The Study of Antioxidant Effects in Melanins Extracted from Various Tissues of Animals

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ABSTRACT : This study was carried out to investigate the antioxidant effects of melanin extracts, including silkie fowl skin melanin (SS-melanin), silkie fowl comb melanin (SC-melanin), sepia ink sac melanin (SE-melanin), octopus ink sac melanin (OC-melanin) and synthetic melanin (SY-melanin). The results showed that with the addition of melanin extracts, linoleic acid peroxide significantly, decreased ($p<0.05$) with the increase in the irradiative time of UV and that OC-melanin had the highest efficiency on antioxidant activity ($p<0.05$). Melanin extract had reducing power and chelating power to Fe^{2+}, which increased with the increase in the different melanin concentration. Therefore, it could be concluded that the antioxidant action of melanin extracts did not come from one single function, but is a result of many characteristic functions. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 2 : 277-281)***

Key Words : Antioxidant, Melanin, Silkie Fowl, Sepia Ink Sac, Octopus Ink Sac

INTRODUCTION

Reactive oxygen tissues (ROS) have become a hot wave in biological and medical research. ROS is produced by normal metabolism or through exposure to other pollutant materials and ionizing radiation. It includes hydroxide free radical (•OH), superoxide free radical (O_2•) and hydrogen peroxide (H_2O_2) etc. These ROS would initiate lipid oxidation and speed up the yield of lipid peroxide (Terao, 1988). Halliwell and Gutteridge (1989) and Ames et al. (1993) also suggested that ROS played an important role in clinical disease, human aging, and food spoiled with oxygen.

The oxidative stress caused by free radicals and ROS would damage lipid and protein lower food quality. Therefore, it is important to decrease oxidative stress by the supplementation with antioxidants. However, it was found that there were some problems with safety of chemical antioxidants, such as BHT and BHA that limited their use (Imaida et al., 1983). The search for the natural antioxidants that could prevent the damage caused by free radical and ROS in natural systems has become a very important research area in biological and medical research.

Silkie fowl is an excellent breed for both medical properties and pet keeping. It is the raw material for Chinese medical food and liquor, so it has a potential for commercial production. Besides, it has a special position in Chinese traditional food, especially in medicinal food preparation. According to Lee (1578) famous Chinese doctor, silkie fowl had many kinds of medicinal effects such as preventing of dizziness, heartache, and inappetence etc. In Japan, there are many silkie fowl food products. These products include silkie fowl medicinal food, silkie fowl powder and blood powder, silkie fowl egg yolk oil, silkie fowl vinegar pickled egg and so on.

In our past research (Lin and Wu, 2000a,b), it was found that silkie fowl meat a had higher content of protein and lower fat compared with that of broiler meat. At the same time, it was found that the collagen content in silkie fowl meat was seven times that of broiler meat. Besides, silkie fowl had higher IMP content and special flavor. In SDS-PAGE analysis, silkie fowl and broiler muscle proteins were similar, but thigh muscle of silkie fowl had an additional band in 45 KD position. And it was found in the measurement of contents of melanin that the band in 45 KD position was the main content of melanin (Wu, 1998). Kan and Kan (1992) found that silkie fowl meat was rich in iron, Ca^{2+} and Zn^{2+}. Blarzino et al. (1999) and Jacobson and Tinnell (1993) have suggested that melanin had an antioxidant effect. The purpose of this study is to investigate the effects of melanin from comb and skin parts of silkie fowl and compare it with ink sac from sepia and octopus, then to measure their antioxidant characteristics and compare them with chemical melanin, and to find out whether melanin extracts had antioxidant effects on oils.

MATERIALS AND METHODS

Sample preparation

Silkie fowl (average weight 1.6-1.8 kg, males) were reared to 14-15 weeks of age with commercial, corn-soybean diets that were obtained from a commercial source. Sepia and octopus were obtained from commercial fish market. Synthetic melanin came from Sigma Co.

