ratio of eumelanin to pheomelanin in animal tissues is usually correlated with the visual phenotypes of their constitutive pigmentation (Taylor, 2002; Ito and Wakamatsu, 2003; Del Bino *et al.*, 2015). Additionally, melanin has different functions in the biosystem, such as photosensitization, metal ion chelation, photoprotection, antiaging activity, as well as resistance against harmful environmental factors (Lukiewicz, 1972; Slominski *et al.*, 2004; Selvakumar *et al.*, 2008; Lu *et al.*, 2014; Mbonyiryivuze *et al.*, 2015).

It is well-known that melanin is one of the essential compounds in shell pigments of molluscan species, but the correlation between melanin types and their visual phenotypes is not well understood. For mollusks, the shells with beautiful forms and colors have attracted the interest of naturalists and collectors for decades (Comfort, 1951). In the shellfish market, the quality and value of animals are usually determined by consumer preferences for shell color (Brake *et al.*, 2004; Guan and He, 2009; Ge *et al.*, 2015a). Furthermore, due to its Mendelian inheritance, the merit of selecting color variants has already been documented in clams, oysters, and scallops (Evans *et al.*, 2009; Zheng *et al.*, 2013; Ge *et al.*, 2015b; Yue *et al.*, 2015). Despite of the great importance of shell color traits, limited information is available on melanin extraction and quantification from pigmented shells. Recently, the rapid advances in next generation sequencing technologies allow us to identify potential genes involved in shell pigmentation of molluscan species (Bai *et al.*, 2013; Ding *et al.*, 2015; Yue *et al.*, 2015). However, how these color-related genes associate with the constitutive pigmentation of molluscan shells (*e.g.*, melanin pigments) are largely unknown. This hampers our understanding of shell color polymorphism and selective breeding on new color variants in molluscan species.

Melanin in mollusks probably is produced in the secretory cells of the mantle edge and is further transported to the prismatic shell layer (Comfort, 1951; Nagai *et al.*,

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2007). A series of reactions in melanin biosynthesis are regulated by tyrosinase, which catalyzes the initial and rate-determining step of melanogenesis (Slominski *et al.*, 2004). To date, a variety of genomic and transcriptomic studies in mollusks revealed that the expression of tyrosinase genes (*tyr*) may contribute to melanin biosynthesis and shell pigmentations in bivalves (Zhang *et al.*, 2012; Bai *et al.*, 2013; Ding *et al.*, 2015; Yue *et al.*, 2015). Although these evidences suggest that the pigmented shells might contain melanin, melanin has not yet been identified and characterized in these molluscan species.

In our selective breeding practice of Yesso scallop *Patinopecten yessoensis*, we obtained a number of shell color variants for this important marine bivalve species in aquaculture and fishery. Meanwhile, according to our transcriptomic study, tyrosine-related proteins have a function in both biomineralization and melanogenesis, which are predicted to be involved in both shell formation and pigmentation of the scallops (Sun *et al.*, 2015). In order to confirm the presence of melanin in the shells of *P. yessoensis*, we performed melanin extraction using hydrochloric acid method, and further characterized the types of melanin pigments, including eumelanin and pheomelanin, using the spectrophotometric method. The aim of the study was to study the effects of different types of melanin on shell color phenotypes in molluscan species, which can facilitate melanin studies related to shell color polymorphism and the selective breeding of bivalves with different shell colors.

2 Materials and Methods

2.1 Shell Samples Collection

The healthy scallop *P. yessoensis* individuals were obtained from a commercial hatchery in Yantai, China (119.97°N, 35.88°E). The scallops were cultured in sand-filtered sea water at 14±2℃ for two weeks as described in the previous study (Sun *et al.*, 2015). Since the presence of dark brown pigment on the left shell and the absence of pigmentation on the right valve, we collected the left shells of *P. yessoensis* (Red box area in Fig.1). Samples from three individuals were mixed and divided into three parts as the biological replicates, with 20 g shell powder in each part. They were used for melanin extraction and identification.

Fig.1 The picture for left and right shells in Yesso scallop *Patinopecten yessoensis*. The pigmented left shell (Red box) was selected to isolate melanin in this study.

2.2 Extraction, Purification, and Identification of Melanin

The shells were cleaned carefully to remove shell inhabitants, and any other particles from the outer shell surface. They were further rinsed thoroughly with clean water and dried completely in air. The cleaned shell samples were grinded into shell powder, and were used as the raw material for melanin extraction and purification.

