Food Chemistry 111 (2008) 745–749

Contents lists available at ScienceDirect

Food Chemistry

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

Isolation and characterization of natural melanin derived from silky fowl (*Gallus gallus domesticus Brisson*)

Si-Rui Chen, Bin Jiang, Jiang-Xia Zheng, Gui-Yun Xu, Jun-Yin Li, Ning Yang *

College of Animal Science and Technology and State Key Laboratory of AgroBiotechnology, China Agricultural University, Beijing 100193, China

ARTICLE INFO

Article history:
Received 17 December 2007
Received in revised form 29 February 2008
Accepted 22 April 2008

Keywords:
Melanin
Eumelanin
Silky fowl
Physicochemical property
Morphology

ABSTRACT

Water-insoluble melanins derived from silky fowl (SF-melanin) were isolated using an enzymatic extraction procedure. The yields of the pigments from periosteum, ovary or testis, trachea, skin and muscle, were 21.3, 13.7, 10.2, 1.1, and 1.0 mg/g, respectively, on a fresh tissue basis. The isolated pigments were identified as melanins according to the Electron Paramagnetic Resonance Spectroscopy spectra. Using synthetic melanin as a calibration, their physicochemical and morphological properties were further characterized by X-ray photoelectron spectroscopy (XPS) and advanced imaging technology. Elemental composition analysis by XPS revealed that the main component of the SF-melanin was eumelanin. The morphological study showed that (1) the SF-melanin displayed ellipsoidal melanosomes which was regarded as representative of the natural material; and (2) the SF-melanin maintained its natural integrity. In conclusion, our enzymatic extraction method yielded highly purified products while maintaining the natural properties of the SF-melanin, which could be chemically, physically, morphologically, defined as eumelanin.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Black-bone silky fowl (*Gallus gallus domesticus Brisson*), known as a marvel of traditional Chinese medicine, lives up to its name with snow-white silky feather but black skin, meat and bones. As a unique chicken breed, the silky fowl, originated from the south of China, is distinguished from broiler and layer chicken according to the genome-wide genetic variation analysis (International Chicken Polymorphism Map Consortium., 2004). Generally, the silky fowl could be differentiated into two phenotypes according to the feather color: white-feather-black-bone, black-feather-black-bone. However, only Taihe black-bone silky fowl (Jiangxi province, China), is designated as main ingredient in Chinese traditional medicine. The orthodox Taihe black-bone silky fowl has ten apparent characteristics: crest, beards, blue comb, green earlobes, white silky feathers, black bones, black muscles, black skin, five toes and feathered shanks. The white silky feather is the unique characterization of the Taihe chicken when compared to the other black-bone silky fowl. In Asian countries, silky fowl is not only a reputed medicine known as "white phoenix", but also a popular healthy food used as an immune booster and a ward against emaciation and feebleness (Tian, Xie, Wang, Wu, Fu, & Lin, 2007). While looking for an explanation for the bird's reputed medical abilities, several aspects have been considered to be contributive including antioxidant contents and amino acid/peptide profiles, e.g., carnosine (Tian et al., 2007). In silky fowl, the melanins are *de novo* synthesized in melanocytes (Muroya, Tanabe, Nakajima, & Chikuni, 2000), which are widely dispersed in the muscle, the surface of bone, trachea, mesentery, digestive canals, ovary, testis and other tissues (Makita & Mochizuki, 1984). Considering this wide distribution of the melanins, people wonder whether silky fowl melanin (SF-melanin), a natural pigment, might be partly responsible. However, information is lacking on the physiochemical property, morphological characterization, and biological activity of SF-melanin.

