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Chemical composition and radical scavenging activity of melanin from *Auricularia auricula* fruiting bodies

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Abstract

Melanin extracted from *Auricularia auricula* fruiting bodies (AAFB) was examined by element analyzer, amino acid analyzer, inductively coupled plasma-optical emission spectrometry. Elemental composition analysis revealed that main component of AAFB melanin was pheomelanin. Amino acid analysis showed that 16 amino acids were found in AAFB melanin and total amino acid content was 321. 63 mg/g. There were 13 detectable metal elements in AAFB melanin, which was rich in Ca, Fe, Cu and Zn. In addition, AAFB melanin exhibited stronger scavenging activities on 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical, superoxide radical and hydroxyl radical with IC_{50} values of 0.18, 0.59 and 0.34 mg/mL, respectively. These results indicated that AAFB melanin might be potentially used as a natural antioxidant.

Keywords: *Auricularia auricula*; melanin; chemical composition; radical scavenging activity.

Practical Application: *A. auricula* melanin can be potentially used as a natural antioxidant.

1 Introduction

It is well known that active free radicals are by-products of normal metabolism which induce oxidative damage to biomacromolecules, including DNA, proteins, membrane lipids and carbohydrates (Wiseman & Halliwell, 1996). In order to reduce the oxidative damage of active free radicals, many synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) with a strong antioxidant capacity are widely used in food industry. However, frequent discovery of potential harmful effects of synthetic antioxidants on human health, such as liver damage and carcinogenesis (Sun & Kennedy, 2010; Yuan et al., 2008), has led to public interest in natural antioxidants as an alternative. Therefore, some natural and safe antioxidants, including ascorbate, tocopherols, flavonoids and phenolic compounds from plants and microorganisms, has been developed and used in food processing to improve body's antioxidant defenses and reduce the oxidative stress to human body.

Melanin is a dark-colored polyphenolic pigment produced from oxidative polymerization of phenolic or indolic compounds by tyrosinase. These natural pigments are synthesized by some fungi, plants, animals and several bacterial species (Dalfard et al., 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 (Wakamatsu & Ito, 2002). In recent studies (Liu et al., 2005; Tu et al., 2009), protein is considered to be an important component in melanins and can affect the function of the assembled pigment. Melanins from different sources possess a number of healthful functions, such as antioxidation (Tu et al., 2009; Wu et al., 2008), anti-HIV activity (Manning et al., 2003; Montefiori & Zhou, 1991), and immunomodulatory activity (Sava et al., 2001). These functions promise natural melanin with great development potential as a healthful food colorant.

Auricularia auricula (*A. auricula*) is a precious macro-fungus distributed in the Northeast Provinces of China and has been used as food and drug in China for a long time. Fruiting bodies of *A. auricula*, a kind of edible black-brown mushroom, are rich in nontoxic melanin and are increasingly popular as a "black food" in China (Zou et al., 2010). Melanin is considered to be one of the most important functional components in these "black food". However, most of this precious macro-fungus product is only used as cuisine materials, and many of its functional components are not fully developed and employed.

In previous research, one melanin had been successfully extracted from *A. auricula* fruiting bodies (AAFB) using ultrasound-assisted extraction technology (Zou et al., 2010). However, up to now, no further investigation has been conducted on physicochemical property and biological activity of AAFB melanin. In this study, chemical composition of AAFB melanin was analysed. Meanwhile, scavenging activity of AAFB melanin against radical was investigated for seeking new biological functional principle used in food industry.

2 Materials and methods

2.1 Materials

Eighty grams dried fruiting bodies of *A. auricula* were purchased from a local market in Dongning City (Heilongjiang Province, China), pulverized and sifted through a 40-mesh sieve.

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The powder (moisture content 12-15% on dry basis) stored in dark bags to prevent from moisture and light. Synthetic melanin was purchased from Sigma Chemicals Co. (St. Louis, USA). All the other chemicals and reagents used in the experiment were of analytical grade.