In each experiment, 20 g dry shell powder was treated with 6 mol L^{-1} HCl, and melanin was extracted by hot reflux method (Liu *et al.*, 2009; Magarelli *et al.*, 2010; Xu and Zhou, 2013). The HCl was slowly added to the shell powder in 250 mL round-bottom flask and stirred continuously to avoid overflow and promote calcium carbonate and other organic matrix in shells to react with hydrochloric acid. The raw melanin residues were purified by acid hydrolysis with 6 mol L^{-1} HCl at 95–100℃ for 2 h in a hot reflux equipment to maximize the yield of melanin. The precipitated products, representing the crude products of melanin, were collected by filtration on a Buchner funnel.

The crude products of melanin were packed and purified in a Soxhlet extractor with petroleum ether (60–80℃ for 4 h) to remove the potential fat content. The products were rinsed thoroughly with clean water, filtered with decompress filter, and dried by oven at 80℃. A small fraction of melanin precipitate was picked and dissolved in 2 mL of NaOH incubating at 85℃ water bath for 1 h. The supernatant was collected by centrifugation at 10000 r min^{-1} for 2 min, which was used for ultraviolet-visible (UV) scan from 190–500 nm at 1-nm intervals using Unico UV-2800 spectrophotometer (Shanghai, China) in a rectangular quartz cuvette.

To determine the accuracy and reliability of spectrophotometric analysis, the same sample was also identified and characterized by high pressure liquid chromatography (HPLC) method according to Ito *et al.* (2011). An aliquot (80 μL) of the supernatant was directly injected into the HPLC system on an Agilent 1100 ChemStation. The C18 column (4.6 mm × 250 mm, 5 μm particle size; Ascentis) was used and the UV detector measured at 269 nm. The mobile phase was 0.1 mol L^{-1} potassium phosphate buffer (pH 2.1)/methanol, 99:1 (v/v). Analyses were performed at 50℃ at a flow rate of 0.7 mL min^{-1}.

2.3 Quantitative Assessment of Pheomelanin by Spectrophotometry

The remaining melanin pellet was suspended in 5 mL of 1 mol L^{-1} NaOH and incubated at 85℃ for 1 h with stirring several times to obtain pheomelanin solution (Ito *et al.*, 1993; Oancea *et al.*, 2009; Kumar and Joy, 2015). The colored supernatant was collected in fresh tubes by centrifugation at 10000 r min^{-1} for 2 min at 4℃. The resulting insoluble precipitate was kept in 4℃ for further eumelanin analysis. The obtained supernatant containing pheomelanin (200 μL) was analyzed by Tecan Infinite 200 PRO (Männedorf, Switzerland) at 405 nm according to

the previous studies (Oancea *et al*., 2009; Kumar and Joy, 2015). The concentration of pheomelanin extracted from shell powder was estimated from the melanin standard curve, which was also determined by measuring the absorbance at 405 nm for a series of concentrations of synthetic melanin (Sigma) dissolved in 1 mol L^{-1} NaOH. Three replicates were analyzed for each measurement.

2.4 Quantitative Assessment of Eumelanin by Spectrophotometry

The alkali insoluble fraction containing eumelanin was re-suspended and adopted as the solubilization condition for eumelanin according to the previous study (Ito *et al.*, 1993). The optical density at 350 nm was measured using the resulting eumelanin solution made by *Sepia* melanin (Melanin from *Sepia officinalis*, Sigma) according to Wakamatsu and Ito (2002). *Sepia* melanin was dissolved in hot sodium hydroxide in the presence of hydrogen peroxide as described above. The standard curve for eumelanin over the concentrations was also made by measuring the absorbance at 350 nm using Tecan Infinite 200 PRO. The relative ratio of eumelanin to pheomelanin was calculated to serve as the indicator of melanin types in scallop shells (Ozeki *et al.*, 1996). Three replicates were made for each measurement. All the data analyses have been performed using EXCEL.

3 Results

3.1 The Extraction and Identification of Melanin

The solid substances extracted from the shells are black-brown powder. The UV spectrophotometry scanning ranged from 190 to 500 nm indicates that the UV spectral property is typical of the absorption profile of melanin (Fig.2). The absorption peak is observed at 297 nm, and then rapidly decreased with the increasing wavelength, which may be due to the presence of many complex conjugated structures in the melanin molecule (Selvakumar *et al.*, 2008). The accuracy and reliability of spectrophotometric analysis was verified by HPLC analysis. As showed in Fig.3, HPLC chromatograms of melanin samples were characterized as 1,3-thiazole-4,5-dicarboxylic acid (TDCA, peak 1), pyrrole-2,3-dicar-boxylic acid (PDCA, peak 2), 1,3-thiazole-2,4,5-tricar-boxylic acid (TTCA, peak 3), and pyrrole-2,3,5-tricarboxylic acid (PTCA, peak 4).