Melanins are irregular pigments widely distributed in tissues of diverse organisms (Harki, Talou, & Dargent, 1997). Melanins have radioprotective and antioxidant properties that provide effective protection to living organisms from ultraviolet radiation (Vinarov, Robysheva, Sidorenko, & Dirina, 2002). They are also considered as important antioxidant in food (Hung, Sava, Makan, Chen, Hong, & Huang, 2002; Sava, Yang, Hong, Yang, & Huang, 2001). In addition, they have been widely used in medicine, pharmacology, cosmetics and other fields (Borshchevskaya & Vasilieva, 1999). It is believed that the melanin production in mammals mainly results from oxidative polymerization of phenolic compounds by tyrosine or catecholamines, which are transformed into the pigmented polymers by tyrosinase or peroxidase (Prota, 1992). Melanins are produced and stored in the melanosomes within the melanocytes that produce two chemically distinct types of melanin pigments, namely the eumelanin and the phaeomelanin (Prota, 1992; Ito, 1993). Avian-derived melanins also contain two basic types: sulfur-free eumelanin and sulfur-containing phaeomelanin (Jimbow, 1994). Ito and Jimbow reported that the content of sulfur and nitrogen

* Corresponding author. Tel.: +86 10 6273 1351; fax: +86 10 6273 2741.
E-mail address: nyang@cau.edu.cn (N. Yang).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.04.053

in eumelanin ranged from 0 to 1%, and 5% to 9%, respectively (Ito & Jimbow, 1983).

Several technologies have been used to obtain melanins, for example, biological extraction from natural sources (animal or plant) (Golounin, 1997; Kerestes, Kerestes, & Venger, 2003), chemical synthesis (Pawelek & Orlow, 1992), and microbiological production (Oloke & Click, 1997). Generally, melanosomes in animal materials are distributed in a biologically environment. The isolation of melanin from such tissues has commonly been achieved by using concentrated acid and sodium hydroxide (Arnaud & Bore, 1981; Filson & Hope, 1957). However, extracted natural melanins exhibit defects because conventional extraction procedures have employed harsh treatments which can modify their physical and chemical properties. In the other hand, chemical synthesized melanins can not fully represent the nature of melanins because they do not contain any proteins as associated *in vivo*. The other critical problem in the study of pigments is the extraction efficiency. Moreover, extraction efficiency varies widely depending upon both physical disruption and chemical extraction. Independent or combined mechanical disruption techniques including steeping, grinding, and ultrasonic baths might be effective if employed appropriately (Hagerthey, Louda, & Mongkronsri, 2006). It is now well documented that natural pigments contain both melanin (different kinds of monomer units that are connected through carbon–carbon bonds) and melanoproteins which are important in defining the assembly of melanins (Kazumasa, Wakamarsu, & Shosukeito, 2002).These monomer units can not be easily split either by chemical methods or by the action of enzymes because of the chemical properties, such as their insolubility over a broad range of pH (Liu et al., 2003). In contrast, the melanoproteins which are spherically surround these monomer units can be modified during the harsh extraction procedures, and it has been demonstrated by electron microscopy imaging (Liu et al., 2003). In recent studies, new enzymatic isolation methods were developed which provided more homogenous and intact products than chemical extraction. These protocols often involve consecutive enzymatic treatments with proteinase K, collagenase, and other kinds of protease to separate the melanin from proteins and other biologic materials (Liu et al., 2003).

The aim of this study was to develop a rapid, cost-effective and efficient procedure for SF-melanin isolation as well as to investigate both physicochemical and morphological characterization of the pigment with sophisticated measures.

2. Experimental

2.1. Materials

Chemical synthetic melanin was purchased from Sigma (St Louis, MO, USA) and used as a standard for both physicochemical and morphological characterization without further purification. Proteinase K and Triton X-100 were purchased from Sigma (St Louis, MO, USA). All other chemicals were of suitable grade. Six Taihe black-bone silky fowl chickens including three hens and three cocks aged 20 weeks, were obtained from National Center for Poultry Performance Testing (Beijing, China). All procedures in this experiment were approved by the Animal Care and Use Committee of China Agricultural University (Beijing, China) and performed in accordance with animal welfare and ethics.

