Preparation and Properties of the Melanin from *Lachnum singerianum*

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***Abstract*—Melanin was isolated from the mycelium of *Lachnum singerianum* by alkaline extraction, acid hydrolysis, and repeated precipitation with a yield of 8.85 g/100 g (dry weight basis). This melanin was soluble in alkaline solutions of NaOH and ammonia, but insoluble in water, HCl and common organic solvents (ethyl acetate, chloroform, ethanol, acetone and ethyl ether, etc.). And it was stable to light (UV light and natural light), reducing agent (sodium sulfite), additives (sodium benzoate, sucrose and sodium citrate) and some metal ions (Na^+, K^+, Mg^{2+}, Mn^{2+}, Ca^{2+} and Al^{3+}), but unstable to hydrogen peroxide, citric acid, Vc, Cu^{2+} and Fe^{3+}, which showed that it has a physico-chemical characteristics of typical melanin. When the concentration of this melanin was 200 mg·L^{-1}, the scavenging rate of ·OH, DPPH· and O_2^{-}· radical were 52.64%, 41.2% and 43.48%, respectively. The IC_{50} values were 180.87 mg·L^{-1}, 240 mg·L^{-1} and 275.27 mg·L^{-1}, respectively. The results showed this melanin has a strong antioxidant activity, which suggested that this melanin can be used as a new antioxidant component in medicine or health products.**

***Keywords*—*Lachnum singerianum*, Melanin, Preparation, Stability, Antioxidant activity.**

I. INTRODUCTION

Melanin is a polyphenolic polymer with an irregular structure that is produced by the oxidation of polyhydric phenols [1]. Generally, natural melanin is only soluble in alkaline solutions, but insoluble in water, acid solutions and common organic solvents. In addition, it can be oxidized by oxidants ($KMnO_4$, NaOCl and H_2O_2), and is resistant to chelate metal ions and has strong ability to absorb UV-VIS light [2]. Accordingly, melanin (water solubility) can not only be used as natural edible melanin additive, but also be widely applied in cosmetics, protection against UV radiation and protection of biopesticides against light, etc [3].

A lot of microorganisms have been reported to produce melanin, which includes intracellular melanin and extracellular melanin [4], [5], [6]. *Lachnum* is a category of saprophytic fungi that is distributed throughout the world [7], [8]. More than 250 species of *Lachnum* have been reported in the world, and some species of them are able to produce antibiotic activity [9], [10]. A few years ago, we discovered that *Lachnum brasiliense* could produce a great amount of melanin under submerged culture conditions, which was stable to temperature, light, food additives, reducing agents, etc [11].

The purpose of this study is to obtain the intracellular melanin from *Lachnum singerianum* YM-292 and reveal its stability and antioxidant activity.

II. Materials and Methods

A. Sporocarps and Mycelium

Sporocarps of *Lachnum singerianum* YM-292 were collected from Huangshan Mountain in Anhui of China, and the strain was isolated and preserved in the Laboratory of Microbial Resources and Application of Hefei University of Technology.

B. Extraction and Purification of the Melanin

The method of Ye et al [12] was used for fermentation of melanin from strain YM-292, and the method of Wang et al [13] with minor improvements was used for extraction of the intracellular melanin. After completion of fermentation, the fermentation broth was drawn and filtered 2-3 times. The obtained mycelium was washed several times with distilled water, and then vacuum dried at 40 ℃ to constant weight. The mycelium was mixed with 0.5 mol·L^{-1} $NH_3 \cdot H_2O$ in the proportion of 1:40 (w/v), extracted in water bath at 80 ℃ for 2 h, and filtered to obtain the intracellular melanin extract. The extract was then adjusted to pH 2-3 with 1 mol·L^{-1} hydrochloric acid solution, and kept still at room temperature for 4 h, followed by centrifugation at 6000 r·min^{-1} for 10 min to obtain the crude product of melanin.

The crude product of melanin was hydrolyzed by 6 mol·L^{-1} HCl solution at 100 ℃ for 4 h, and then centrifuged at 6000 r·min^{-1} for 10 min. The sediment was rinsed repeatedly with the deionized water until the pH value of the supernatant was 7, re-dissolved in 1 mol·L^{-1} $NH_3 \cdot H_2O$, evaporated with the rotary evaporator (Shanghai Qingpu Huxi Instrument Factory, Shanghai, China) to remove NH_3 until the pH value was around 7.5, and then extracted in turn with chloroform, ethyl acetate and absolute ethanol. The extract liquid was adjusted to pH 2-3 with 1 mol·L^{-1} HCl solution, and 4 h later, centrifuged at 6000 r·min^{-1} for 10 min. The supernatant was discarded. The sediment was rinsed repeatedly with the deionized water until the supernatant gave a negative Cl^- test. The purified melanin was obtained after vacuum freeze-drying.