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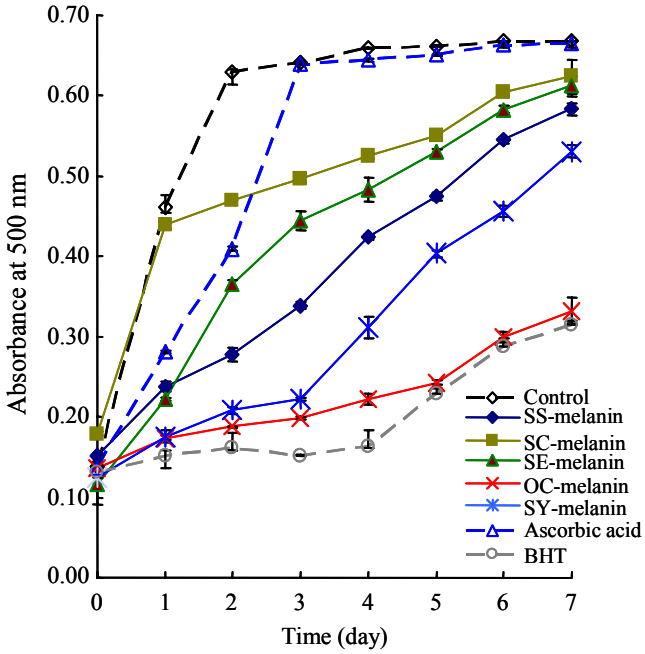


Figure 1. Antioxidant activity of melanin extracted from various tissues of animals as measured by the thiocyanate method.

Extraction of melanin from silkie fowl, sepia and octopus

Melanin was extracted from silkie and comb of silkie fowl and from ink sac of sepia and octopus as described by Harki et al. (1997). 20 g of samples were homogenised in 200 ml of 1 M KOH using a Waring Blender (8,000 rpm for 10 min) and then sonicated with an Ultrasonic disintegrator (12 K C/S for min, Model VCX750, Sonics, USA). The melanin was extracted from the cell debris by treatment with hot alkali (1 M KOH at 100°C for 5 h) under reflux in an atmosphere of nitrogen. After filtration, the dark brown filtrate was acidified to pH 2. The precipitate was collected by centrifugation (10,000 rpm for 10 min) and washed with distilled water.

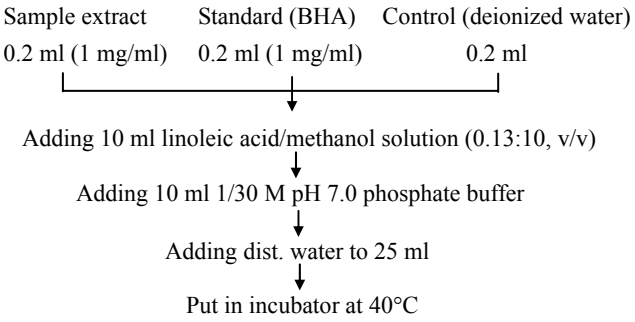
The crude melanin was hydrolysed with 40 ml of 7 M HCl for 2 h at 100°C. The non-hydrolysable residue was collected by centrifugation (1,000 rpm for 10 min) and washed in 0.01 M HCl solution and then in distilled water.

The non-hydrolysable melanin was re-dissolved in 20 ml of 1 M KOH and 8 ml of chloroform were added to it, followed by 0.8 ml of 1-butanol. The mixture was shaken for 30 min under nitrogen and centrifuged (6,000 rpm for 10 min).

The chloroform phase was discarded and the operations were repeated twice on the alkaline melanin solution. Finally, the melanin was precipitated by acidification and washed with distilled water. The precipitate was dried in a desiccator and kept under nitrogen as "pure" melanin.

Determination of antioxidant activity

Ferric thiocyanate method : Ferric thiocyanate method was adopted to evaluate the antioxidant activity as described by Mitsuda et al. (1966). In 2.5 ml of 0.02 M linoleic acid emulsion, 1 mg melanin and 2 ml of 0.2 M phosphate sodium buffer (pH 7.0) were added. After mixing, it was put in UV box about 30 cm away from the ultraviolet light (GL-15, Sankyo Denki, Japan). At every stable storage time, it was taken out to measure linoleic acid peroxide.



Measuring oxidant level each 24 h by
(1) taking 2 ml liquid mentioned above
(2) adding 9.4 ml of 75%methanol (Merck)
(3) adding 0.2 ml of 30% NH_4(SCN) liquid
(4) adding 0.2 ml of 0.02 M $FeCl_2$ solution
(5) allowing it react for 3 minutes and then measuring its absorbance at 500 nm (Spectrophotometer U-2000, Hitachi, Japan).

Reducing power : Determination of reducing power was carried out as described by Oyaizu (1986). The method of measuring reducing power was by making formation of Prussian blue $\{Fe_4[Fe(CN)_6]_3\}$ as an index. The complex compound had biggest absorbance at 700 nm determining with spectrophotometer (U-2000 Hitachi). If the absorbance was higher, it meant that the reduction was stronger.