2.2. Methods

2.2.1. Extraction Procedures

2.2.1.1. Sample preparation. Fresh tissue samples from the silky fowl were the skin, muscle, ovary or testis, trachea, and periosteum. Briefly, adequate tissues were dissected into small pieces, and then precleared of blood by stirring in phosphate-buffered saline (PBS, pH 6.8) at room temperature. After centrifugation at 3300*g* for 15 min, the material was washed with distilled water and suspended in 0.1 M phosphate buffer (pH 6.8) with 2% Triton X-100 to remove grease. After further centrifugation at 100,000g for 15 min, the pellets were washed with distilled water for 6 times to isolate the insoluble material. This insoluble material was homogenized to powder in liquid nitrogen by pestle and mortar. The following enzymatic extraction was carried out based on a method reported by Liu with modifications (Liu et al., 2003).

2.2.1.2. Enzymatic extraction. (1) An aliquot of the powder (5 g) was added to 300 ml of 0.1 M phosphate buffer, pH 7.4, together with 500 mg of dithiothreitol (DTT). This solution was stirred for 23 h at 37 °C under argon. (2) Proteinase K (3.86 U/mg) and DTT (300 mg) were added and allowed to act for 10 h at 37 °C. The solution was centrifuged at 3300*g* for 15 min. The pellet was rinsed for 6 times with distilled water, and then suspended in 100 ml phosphate buffer. (3) Proteinase K (1.3 U/mg) and DTT (40 mg) were added and allowed to react for 10 h as before. (4) The solution was centrifuged, and the pellet was suspended in 50 ml deaerated buffer with 2% Triton X-100. (5) The suspension was then stirred for 4 h. Ultracentrifugation (100,000*g*) was used to pellet the SF-melanin. (6) The final pellet was washed for 6 times with distilled water and dried over NaOH under argon, weighed and stored at −20 °C. Melanin content was expressed as the amount of pigment per gram of fresh tissue.

2.2.2. Physicochemical characterization of the SF-melanin by EPR and XPS spectra

The samples (SF-melanin, synthetic-melanin) were weighed and placed in quartz tubes with same diameter and analyzed by EPR spectroscopy. EPR spectra were registered at 25 °C by EMX-8 (Bruker BioSpin Corp, Germany) on the 9.5400 Hz high frequency. The chemical contents of these samples were performed on the XPS spectra. XPS spectra were obtained using metal tablets on PHI Quantera SXM (Pekin-Elmer PHI Corp, USA).

2.2.3. Morphological characterization of the SF-melanin by SEM and TEM imaging

The morphological properties of the samples were observed by advanced imaging technology. Scanning electron microscopy (SEM) images were captured by a KYKY2800 SEM (KYKY technology development LTD Corp, China) operated at 20 kV/25 kV, working distance at 6.0 mm. Transmission electron microscopy (TEM) images were collected by N-800 TEM (Hitachi Corp, Japan) operated at 100 kV.

2.2.4. Statistical analysis

Data of the melanin contents for the silky fowl tissues were analyzed by a two-way Analysis of Variance (ANOVA) using the General Linear Model procedure (Statistics Analysis System). The main effect of the melanin contents for tissues was further tested by the Tukey's significant difference test. The probability level at $p \leqslant 0.05$ was considered significant.

3. Results and discussion

3.1. Sample preparation

Enzymatic extraction is the primary isolation procedure for melanin and is also widely applied to food processing industry. In silky fowls, most of the internal organs are infiltrated with melanocytes where melanosomes produce and store melanins. Extraction efficiency of melanin from these tissues depends on structural

resistance to disruptions, extraction time, and mechanical disruption protocols. Tight junction-associated proteins between melanocytes and other cells, e.g., keratinocytes, differ among tissues such as skin, musle, hair and periosteum, which may result in different structural resistance to enzymic disruptions. An optimized combination of enzymes might decrease this structural resistance. Muroya et al. (2000) have extracted melanin from ovary and testis using Collagenase II and trypsin III. Long-duration extractions can increase the formation of pigment degradation products and isomerization (Cartaxana & Brotas, 2003). To reduce these disadvantaged changes of melanin, proper physical disruption of tissues is of vital importance so that most of the tissues that hold melanin can be easily reached and digested by enzyme. In the present study, we developed an improved procedure which incorporated the tissue grinding in liquid nitrogen prior to enzymatic digestion. When validating an extraction technique, parameters like extractability, fidelity, compatibility, precision, simplicity and safety are very important (Wright, Jeffrey, & Mantoura, 1997). Heat produced during grinding and sonication can also increase pigment degradation products and stimulate pigment activity (Wright et al., 1997). In our extraction, liquid nitrogen was used to reduce the heat production in the procedure of mechanical disruption. At the same time, SF-melanin could be easily separated from the cells since melanocytes have been intensely broken during manual grinding in liquid nitrogen.