C. Identification of the Melanin

The purified melanin of 0.02 g was dissolved in 0.5 mol·L^{-1} $NH_3 \cdot H_2O$ solution, heated in water bath at 50 ℃ for 1 h, evaporated in the rotary evaporator to remove NH_3 until the pH value was around 7.5, and then diluted to 100 mL. The spectra were recorded from 190 to 800 nm using a 1 cm quartz cell.

D. Stability of the Melanin

Purified melanin of 0.01 g was dissolved in 10 mL NaOH solution (pH9), ammonia water, DMSO, HCl (1 mol·L^{-1}), distilled water, absolute ethanol, chloroform, ethyl acetate, acetone, ethyl ether and xylene respectively and shaken. After 1h, the absorbance value (A) at λ_{max} was measured.

The method of Pan et al [14] with minor modifications was used. The stability of YM-292 intracellular melanin under different temperature, pH and light conditions and with the addition of different metal ions, oxidants, reducing agents and food additives were investigated. Each experiment was repeated three times. The results were expressed by Mean±SD. SPSS11.5 software was used for analysis of variance. And $P < 0.05$ indicated a significant difference.

E. Antioxidant Activity of the Melanin

·OH scavenging assay

·OH scavenging activity was determined using a modified method of Wang et al [15]. Orthophenanthroline solution (5×10^{-3} mol·L^{-1}) of 1.5 mL was taken and added with 2.0 mL of phosphate buffer (pH 7.4, 0.05 mol·L^{-1}) and evenly mixed, then added with 1.0 mL of

7.5×10^{-3} mol·L^{-1} $FeSO_4$ solution, evenly mixed immediately after the addition of each tube, and then added with 1 mL of H_2O_2 (0.1%), and finally supplemented with distilled water to a total volume of 10 mL. This reaction solution was kept at 37 ℃ for l h, and $A_{damaged}$ was measured at 536 nm. The ·OH scavenging abilities of the melanin and Vc solutions were determined with the same method as described above. Melanin solutions or Vc solutions of different concentrations were added, and then added with H_2O_2. After being kept warm at 37 ℃ for 1 h, $A_{sampled}$ was measured. And the undamaged tubes were not added with H_2O_2, melanin solution or Vc solution.

·OH scavenging rate(%)=[($A_{sampled}$ - $A_{damaged}$) / ($A_{undamaged}$ - $A_{damaged}$)]×100.

DPPH· scavenging assay

DPPH· scavenging activity was determined using a modified method of Turkoglu et al [16]. Prepared the absolute ethanolic DPPH· solution (0.1 mmol·L^{-1}) and different concentrations of absolute ethanolic melanin solution. DPPH· solution of 1 mL was taken and added with 3 mL of different concentrations of melanin solution, evenly mixed. After 30 min incubation period in the dark, the absorbance of mixture was measured at 517 nm. Absolute ethanol solution replaced the sample solution as blank controller, and BHT serve as positive controller. DPPH· scavenging activity in percent (I%) was calculated in following way:

I% =(A_{blank} –A_{sample}/A_{blank})×100%;

O_2^{-}· scavenging assay

Pyrogallol oxidation method was adopted [17]. Pyrogallol can quickly oxidated by itself in alkaline conditions, generating series product that is absorbed at near 318 nm as well as releasing O_2^{-}·. Method: Tirs-HCl (pH=8.2, 50 mmol·L^{-1}) of 3mL was taken and added with melanin solutions of different concentrations and evenly mixed, this solution was kept at 25 ℃ for 20 min then pyrogallol solution (7 mmol·L^{-1}) of 0.3mL preheated at 25 ℃ was added to react exactly 4 min and HCl (10 mmol·L^{-1}) of 1 ml was added to terminate reaction. A_1 was measured at 318 nm.

O_2^{-}·scavenging rate(%) = [1-(A_1-A_1')/ A_0]×100%

Where A_1' is the absorbance of water replacing reaction reagent; A_0 is the absorbance of water replacing sample solution.

III. Results and Discussion

A. Preparation of LSM

Lachnum singerianum YM-292 intracellular melanin, LSM, was extracted from the mycelium of *Lachnum singerianum* YM-292, and the purified melanin of 8.85 g/100 g (dry weight basis) was obtained by alkaline extraction, acid hydrolysis, repeated precipitation, and then being freeze-dried.

B. Identification of LSM

Fig.1 showed LSM had a maximum absorption peak at 210 nm, and exhibited an exponential decrease in the region of 210-600 nm, showing the melanin was similar to the melanin synthesized with enzymatic reaction [18].