2.2 Isolation and purification of AAFB melanin

The extraction process of melanin was carried out according to the method of Zou et al. (2010), fruiting bodies powder of *A. auricula* was washed with running water at a ratio of 30 mL/g (water/raw materials) for 5 min, followed by centrifugation at 4000 rpm for 5 min. The precipitate was immersed into water at a liquid-solid ratio 43 mL/g and the initial pH was adjusted to 12.0 with 1 M NaOH. Then, the mixture was put into a conical flask and sonicated at 43 °C for 36 min by an ultrasound cleaning bath (Kunshan ultrasound instrument Co. Ltd., KQ250-DB, Kunshan, China) working at a frequency of 40 kHz and a ultrasound intensity of 0.5 W/cm^2 (the internal dimensions: 300 × 240 × 150 mm). Afterward, the sample was centrifuged at 4000 rpm for 5 min and the supernatant containing melanin was obtained.

Purification of melanin was performed as described by Wu et al. (2008) with proper modification. Melanin extract was first adjusted to pH 2.0 with 3 M HCl to precipitate melanin, followed by centrifugation at 10000 rpm for 20 min and the pellet was collected. The crude melanin was hydrolyzed with 7 M HCl at 100 °C for 2 h. The non-hydrolysable melanin collected by centrifugation (10000 rpm, 20 min) was washed with chloroform, ethyl acetate and ethanol, and then dissolved in 1 M NaOH. The pH value of water phase was adjusted to 2.0 with 3 M HCl. After centrifugation (10000 rpm, 20 min), the pellet was washed with deionized water for three times. Finally, the purified melanin was lyophilized and stored at -20 °C.

2.3 Chemical composition of AAFB Melanin

Elemental composition analysis

The amounts of C, N, H, O, and S in the AAFB melanin were determined with an element analyzer (Elementar analysen systeme, Vario EL III, Hanau, Germany) according to the national standard method of the People's Republic of China (GB/T 19145-2003).

Amino acid composition analysis

Melanin (100 μg) was heated in 0.4 mL of 6 M HCl at 120 °C for 24 h in an evacuated and sealed tube. The hydrolysate was evaporated to dryness in a vacuum and was dissolved in 800 μL of a pH 2.2 buffer for amino acid composition analysis using an amino acid analyzer (Hitachi Ltd., L-8800, Tokyo, Japan).

Metal composition analysis

Melanin (500 mg) was weighed into a beaker, digested in 7 mL of HNO_3-H_2O_2 (5:2) for 10 min, and the mixture heated to near dryness. After cooling, the residue was treated with 0.1 M HNO_3 and brought to 50 mL with bidistilled water. Metal composition was determined with an inductively coupled plasma-optical emission spectrometry (ICP-OES) (Teledyne Leeman Labs Ltd., Prodigy, Hudson, NH, USA).

2.4 Assessment of radical scavenging activity of AAFB melanin

Assay of scavenging activity on 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical

Assessment of the scavenging abilities of AAFB melanin and BHT on DPPH radical was performed by the method previously described by Tu et al. (2009) with a minor modification. Melanin or BHT solutions (2 mL) in 95% ethanol at different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) were added to 2 mL 0.2 mM solution of DPPH in 95% ethanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and then the absorbance was measured at 517 nm against a blank (95% ethanol solution). The scavenging ability of DPPH radical was calculated using the formula given below. IC_{50} (Inhibitory concentration) was the concentration of the sample required to scavenge 50% of DPPH radicals (Equation 1).

$$\text{Scavenging activity (\%)} = [1-(A_1-A_2)/A_0] \times 100 \quad (1)$$

where A_0 was the absorbance of DPPH solution without sample (95% ethanol, instead of sample solution), A_1 was the absorbance of the test sample mixed with DPPH solution and A_2 was the absorbance of the sample without DPPH solution (95% ethanol, instead of DPPH solution).