3.2. Melanin contents in silky fowl tissues

The melanin contents with our enzymatic procedure from different tissues were shown in Table 1, which corresponded well with previous anatomical observation of the melanocyte distribution in silky fowl (Makita & Mochizuki, 1984). The melanin contents of SF-melanin in different tissues differed notably. The melanin contents of the melanin in periosteum severed from femur was 21.3 mg per gram of tissue and was the highest of all tissues examined ($p < 0.05$). The hyperpigmentaion in the periosteum depended on the expansive distribution process of the melanocyte but not on the special biosynthesis and characteristics of the melanin (Muroya, Tanabe, Nakajima, &Chikuni, 2000). Pigmentation in the gonads (ovary or testis) and trachea (13.7 and 10.2 mg/g, respectively) were significantly lower than in the periosteum, but significantly higher than in the other tissues. The melanin contents of muscle and skin were 1.0 mg/g and 1.1 mg/g, respectively. The melanin contents in liver, pectoralis, gizzard, heart and other tissues were less than 1.0 mg/g. Our study showed that periosteum, gonads (ovary or testis) and trachea were the most abundant in melanin. It is noted that meat, viscera and bone of Taihe black-bone silky fowl are the very tissues used in traditional Chinese medicine. Melanins are known for the radioprotective and antioxidant properties that protect the living organisms from ultraviolet radiation and free radical species (Vinarov et al., 2002). Tea melanins can chelate metal, terminate the onset the free radical species reactions (Sava et al., 2001), and prevent lipid peroxidation (Hung et al., 2002). Taihe black-bone silky fowl is a traditional Chinese medicine used in gynecology, diabetes, osteoporosis, early aging and other nutritional disorder. The abundant melanin may be beneficial for its nourishment and medicinal functions. As a high-quality chicken breed abundant in melanin, black-bone silky fowl would be good animal resources of natural bio-active compounds for lasting health benefits.

Table 1
Comparison of melanin contents in different tissues of the silky fowl

Tissue	Melanin contents (mg/g)*
Periosteum	21.3 ± 2.0[a]
Ovary or Testis	10.7 ± 3.5[b]
Trachea	10.2 ± 1.2[b]
Skin	1.1 ± 0.3[c]
Muscle	1.0 ± 0.3[c]

* Six silky fowls were used. Pigment content of each tissue for each chick was measured in duplicate. Means with different letters are significantly different ($p < 0.05$).

3.3. Physicochemical and morphological characterization of melanin from silky fowl

3.3.1. Electron paramagnetic resonance signal of the melanins

The electron paramagnetic resonance (EPR) spectra of the SF-melanin and the synthetic melanin (Sigma) are presented in Fig. 1. The patterns appeared nearly identical to those exhibited by other melanins reported in the literature, such as the water-soluble microbial melanin (Aghajanyan, Hambardzumyan, Hovsepyan, Asaturian, Vardanyan, & Saghiyan, 2005) and the melanin from tetrahydropapaveroline (THP-melanin) (Mosca, Blarzino, Coccia, Foppoli, & Rosei, 1998). The spectrum of SF-melanin was a slightly asymmetric singlet without hyperfine structure. Linewidth was about 7 Guass, and $g = 2.002$. Data of EPR spectra are presented in Table 2. Both ΔH and g of SF-melanin were higher than synthetic melanin, the concentration of paramagnetic centers in synthetic melanin (7.446×10^{17} spin/g) was about 58.4% of the value of the SF-melanin (12.743×10^{17} spin/g). However, a single narrow EPR line without any structure is not a unique feather of melanin. What differentiates melanin pigments from such non-melanin material is the reaction of the EPR signal of melanin on pH, light and Zinc ions. To identify whether the EPR signal was solely attribute to the melanin, a serial gradient dilution seemed necessary as used in determination of the water-soluble *Bacillus thuringiensis* melanin (Aghajanyan et al., 2005). It is noted that SF-melanin is a water-insoluble dioxyphenylalanine melanin, which can not be distinguished from the charcoal-like material. Though its physical property was performed on its aqueous solution, the molecular structure of this SF-melanin is warranted to be defined with more sophisticated imaging technologies.