Determination of chelating effect on Fe^{2+} : Determination of chelating effect on Fe^{2+} was carried out as described by Dinis et al. (1994). Its principle was to use color reaction of the complex of Fe^{2+} and ferrozine at 562 nm. Chelating effect of samples on Fe^{2+} can be measured. When samples chelated Fe^{2+}, it would make the absorbance at 562 nm lower down. Therefore, when the absorbance was lower, the chelating effect was higher.

Statistical analysis

Data were statistically analyzed by using the General Linear Model Procedure of SAS. Institute (Statistical Analysis System, Ver. 8.01 for Windows, 2000). The significance of treatment effects was solved using the Duncan's new multiple range tests.

RESULTS AND DISCUSSION

Antioxidant activity of melanin extracted from silkie

Table 1. Comparison of antioxidant effect of melanin extracted from silkie fowl, sepia octopus and synthetic-melanin, ascorbic acid and BHT on peroxidation of linoleic acid as measured by the thiocyanate method after incubation for 2 days and 7 days

Sample***	Inhibition % * (two days)	Inhibition % (seven days)
Control	0 **	0
SS-melanin	55.86±1.41 ^{d}	12.71±1.14 ^{c}
SC-melanin	25.37±0.14 ^{a}	6.67±1.37 ^{b}
SE-melanin	41.99±0.37 ^{c}	8.47±0.12 ^{b}
OC-melanin	70.15±0.42 ^{f}	50.32±0.15 ^{e}
SY-melanin	66.92±0.71 ^{e}	20.59±1.12 ^{d}
Ascorbic acid	34.95±0.40 ^{b}	0.64±0.57 ^{a}
BHT	74.43±2.94 ^{g}	52.86±0.79 ^{e}

* Inhibition of peroxidation (%)=Capacity to inhibition the peroxide formation in linoleic acid=[1-(absorbance of sample at 500 nm)/(absorbance of control at 500 nm)]×100. A high inhibition % indicated a high antioxidant activity.

** Each value is the mean±standard deviation of three replicate analyses. Values in a column with the different superscripts letters are significantly different ($p<0.05$).

*** The concentration of samples was 1 mg/ml.

fowl, sepia and octopus as measured by thiocyanate method

Ferric thiocyanate method was adopted to evaluate the antioxidant activity. Its principle was to make use of the high energy of hydroperoxide in initial period formed by lipid oxidation that oxidized Fe^{2+} to Fe^{3+}. This Fe^{3+} reacted with SCN^{-} to become red Fe $(SCN)^{2+}$. The complex compound had the biggest absorbance at 500 nm wavelength. When the level of lipid oxidation was higher, the amount of hydroperoxide was more and the color was darkness. Therefore, by measuring the absorbance at 500 mm wavelength, we could know the antioxidation of the material (Mitsuda et al., 1966).

Blarzino et al. (1999) and Jacobson and Tinnell (1993) have suggested that melanin had antioxidant effect. To find out whether melanin extracts had antioxidant effect on oil, this study attempted to investigate the inhibiting effect of different kinds of melanin extracts on lipid oxidation by taking linoleic acid measured by ferric thiocyanate method.

In Figure 1, the antioxidant effect of different kinds of melanin extracts on linoleic acid for 7 days at 37°C was shown. The results showed that peroxide of linoleic acid increased with the increasing storage time, but the addition of melanin extracts and BHT would decrease the yield of peroxide of linoleic acid ($p<0.05$). During storage time, control treatment had the highest yield of lipid peroxide on the 2nd day. However, BHT was a long-effect antioxidation, so it could maintain antioxidant effect for 7 days. Ascorbic acid reached the highest yield of peroxide of linoleic acid in 3 days. But adding melanin extracts to inhibit the yield of peroxide had significant effects ($p<0.05$).

Table 1 showed the percentage of yield of lipid peroxide on the 2nd day and the 7th day inhibited by different kinds of melanin extracts. In adding melanin treatments, OC-melanin had the best antioxidant effects. During storage time, it could significantly decrease the yield of peroxide, and even to the 7th day, it could inhibit 50.32% of the yield of lipid peroxide that did not have significant differences compared to 52.86% by BHT. Then the strength in order were SY-melanin (20.59%)>SE-melanin (8.47%)>SC-melanin (6.67%). Although these 4 kinds of melanin extracts did not have long- antioxidant effect like BHT, they had significantly higher inhibiting effect on oxidation of linoleic acid than ascorbic acid did ($p<0.05$). In addition, in the report of Rózanowska et al. (1999), it was suggested that melanin structure had the characteristics of oxidation-reduction, so it could scavenge free radicals.