Assay of scavenging activity on superoxide radical

The superoxide radical-scavenging activities of AAFB melanin and BHT were evaluated according to the method detailed by Martinez et al. (2001) Each 3 mL of reaction mixture contained 50 mM of sodium phosphate buffer, pH 7.8, 13 mM of methionine, 2 μM of riboflavin, 100 μM of ethylene diamine tetraacetic acid (EDTA), 75 μM of nitroblue tetrazolium (NBT), and 1 mL of melanin or BHT of different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). The production of blue formazan was then followed by monitoring the increase in absorbance at 560 nm after a 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with reaction mixture were kept in the dark and served as a blank. The scavenging activity of superoxide radical was calculated using the formula given below. IC_{50} (Inhibitory concentration) was the concentration of the sample required to scavenge 50% of superoxide radicals (Equation 2).

$$\text{Scavenging activity (\%)} = (A_C-A_S)/A_C \times 100 \quad (2)$$

where A_C was the absorbance of the control (deionized water, instead of sample), and A_S was the absorbance of the test sample mixed with reaction solution.

Assay of scavenging activity on hydroxyl radical

The scavenging ability of AAFB melanin on hydroxyl radical was determined by the method previously reported by Sun & Kennedy (2010). Reaction mixtures in a final volume

of 1.0 mL contained deoxyribose (60 mM), phosphate buffer (pH 7.4, 20 mM), ferric trichloride (100 μM), EDTA (100 μM), H_2O_2 (1 mM), and different concentrations of melanin or BHT (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). The reaction solution was incubated for 1 h at 37 °C, and then 1mL of 1% thiobarbituric acid (TBA) and 1mL of 20% (v/v) HCl were added to the mixture. The mixture was boiled for 15 min and cooled on ice. The absorbance of the resulting mixture was measured at 532 nm. The scavenging activity of hydroxyl radical was calculated according to the formula given below. IC_{50} (Inhibitory concentration) was the concentration of the sample required to scavenge 50% of hydroxyl radicals (Equation 3).

$$\text{Scavenging activity (\%)} = (A_B\text{-}A_S) / A_B \times 100 \quad (3)$$

where A_B was the absorbance of the control (deionized water, instead of sample), and A_S was the absorbance of the test sample mixed with reaction solution.

2.5 Statistical analysis

The experimental results were expressed as means ± standard deviation (SD) of triplicates. Statistical analysis was performed using Fisher's *F*-test and $p<0.05$ was regarded as significant.

3 Results and discussion

3.1 Element composition of AAFB melanin

Melanins present in pigmented tissues generally appear to be mixtures or copolymers of eumelanins and pheomelanins (Chen et al., 2008; Ito & Fujita, 1985). Pheomelanin contains more sulfur than eumelanin. In this study, the amounts of C, H, N, O, and S in AAFB melanin were quantified (Table 1). The experimental results showed that the sulfur content of AAFB melanin was 5.44%. Thus, elemental composition of AAFB melanin was very similar to that of pheomelanin. Fruiting bodies of *A. auricula* grow on surfaces of withered woods in natural environments. Therefore, withered woods might be source of sulfur in AAFB melanin. The contents of sulfur and nitrogen in AAFB melanin were lower than in pheomelanin. The contents of carbon and oxygen in AAFB melanin were higher than in pheomelanin (Table 1). Thus, the structure of AAFB melanin might contain more aliphatic groups or carboxylatecontaining monomers than pheomelanin.

3.2 Amino acid composition of AAFB melanin

The amino acid analysis showed that 16 amino acids were found in AAFB melanin and total amino acid content was 321.63 mg/g, accounted for 32.16% of the mass of the melanin sample (Figure 1). Previous studies have shown that total amino acid contents of melanins extracted from sepia (Chedekel et al., 1992), black-bone silky fowl (Tu et al. 2009), black-hair, and red-hair (Liu et al., 2005) were 6-8%, 25.4%, 14.6%, and 44%, respectively. This indicated that protein was an important component in melanin. In previous reported literatures, the term "melanin" was defined loosely and it might generally contain a certain amount of protein. These proteins which spherically surround monomer units of melanins could affect the function of melanins (Liu et al., 2005; Tu et al., 2009). The extensive proteolytic digestion should be avoided because the intactness of melanin might be destroyed during extraction process using proteases. The future work is required to delineate the role of protein in the structure and property of AAFB melanin.