3.3.2. Chemical analysis and comparison of element composition

Melanins presented in pigmented tissues generally appeared to be mixtures or copolymers of eumelanins and phaeomelanins (Prota, 1992). In the present work, the element composition of two melanins from different sources had general similarity but specific dissimilitude (Table 3). The results suggested that the main com-

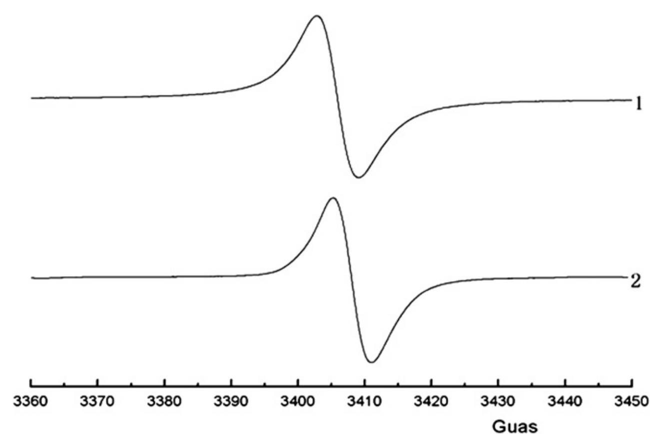

Fig. 1. EPR spectra of melanins: (1) synthetic melanin and (2) SF-melanin.

Table 2
EPR parameters of both SF-melanin and synthetic melanin

Source	Sample weight (mg)	g	ΔH
Synthetic melanin	7.0	2.001	6.351
SF-melanin	10.3	2.002	7.000

EPR: electron paramagnetic resonance; SF-melanin: melanin from silky fowl tissues; and g, ΔH are parameters.

Table 3
The element composition of melanins

Source[a]	Element composition (%)[b]			
	Carbon (C)	Nitrogen (N)	Oxygen (O)	Sulfur (S)
Synthetic melanin	69.72	5.08	24.71	ND[c]
SF-melanin	78.86	5.67	15.15	0.31

[a] Pigment samples from four silky fowl chicken tissues were mixed and performed on the XPS spectra.
[b] The element composition of pigment samples were measured in duplicate. Data we used were means.
[c] ND indicates the element could not be detected (the element content was less than 0.1%).

ponent of SF-melanin was eumelanin. Eumelanins are highly heterogenous polymers derived from oxidative coupling of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid (Ito, 1986). Cheun suggested that the melanin domains contained many layers connected by covalent bonds formed by the functional groups from the monomers at the edge of the domain, with which the melanin domain spherically was surrounded by the melanoproteins (Cheun, 2004). We speculate that sulfur in SF-melanin might have two possible sources: (1) sulfur-containing phaeomelanins or copolymers of eumelanins and phaeomelanins; (2) sulfur-containing aminophenol in the melanoproteins. The contents of both nitrogen and carbon in the SF-melanin were higher than that of the synthetic melanin. On the contrary, the content of oxygen in SF-melanin was lower than that in synthetic melanin. This result indicated that the compactness of layers within SF-melanin should be higher than that within synthetic melanin.

3.3.3. The morphological properties of melanins

A variety of imaging technologies such as scanning probe microscopies have provided new insights into the morphology of the pigment assembly (Liu & Simon, 2003a). In the present study, the morphological properties of SF-melanin and synthetic melanin were significantly different. The synthetic melanin presented amorphous morphology, while SF-melanin with enzymatic procedure exhibited spherical and/or ellipsoidal melanosomes (Figs. 2 and 3).