From the above, it is clear that melanin could decrease the formation of lipid peroxide, so it had antioxidative effects on lipid.

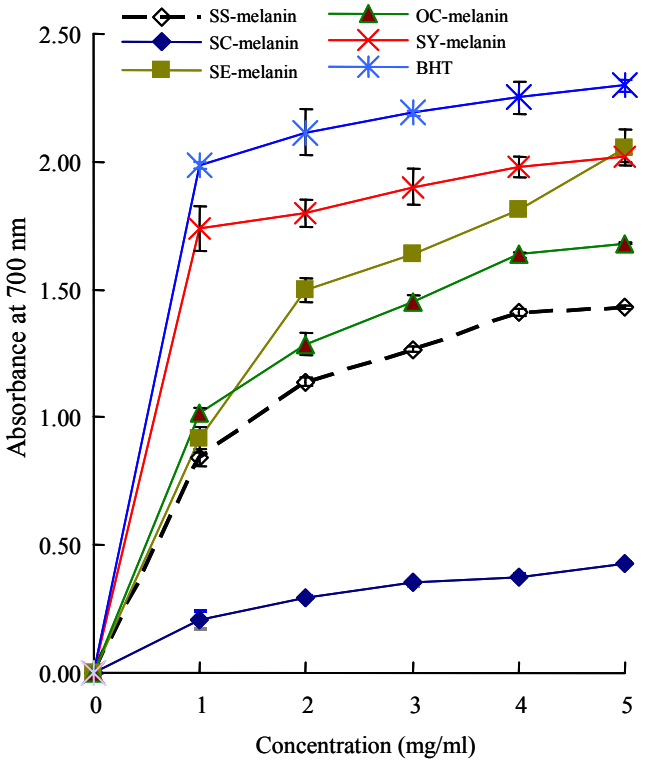


Figure 2. Reducing power of melanin extracted from various tissues of animals with different melanin concentration.

Reduction effect of melanin extracted from silkie fowl, sepia and octopus

Figure 2 showed the reducing power of melanin extracts. The results showed that the reducing power would rise with the increase in the concentration of melanin extracts. When 5 mg/ml of melanin extracts were added, the strength of the reducing power was in order as SE-melanin SY-melanin >OC-melanin>SS-melanin>SC-melanin. The absorbance

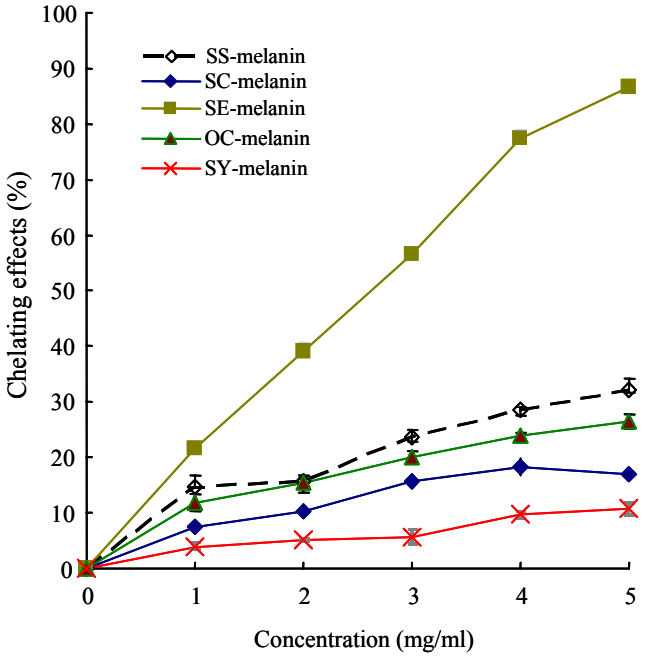


Figure 3. Chelating effects of melanin extracted from various tissues of animals on Fe^{2+} ion.

values were 2.05, 2.02, 1.68, 1.43 and 0.43. Among them, SE-melanin and SY-melanin had the strongest reducing power, and SC-melanin had the weakest. Being an electron donor, melanin could react with free radical to form a more stable product, thus, ending the chain reaction of the free radical. Therefore, melanin extracts had reducing power that would be influenced by the quantity of unpaired electrons in the structure. Bochenek and Gudowska-Nowak (2003) have suggested melanins are composed of indolic unit derived from the oxidation of tyrosine: among those 5,6-dihydroxyindole (DHI or HQ) together with their oxidized form and semiquinone (SQ) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) are usually accepted as main components of natural melanins and their synthetic analogues (Prota, 1992). Ability to bind transition metal ions, redox properties and presence of persistent free radical centers in the melanins structure are usually mentioned as the main features of melanins (Sarna and Swartz, 1998).