Table 1. Element composition of AAFB melanin.

Source	Element composition (%)				
	C	H	N	O	S
AAFB melanin	49.56	4.41	6.35	34.24	5.44
Eumelanin*	56.45	3.15	8.49	31.82	0.09
Pheomelanin*	46.24	4.46	9.36	30.16	9.78

*As reported by Ito & Fujita (1985).

3.3 Metal composition of AAFB melanin

There were 13 detectable metal elements in AAFB melanin, as found by ICP-OES (Figure 2). The experimental results showed that AAFB melanin was rich in Ca, Fe, Cu and Zn and their contents were 2.12, 1.43, 0.19, and 0.18 mg/g, respectively. The other investigator had reported that melanin extracted from the

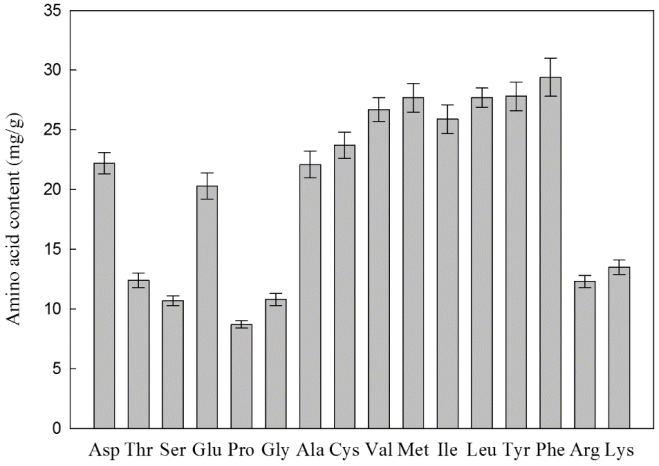

Figure 1. Amino acid composition of AAFB melanin. Values are means ± SD of three independent determinations.

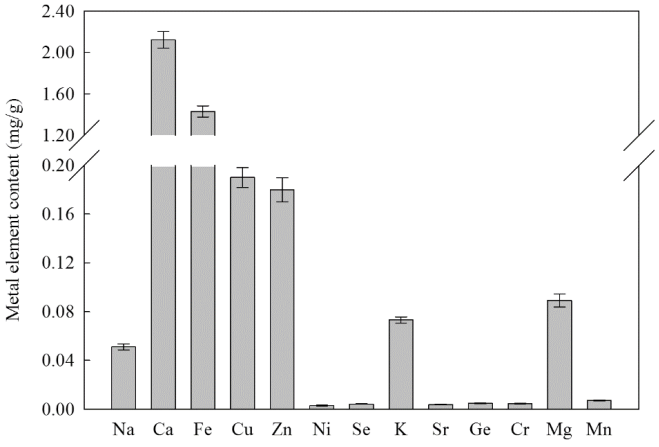

Figure 2. Metal composition of AAFB melanin. Values are means ± SD of three independent determinations.

muscles of black-bone silky fowl was rich in Ca, Fe, Mg and Zn (Tu et al. 2009), which was similar to the result obtained in this study. This indicated that melanins from different sources could serve as a reservoir of metal ions, such as Ca (II), Fe (II), Fe (III), Cu (II), Zn (II), and Mg (II). Therefore, the ability of binding various metal ions was one of the most typical characteristics of melanin.

3.4 DPPH radical scavenging activity of AAFB melanin

Antioxidant properties, especially radical scavenging activities, were very important because of the deleterious role of free radicals in foods and biological systems. Excessive formation of free radicals accelerated the oxidation of lipids in foods and induced severe damage to adjacent biomolecules (Peksel et al., 2010). DPPH assay was the simplest and most accurate method to evaluate the radical scavenging ability of antioxidants. Under ethanol solution, antioxidants could interaction with DPPH radical and transfer an electron or hydrogen atom to DPPH radical, thus neutralizing its free radical character (Herchi et al., 2014).