Fig. 2A presents a scanning electron microscopy (SEM) image of the synthetic melanin, while higher magnification image is shown in Fig. 2B. The synthetic melanin appeared as chunks of amorphous pseudocubic materials lacking a crystalline structure. Fig. 2C is the image of purified SF-melanin which formed fine-grained aggregates of tiny crystals. Higher magnification image revealed the surface of these crystals was not smooth, as shown in Fig. 2D. These isolated melanosomes might aggregate into clusters when dried. The SF-melanin contained many granules which had spherical and/or ellipsoidal shape in common as exhibited also by other melanins such as *Sepia* melanin (Liu & Simon, 2003b), human hair eumelanin (Liu et al., 2003). Fig. 3 shows the transmission electron microscopy (TEM) images of synthetic melanin and SF-melanin. The TEM images confirmed the amorphous morphology and spherical crystalline structure for synthetic melanin and SF-melanin, respectively. In Fig. 3B, the granules were in spherical and/or ellipsoidal shape which was consistent with the result from Fig. 2D. The melanosomes appeared fairly uniform in shape, and were ellipsoidal with a length of ~300 nm and an aspect ratio of ~2.5.

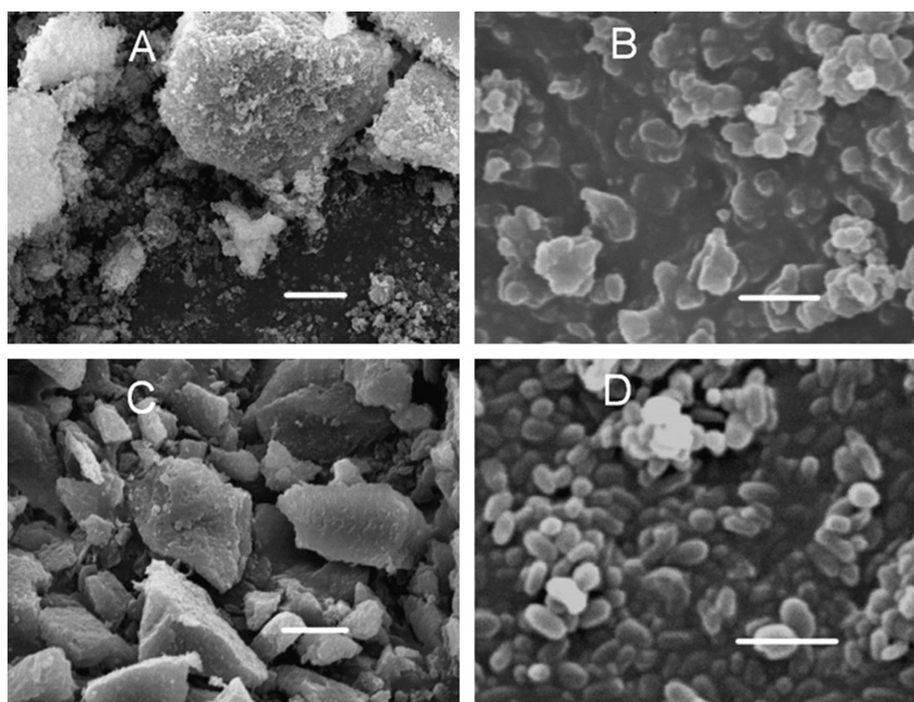

Fig. 2. SEM images of synthetic melanin and SF-melanin deposited on conductive wafer. (A) Synthetic melanin; (B) higher resolution scan of a region shown in (A); (C) SF-melanin; and (D) higher resolution scan of a region shown in (C). The scale bars are 10μm on the low magnification images (left) and 1μm on the higher magnification images (right).