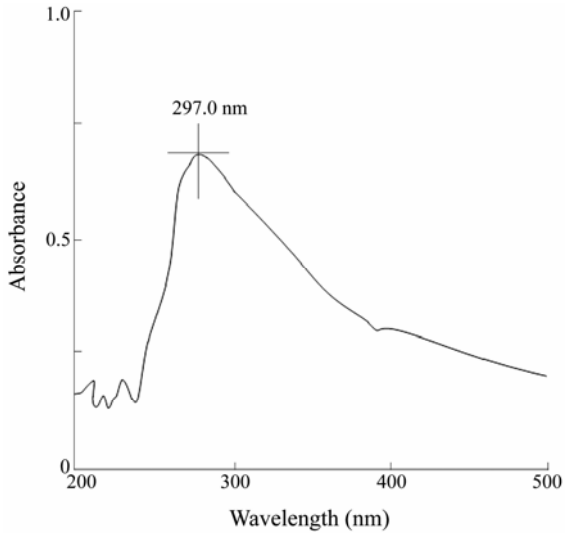

Fig.2 UV spectrum of melanin extracts of shells in scallop *P. yessoensis*.

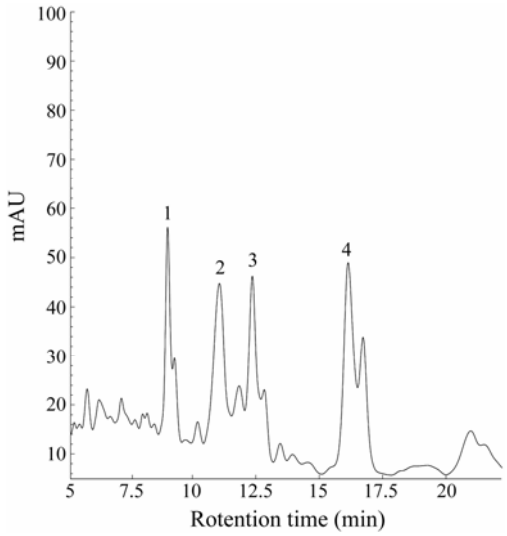

Fig.3 HPLC chromatograms of the extracted melanin, with TDCA (peak 1), PDCA (peak 2), TTCA (peak 3), PTCA (peak 4).

3.2 Quantitative Assessment of Pheomelanin and Eumelanin by Spectrophotometry

Based on the 405 nm absorbance of four serial dilutions of synthetic melanin (0.001, 0.01, 0.1, and 0.5 mg mL^{-1}), a linear standard curve ($y=7.497x+0.1177$, $R^2=0.998$) is plotted for the quantification of pheomelanin in Fig.4. The UV spectrophotometry scanning for pheomelanin was showed in Fig.5A, with the absorption peak at 315 nm followed by rapid decreasing. The UV absorbance of alkali-soluble pheomelanin solution is observed at 1.274± 0.01 A (mean ± SD). Therefore, the concentration of pheomelanin is estimated to be (9.646 ± 0.270) mg mL^{-1} according to the standard curve, resulting in a total of 48.23 ± 1.350 mg (9.646 mg mL^{-1} × 5 mL) of pheomelanin.

A linear standard curve for eumelanin is also obtained according to the 350 nm absorbance of the same four serial dilutions of Sepia melanin, $y=7.275x+0.3251$ ($R^2=$ 0.973; Fig.6). The UV spectrophotometry scanning for eumelanin was showed in Fig.5B, with the same but wider peak at 315 nm followed by rapid decreasing. Because the absorbance values for eumelanin resolution are beyond the upper limit for UV detection (about 4.0), four-fold dilution with 1 mol L^{-1} NaOH is made for the absorbance measurement of eumelanin. The UV absorbance of the diluted eumelanin solution is 2.587 ± 0.041 (absorbance, mean ± SD), which results in an average concentration of (31.530 ± 1.181) mg mL^{-1} for eumelanin. To sum up, there is a total of (157.65 ± 5.905) mg (31.530 mg mL^{-1} × 5 mL) of eumelanin. Therefore, the present results reveal that scallop shell comprises approximately

76.6% of eumelanin and 23.4% of pheomelanin. The relative ratio of eumelanin to total melanin is estimated to be about 0.8, indicating the eumelanin-rich pigment in the colored shells.