The scavenging activities of AAFB melanin and BHT of various concentrations on DPPH radical are given in Figure 3. The scavenging activities of AAFB melanin and BHT increased with a concentration increase. Of all the concentrations tested (0.2-1.0 mg/mL), AAFB melanin showed significantly higher (p<0.05) activities than BHT. Meanwhile, IC_{50} values of AAFB melanin was significant lower (p<0.05) than BHT (Table 2). The experimental results indicated that AAFB melanin had a stronger scavenging activity on DPPH radical than BHT at an identical concentration.

3.5 Superoxide radical scavenging activity of AAFB melanin

Although the superoxide was a relatively weak oxidant, its combination with other reactive species, such as nitric oxide and hydroxyl, might yield stronger reactive species,which possessed greater oxidative ability than the precursor to initiate lipids peroxidation (Miguel et al., 2014; Peksel et al., 2010). Furthermore, superoxide was also known to indirectly induce lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radical (Sun & Kennedy, 2010). The hydrogen-donating ability is a primary index of antioxidants that donate hydrogen to superoxide radicals, resulting in detoxification and inhibition of the propagation phase of lipid oxidation.

The results of the scavenging superoxide radical of AAFB melanin and BHT are shown in Figure 4. AAFB melanin possessed higher (p<0.05) superoxide radical scavenging activity than BHT in a concentration-dependent manner. As was shown in Table 2, IC_{50} values of AAFB melanin with superoxide radical scavenging activity were significant lower (p<0.05) than BHT. At an identical concentration, scavenge activity of AAFB melanin on superoxide radical was stronger to that of melanin from black-bone silky fowl and synthetic melanin (Tu et al., 2009).

Table 2. IC_{50} values of AAFB melanin with antioxidant activities.

Index	IC_{50} values* (mg/mL)	
	AAFB melanin	BHT
Scavenging DPPH radical	0.18±0.03^b	0.57±0.05^a
Scavenging superoxide radical	0.59±0.04^b	0.83±0.07^a
Scavenging hydroxyl radical	0.34±0.04^b	0.61±0.03^a

*The IC_{50} value was defined as the concentration of AAFB melanin or BHT to scavenge 50% of radicals under the assayed condition. Values are means ± SD of three independent determinations. Values with different letters in the same line are significantly different (p<0.05).

3.6 hydroxyl radical scavenging activity of AAFB melanin

Among all reactive oxygen radicals, hydroxyl radical was known as the most powerful radical. It could induce severe damage to adjacent biomolecules in the body, which result in cell damage that caused ageing, cancer and several other diseases (Yang et al., 2014). The removal of hydroxyl radical was probably one of the most effective ways to defense oxidative damage of human body. Therefore, hydroxyl radical scavenging activity was considered to be one of the most important antioxidant mechanisms.

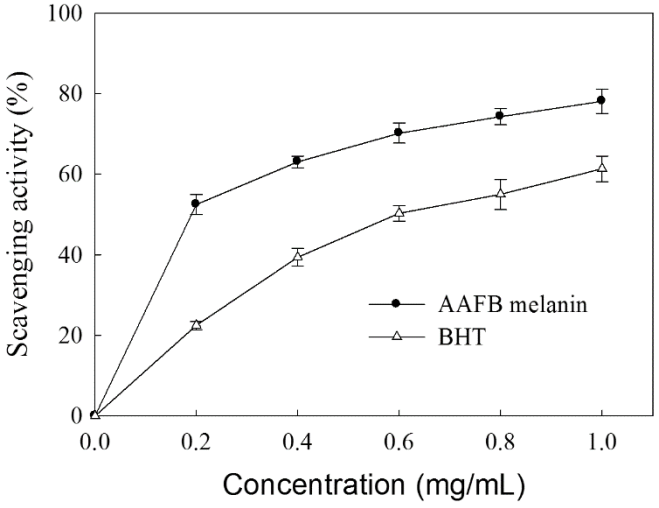

Figure 3. Scavenging effect of AAFB melanin on DPPH radical. Values are means ± SD of three independent determinations.