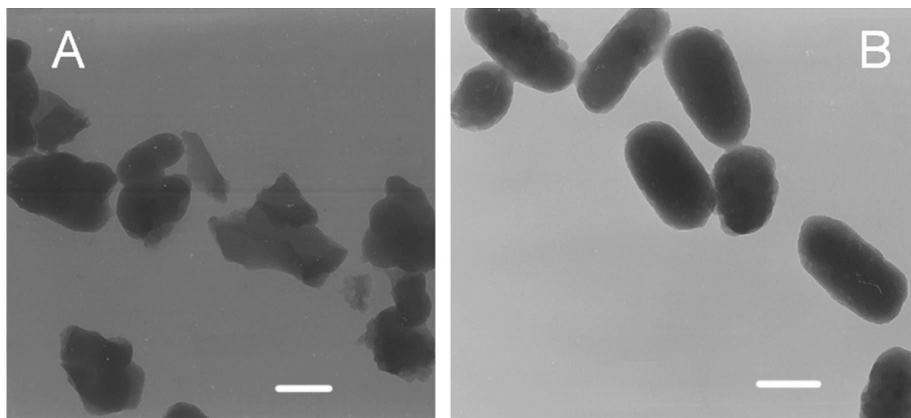

Fig. 3. TEM images of melanins: (A) synthetic melanin and (B) SF-melanin. The scale bars are 200 nm on the images.

4. Conclusions

Herein, we have performed a detailed physical, chemical and spectroscopic characterization of SF-melanin by a new enzymatic extraction. According to the EPR criteria for melanin, this product could be identified as melanin or melanin-like materials based on its characteristic EPR signals. Depending on sulfur and nitrogen content, eumelanin was the main component of SF-melanin. Melanin from silky fowl tissues displayed spherical and/or ellipsoidal melanosomes which were regarded as representative of the natural material. The results obtained provided a rapid, simple and inexpensive method for the large production of natural melanin from animal materials and might have widespread applications. The current study also revealed the potential power in black-bone silky fowl that would help protect health and reduce the risk of disease. We suggest that black-bone silky fowl could be considered as a promising animal source of natural food antioxidants.

Acknowledgements

We would like to acknowledge Yi Wang for many valuable suggestions regarding the experimental design and critical review of the manuscript. The current research was supported in part by the National Basic Research Program of China (2006CB102102) and the National High Technology Development Plan of China (2006AA10A121).

References

Aghajanyan, A. E., Hambardzumyan, A. A., Hovsepyan, A. S., Asaturian, R. A., Vardanyan, A. A., & Saghiyan, A. A. (2005). Isolation, purification and physicochemical characterization of water-soluble Bacillus thuringiensis melanin. *Pigment Cell Research, 18*, 130–135.

Arnaud, J. C., & Bore, P. (1981). Isolation of melanin pigments from human hair. *Journal of the Society of Cosmetic Chemists, 32*, 137–152.

Borshchevskaya, M. I., & Vasilieva, S. M. (1999). The development of knowledge of biochemistry and pharmacology of melanin pigments. *Voprosi medicinskoy khimii, 1*, 1–12.

Cartaxana, P., & Brotas, V. (2003). Effects of extraction on HPLC quantification of major pigments from benthic microalgae. *Archiv für Hydrobiologie, 157*, 339–349.

Cheun, W. L. (2004). The chemical structure of melanin. *Pigment Cell Research, 17*, 422–424.

Filson, A., & Hope, J. (1957). Isolation of melanin granules. *Nature, 179*, 211–212.

Golounin, A. V. (1997). The method of melanin preparation. Russian patent N2083214.

Hagerthey, S. E., Louda, J. W., & Mongkronsri, P. (2006). Evaluation of pigment extraction methods and a recommended protocol for periphyton chlorophyll a determination and chemotaxonomic assessment. *Journal of Phycology, 42*, 1125–1136.

Harki, E., Talou, T., & Dargent, R. (1997). Purification,characterisation and analysis of melanin extracted from Tuber melanosporum Vitt. *Food Chemistry, 58*(1), 69–73.

Hung, Y-C., Sava, V. M., Makan, S. Y., Chen, T-HJ., Hong, M-Y., & Huang, G. S. (2002). Antioxidant activity of melanins derived from tea: Comparison between different oxidative states. *Food Chemistry, 78*(2), 233–240.