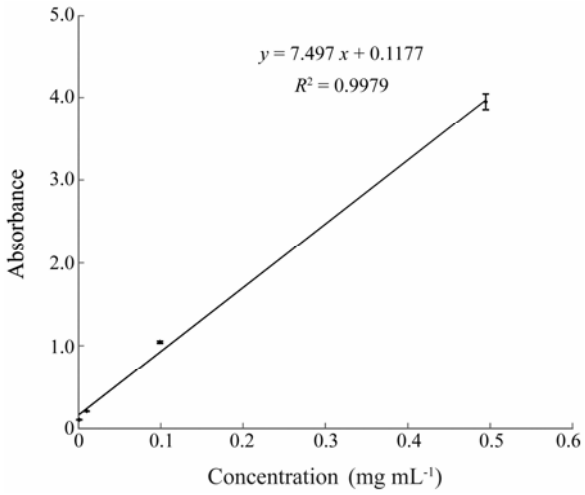

Fig.4 The linear standard curve plotted for pheomelanin quantification based on the 405 nm absorbance of four serial dilutions of synthetic melanin (0.001, 0.01, 0.1 and 0.5 mg mL^{-1}).

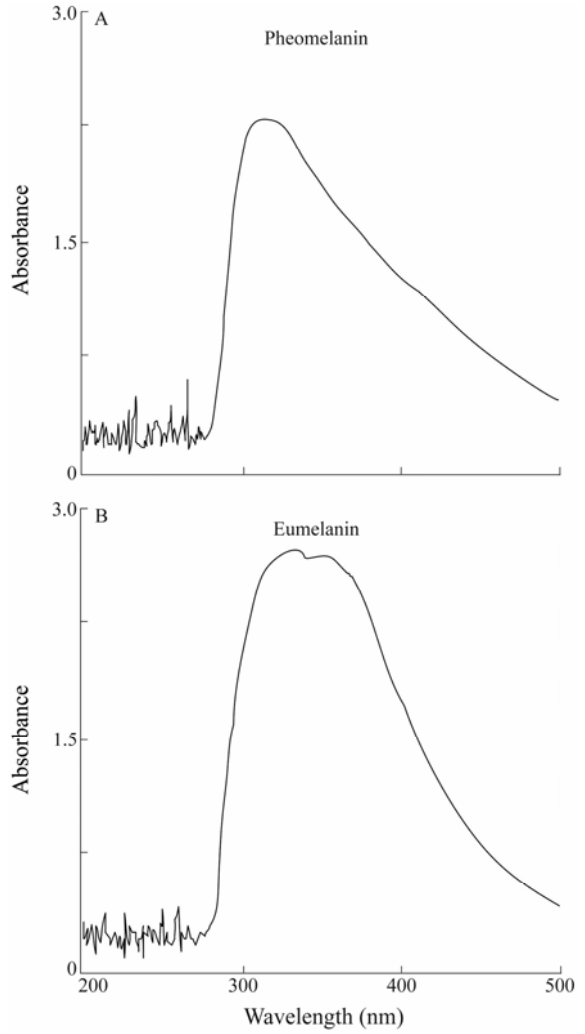

Fig.5 UV spectrum scanning for pheomelanin (A) and eumelanin (B).

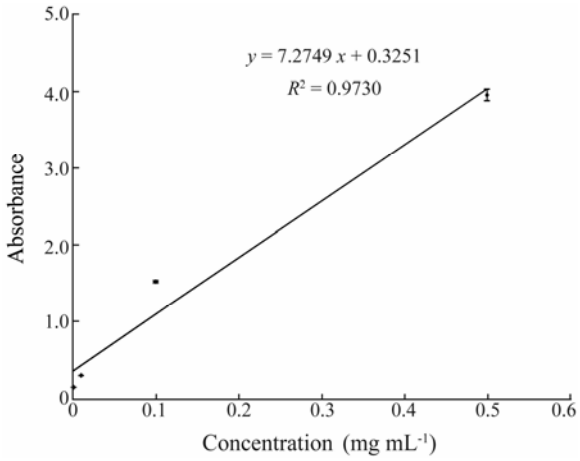

Fig.6 The linear standard curve plotted for eumelanin quantification based on the 350 nm absorbance of four serial dilutions of *Sepia* melanin (0.001, 0.01, 0.1 and 0.5 mg mL^{-1}).