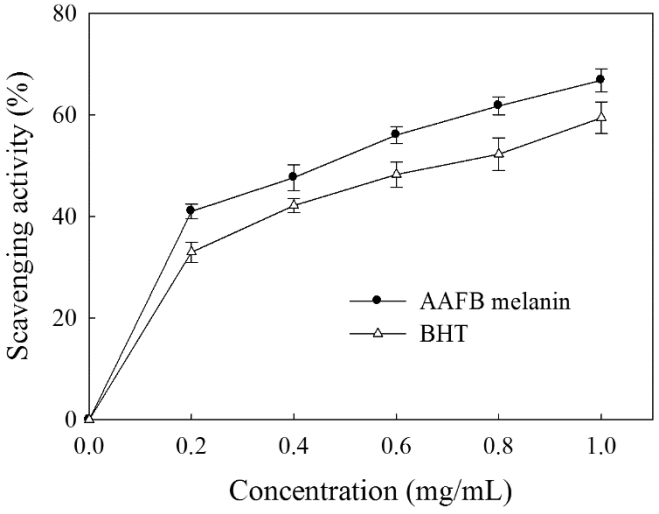

Figure 4. Scavenging effect of AAFB melanin on superoxide radical. Values are means ± SD of three independent determinations.

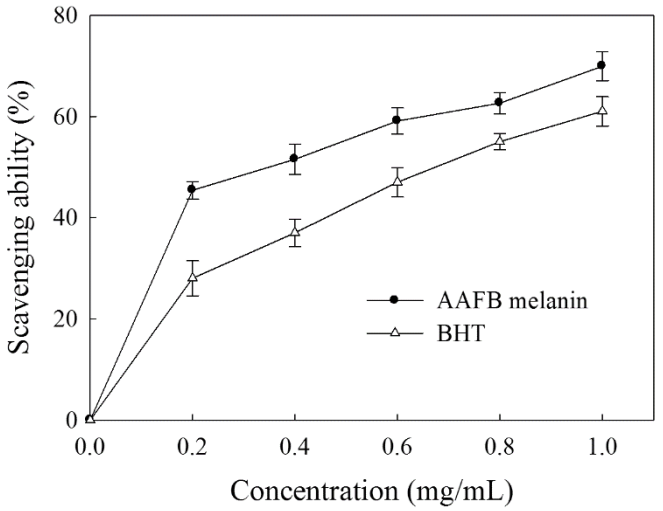

Figure 5. Scavenging effect of AAFB melanin on hydroxyl radical. Values are means ± SD of three independent determinations.

The scavenging abilities of AAFB melanin and BHT on hydroxyl radical are shown in Figure 5. With the increase of concentration, the scavenging abilities of AAFB melanin and BHT on hydroxyl radical also increased. At the concentration range of 0.2-1.0 mg/mL, AAFB melanin showed significantly stronger (p<0.05) scavenging activities than BHT. As was shown in Table 2, IC_{50} values of AAFB melanin was significant lower (p<0.05) than BHT. These results suggested that AAFB melanin was better natural antioxidant than BHT in scavenging hydroxyl radical.

In recent studies, Chiarelli-Neto et al. (2011) found that singlet oxygen induced hair damage was produced by photosensitization of melanin. However, Brenner & Hearing (2009) and Geng et al. (2008) reported that melanin was the most important photoprotective factor and had antioxidant and radical scavenging properties. Therefore, the role of melanin in photosensitization was still controversial. The future research on photo-stability of melanin would be helpful to fully understand the mechanism of photosensitization in organism and delineate the role of melanin in the photoprotection.

4 Conclusions

Chemical composition and radical scavenging activity of AAFB melanin were examined and analysed. The experimental results indicated that AAFB melanin consisted mostly of pheomelanin. AAFB melanin contained 16 amino acids and 13 metal elements. It was rich in Ca, Fe, Cu and Zn. The antioxidant activities of AAFB melanin were evaluated by DPPH radical, superoxide radical, and hydroxyl radical scavenging assay. AAFB melanin exhibited stronger antioxidant activity compared to BHT. Results from this study indicated that AAFB melanin could be potentially used as a natural antioxidant.

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