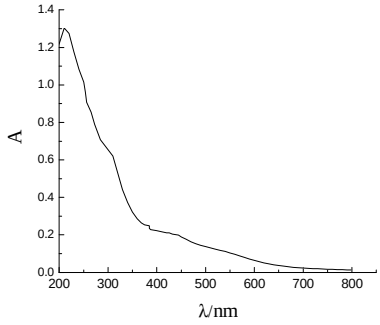

Fig.1. UV-Vis absorption spectrum of LSM

C. Stability of LSM

LSM was soluble in NaOH solution and ammonia water, the solution being brownish black, but insoluble in water, HCl, ethyl acetate, chloroform, ethanol, acetone, ethyl ether and xylene. Its solubility was very similar to that of the typical melanin [19], [20].

After treatment of UV light, natural light and dark condition, the absorbances of LSM solutions all declined to a certain extent, which declined by 0.008 after UV irradiation for 15 days, and declined by 0.005 and 0.004, respectively, under natural light and dark conditions, with no obvious change in the color of the melanin solutions. Therefore, LSM is very stable to light (Table I), and its light stability was equivalent to that of pigment extracted from O. fragrans' seeds previously reported [14].

TABLE I

EFFECT OF LIGHT TO THE STABILITY OF LSM

Absorbance	Light source		
	UV-light	Nature light	Dark
0d	0.990±0.001	0.990±0.001	0.990±0.001
3d	0.990±0.002	0.988±0.001	0.990±0.001
6d	0.988±0.002	0.987±0.004	0.989±0.002
9d	0.985±0.004	0.987±0.003	0.988±0.003
15d	0.982±0.003	0.985±0.001	0.986±0.002

Values are mean±s.d. (n =3).

Natural pigments are usually easy to be oxidized by strong oxidants such as $KMnO_4$, $K_2Cr_2O_7$ and NaOCl and reduced by reducing agents such as Na_2SO_3, thus changing their chemical structures. With the increase of the H_2O_2 concentration and prolongation of time, the color of LSM solution gradually faded, and the absorbance declined. When the H_2O_2 concentration reached 2%, the absorbance value declined by 0.106 after 1.0 h (with a residual rate of 84.48%) (Table II), indicating that LSM was easy to be oxidized and faded by H_2O_2.

TABLE II

EFFECTS OF H_2O_2 ON STABILITY OF LSM

Time	Absorbance			
	0	0.5%	1.0%	2%
0h	0.746±0.002	0.734±0.002	0.719±0.001	0.683±0.001
0.5h	0.745±0.002	0.719±0.002	0.699±0.002	0.632±0.002
1.0h	0.745±0.002	0.702±0.001	0.668±0.001	0.577±0.001

Values are mean±s.d. (n =3).

TABLE III

EFFECTS OF Na_2SO_3 ON STABILITY OF LSM

Absorbance	Time		
	0h	24h	48h
0mg/LNa_2SO_3	0.626±0.002	0.625±0.002	0.623±0.001
50mg/LNa_2SO_3	0.617±0.001	0.612±0.001	0.610±0.003
100mg/LNa_2SO_3	0.613±0.002	0.610±0.003	0.608±0.001
200mg/LNa_2SO_3	0.611±0.001	0.607±0.001	0.605±0.001
400mg/LNa_2SO_3	0.608±0.002	0.604±0.001	0.601±0.001

Values are mean±s.d. (n =3).

As was shown in Table III, with the increase of the concentration of the Na_2SO_3 solution and the prolongation of time, the absorbance value of LSM solution remained almost unchanged. And when the Na_2SO_3 concentration reached to 400 $mg \cdot L^{-1}$, the absorbance value merely declined by 0.007 after 48 h. The result indicated that LSM had a strong resistance to reduction.

Above effects of light, H_2O_2 and Na_2SO_3 on stability of LSM were basically consistent with that of the melanin reported by Tu et al [2].

Table IV indicated that the absorbance of the melanin solution was increased when

mixed with Mn^{2+}, Ca^{2+} and Al^{3+}, while slightly decreased in Na^{+}、K^{+}、Mg^{2+}. But a large amount of precipitate was produced in LSM solution with the addition of Fe^{3+} and Cu^{2+}, which showed that this melanin was extremely unstable to Fe^{3+} and Cu^{2+} and should be kept away from direct contact with Fe^{3+} and Cu^{2+} when being used.

As shown in Table, with the prolongation of time, the absorbance value of LSM solution gradually increased after being added with sucrose and sodium benzoate, indicating that sucrose and sodium benzoate could increase the color (or protect the color) of LSM. But after LSM solution was added with citric acid and Vc, the absorbance value declined by 0.297 and 0.168 after 48 h (the residual rates being 50.3% and 72%, respectively), respectively, which showed that LSM was extremely unstable to citric acid and Vc. The results were similar to that of melanin from *Plectania* sp [21].

TABLE IV

EFFECTS OF METAL-ION ON STABILITY OF LSM.