4 Discussion

As one of the essential pigments in molluscs, melanin exists both as a part of the general color mosaics and in association with photo-receptor mechanisms (Comfort, 1951). Among molluscan species, eumelanin isolated from the ink sac of *Sepia officinalis* is the most striking example of melanin, which is commonly used as a model to study the spectroscopy, and morphology of this class of black pigments (Liu *et al.*, 2003). Melanin is often difficult to isolate due to their intractable chemical properties, the heterogeneity in their structural features, and the lack of simple methods that allow the identification of their chemical forms (Wakamatsu and Ito, 2002; d'Ischia *et al.*, 2013). In this study, melanin was successfully extracted and purified from the scallop shells by using the hydrochloric acid method. The isolated melanin, which was verified by spectrophotometric measurement and HPLC analysis, showed the typical characteristics of melanin absorption spectra and HPLC chromatograms (Cockell and Knowland, 1999; Wakamatsu and Ito, 2002; Wakamatsu *et al.*, 2003). The two main methods have been developed for melanin isolation from skin and hairs, including the acid/base extraction and enzymatic extraction (Ito and Wakamatsu, 2003; Liu *et al.*, 2003). Hydrochloric acid method has been widely used for isolating the insoluble melanin pigment from human hair, feather, and skin (Ito and Wakamatsu, 2003). The present results indicate the feasibility of hydrochloric acid method in isolating melanin from scallop shells. For molluscan shells, they are almost always composed of polymorphs of calcium carbonate, either calcite or aragonite, which can dissolve in acid solutions and produce calcium chloride and carbon dioxide, while melanin could not react with acid solutions (Arnaud and Bore, 1981; Liu *et al.*, 2003). The hydrochloric acid method described in this study is a useful tool for isolating melanin from pigmented shells of other molluscan species.

Because of the alkaline-soluble property of pheome-

lanin, melanin samples are usually dissolved by hot NaOH for the quantification analysis of pheomelanin by spectrophotometric measurement at 405 nm (Oancea *et al*., 2009; Kumar and Joy, 2015). However, the method could not be used for hair or feather samples because of the insolubility of eumelanin in these tissues (Ito *et al.*, 1993; Ito *et al.*, 2011; Ito and Wakamatsu, 2003). For the quantification analysis of eumelanin in human hair, the insoluble eumelanic pigments can be solubilized in hot NaOH in the presence of H_2O_2 and then measured for its absorbance at 350 nm (Wakamatsu and Ito, 2002; Ito and Wakamatsu, 2003). Therefore, in this study, we made the eumelanin solution by using the treatment of alkaline hydrogen peroxide to quantify eumelanin in the scallop shells by the spectrophotometric method (Wakamatsu and Ito, 2002).

It is the quantity and the ratio of eumelanin to total melanin that mainly determines the color of hair, skin, and eyes (Ozeki *et al.*, 1996; Ito and Wakamatsu, 2003). For human hair, a ratio above 0.25 indicates the presence of a eumelanin-rich pigment, whereas a ratio below 0.15 is consistent with a pheomelanin-rich pigment (Ito and Fujita, 1985; Ozeki *et al.*, 1996). The present results reveal that in the scallop shells the quantity of eumelanin is more than three times of pheomelanin (76.6% eumelanin *vs*. 23.4% pheomelanin). The ratio of eumelanin to total melanin (about 0.8) in scallop shells is relatively higher than that in human hair, which means eumelanin is a rich pigment in scallop shells and reflects the distinct type of melanogenesis. Although HPLC (High Performance Liquid Chromatography) is an efficient method for quantitatively analyzing eumelanin and pheomelanin in animal tissues (Ito and Wakamatsu, 2003; Liu *et al.*, 2003), the present spectrophotometric method is relatively more convenient to determine the types of melanin pigment in molluscan shell samples, which would be suitable for routine and comparative purposes on large numbers of samples.

5 Conclusions

In the present study, we isolated melanin from the pigmented shells using hydrochloric acid method, and characterized the types of melanin pigments by the spectrophotometric method. The present findings not only demonstrate the feasibility and practicality of hydrochloric acids method in isolating melanin from molluscan shells, but also reveal that the brown-pigmented shells of scallops comprise approximately 76.6% of eumelanin and 23.4% of pheomelanin. The ratio of eumelanin to total melanin indicates the presence of eumelanin-rich pigment in scallop shells. The present results will promote melanin studies related to shell color polymorphism and shellfish selective breeding on color variants for aquaculture.

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