OC-melanin and SS-melanin had strong antioxidant effects, but did not reach the same results in reducing power. This could be because, in this system, OC-melanin and SS-melanin could not afford enough electrons to stop the yield of Prussian blue. Therefore, the antioxidant mechanism of OC-melanin and SS-melanin could not completely come from reducing power, but from the total expressions of other functions, such as affording hydrogen groups and chelating power on metal.

Table 2. Comparison of chelating effects of control, EDTA, synthetic-melanin, and melanin extracts from silkie fowl, sepia and octopus on Fe^{2+} ion

Sample	Chelating effects (%)*	
	1.0 mg	5.0 mg
Control	0 **	0
SS-melanin	14.66±1.98 ^{c}	32.24±1.94 ^{c}
SC-melanin	7.46±1.19 ^{d}	16.91±0.66 ^{e}
SE-melanin	21.55±2.29 ^{b}	86.70±9.89 ^{b}
OC-melanin	11.89±1.49 ^{c}	26.94±1.39 ^{d}
SY-melanin	3.94±0.72 ^{e}	11.93±1.30 ^{f}
EDTA	49.32±2.35^{a}	99.11±1.78 ^{a}

* Chelating effects %=Capacity to Chelating the Fe^{2+} ion=[1- (absorbance of sample at 562 nm)/(absorbance of control at 562 nm)]×100.

** Each value is the mean±standard deviation of three replicate analyses. Values in a column with the different superscripts letters are significantly different ($p<0.05$).

Chelating power of melanin extracted from silkie fowl, sepia and octopus on Fe^{2+}

Active oxidant function of metal ions often was the main factor to make lipid peroxide. By reaction of redox cycle, only a few metal ions could efficiently produce free radical and speed up the progress of lipid oxidation (Brown, 1988; Gordon, 1990; Decker et al., 1992). In many metal ions, Fe^{2+} always was the most effective oxidation accelerator that would improve the progress of lipid oxidation (Yamaguchi et al., 1988). Using the most absorbance of complex compound of Fe^{2+} and ferrozine at 562 nm, the samples could be measured chelating power to Fe^{2+}. When the samples chelated Fe^{2+}, they could lower the absorbance at 562 nm.

$$Fe^{2+}+\text{Ferrozine} \rightarrow \text{Ferrozine-}Fe^{2+}\text{ complex (violet)}$$

Figure 3 showed the chelating power on Fe^{2+} of melanin extracts of various tissues of animals.

The results showed that the chelating effect would increase with the increasing of melanin concentration and that SE-melanin had the highest yield of chelating effect. In Table 2, it was shown that the chelating comparison of control, EDTA and melanin in 1.0 mg and 5.0 mg extracted from different kinds of tissues. It was known that when the concentration of melanin was low (1mg), the strength of chelating power was in order as SE-melanin (21.55%)>SS-melanin (14.66%)>OC-melanin (11.89%)>SC-melanin (7.46%)>SY-melanin (3.94%). When the concentration was up to 5 mg, except chelating power of SE-melanin, which reached 86%, the other melanin extracts were in order as SS-melanin (32.24%) OC-melanin (26.94%)>SC-melanin (16.91%) SY-melanin (11.93%). EDTA was a very strong chelating agent. When the concentration of EDTA was 1mg, the strength of chelating power was 49.32%. When the concentration was up to 5 mg, the strength of chelating power was up to 99.11%. This result was the same as the

melanin. However, the control lot had no chelating effect. The chelating power of EDTA and melanin extracts would rise with the increase in the concentration. The results showed that melanin extracts had chelating power on Fe^{2+}. Jara et al. (1990) pointed out that function group of melanin could have cross linking with ion and chelating power. Riley (1997) stated that in melanin structure there was 2-carboxyl indole that could link with metal ion that made melanin to have chelating power on Fe^{2+}, the same result as in this study.

From the above results, it was proved that melanin extracts had reducing power and chelating power on Fe^{2+} that would rise with the increasing concentration of samples. Therefore, it was inferred that the antioxidant effects of melanin extracts did not come from one single mechanism, but was a result of many characteristic functions.

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