International Chicken Polymorphism Map Consortium (2004). A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. *Nature, 432*, 717–722.

Ito, S. (1986). Reexamination of the structure of eumelanin. *Biochimica et Biophysica Acta, 883*, 155–161.

Ito, S. (1993). High-performance liquid chromatography (HPLC) analysis of eu- and pheomelanin in melanogenesis control. *Journal of Investigative Dermatology, 100*, 1665–1715.

Ito, S., & Jimbow, K. (1983). Quantitative analysis of eumelanin and phaeomelanin in hair and melanomas. *Journal of Investigative Dermatology, 80*, 268–272.

Jimbow, K. (1994). Current update and trends in melanin pigmentation and melanin biology. *The keio Journal of Medicine, 44*, 9–18.

Kazumasa Wakamarsu & Shosukeito (2002). Review: Innovative technology advanced chemical method in melanin determination. *Pigment Cell Research, 15*, 174–183.

Kerestes, J. J. R., Kerestes, J., & Venger, L. A. (2003). Biologically active fraction of vegetable melanin, process for its production and its use. USA patent 6,576,268.

Liu, Y., Kempf, V. R., Nofsinger, J. B., Weinert, E. E., Rudnicki, M., Wakamatsu, K., et al. (2003). Comparison of the structural and physical properties of human hair eumelanin following enzymatic or acid/base extraction. *Pigment Cell Research, 16*, 355–365.

Liu, Y., & Simon, J. D. (2003a). Isolation and biophysical studies of natural eumelanins: Application of imaging techniques and ultrafast spectroscopy. *Pigment Cell Research, 16*, 606–618.

Liu, Y., & Simon, J. D. (2003b). The effect of preparation procedures on the morphology of melanin from the ink sac of sepia officinalis. *Pigment Cell Research, 16*, 72–80.

Makita, T., & Mochizuki, S. (1984). Distribution of pigment cells in tissues of silky fowl I. Light microscopic observations. *Yamaguchi Journal of Veterinary Medicine, 11*, 17–38.

Mosca, L., Blarzino, C., Coccia, R., Foppoli, C., & Rosei, M. A. (1998). Melanins from tetrahydroisoquinolines: Spectroscopic characteristics, scavenging activity and redox transfer properties. *Free Radical Biology and Medicine, 24*, 161–167.

Muroya, S., Tanabe, R-I., Nakajima, I., & Chikuni, K. (2000). Molecular characteristics and site specific distribution of the pigment of the silky fowl. *The Journal of Veterinary Medical Science, 62*, 391–395.

Oloke, J. K., & Click, B. R. (1997). A new, pigment-producing, strain of *Bacillus thuringiensis. PCT WO 97/41235.*

Pawelek, J. M., & Orlow, S. J. (1992). Soluble melanin. US patent N5618519.

Prota, G. (1992). *Melanin and melanogenesis*. New York: Academic.

Sava, V. M., Yang, S-M., Hong, M-Y., Yang, P-C., & Huang, G. S. (2001). Isolation and characterization of melanic pigments derived from tea and tea polyphenols. *Food Chemistry, 73*(2), 177–184.

Tian, Y-G., Xie, M-Y., Wang, W-Y., Wu, H-J., Fu, Z-H., & Lin, L. (2007). Determination of carnosine in Black-Bone Silky Fowl (*Gallus* gallus domesticus Brisson) and common chicken by HPLC. *European Food Research and Technology, 226*, 311–314.

Vinarov, A. U., Robysheva, Z. N., Sidorenko, T. E., & Dirina, E. N. (2002). Biotechnology of Pigment Melanin. In *Proceedings of the 1st international congress "biotechnology-state of the art and prospects of development" (pp. 96).*

Wright, S. WS. W., Jeffrey, S. WS. W., & Mantoura, R. F. CR. F. C. (1997). Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. In S. W. Jeffrey, R. F. C. Mantoura, & S. W. Wright (Eds.), *Phytoplankton pigments in oceanography: Guidelines to modern methods* (pp. 261–282). Paris: The Unesco Press.