Metal -ion	Absorbance				
	0h	0.5h	1h	1.5h	2h
control	0.567± 0.002	0.563± 0.003	0.559± 0.002	0.553± 0.002	0.547± 0.003
Na^{+}	0.562± 0.001	0.558± 0.002	0.550± 0.001	0.549± 0.001	0.543± 0.002
K^{+}	0.561± 0.001	0.555± 0.001	0.549± 0.001	0.543± 0.001	0.540± 0.001
Mg^{2+}	0.562± 0.002	0.559± 0.002	0.557± 0.001	0.554± 0.003	0.552± 0.001
Fe^{3+}	—	—	—	—	—
Mn^{2+}	0.588± 0.002	0.604± 0.001	0.600± 0.002	0.600± 0.002	0.604± 0.002
Ca^{2+}	0.601± 0.001	0.605± 0.002	0.600± 0.002	0.599± 0.001	0.592± 0.001
Cu^{2+}	—	—	—	—	—
Al^{3+}	0.664± 0.001	0.658± 0.002	0.653± 0.001	0.646± 0.003	0.625± 0.002

Values are mean±s.d. (n =3). "—", deposit.

TABLE Ⅴ

EFFECTS OF FOOD ADDITIVES ON STABILITY OF LSM

Absorbance	Food additives		
	0h	24h	48h
sucrose	0.620±0.001	0.616±0.002	0.638±0.001
citric acid	0.598±0.002	0.481±0.001	0.301±0.001
Vitamin C	0.597±0.001	0.517±0.001	0.429±0.003
Sodium Citrate	0.596±0.002	0.590±0.001	0.588±0.001
Sodium benzoate	0.618±0.001	0.618±0.001	0.620±0.002

Values are mean±s.d. (n =3).

D. Antioxidant activity of LSM

The scavenging activity of LSM on ·OH

The phenolic hydroxyl group has a strong proton donating power. The more the hydroxyl group is, the more hydrogen ions are provided to combine with more active free radicals. As shown in figure 2, when the concentration of LSM was 200 $mg \cdot L^{-1}$, the scavenging rate of ·OH was 52.64%, the hydroxyl radical scavenging activity of LSM was higher than that of *Plectania* YM-421 melanin reported by Ye M. et al [21]. The IC_{50} value (the effective concentration at which the scavenging rate was 50%) of LSM and Vc was 180.87 $mg \cdot L^{-1}$ and 165.43 $mg \cdot L^{-1}$ respectively, it indicated that the hydroxyl radical scavenging activity of LSM lower than that of Vc.

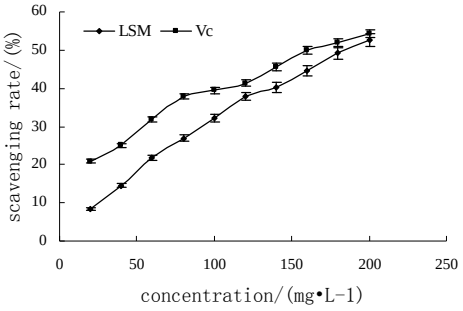

Fig.2. The scavenging activity of LSM on ·OH

The scavenging activity of LSM on ·DPPH

DPPH· was a kind of very stable radical centered on nitrogen and had a maximum absorption peak at 517 nm. When DPPH· reaction system mixed with antioxidant, the number of DPPH· decreased, and the absorbance at 517 nm constantly reduced until it reached stability. Consequently, The scavenging rate of antioxidant on ·DPPH· may be measured at 517 nm. As shown in figure 3, with the increase of LSM concentration, the DPPH· scavenging activity of LSM increased gradually, and there was a good linear relationship between the two($y=0.1848x+5.6467$, $R^2=0.9934$).The scavenging rate on DPPH· radical of LSM reached 41.2% at 200 mg·L^{-1}, the IC_{50} value was 240 mg·L^{-1}, and it was higher than that of BHT (43.39 mg·L^{-1}) and melanin from the muscles of Taihe Black-bone silky fowl (37.3 ±2.62 mg·L^{-1}) [2].

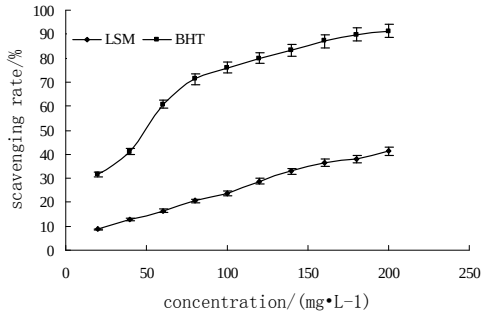

Fig.3. The scavenging activity of LSM on ·DPPH

The scavenging activity of LSM on O_2^-·

Figure 4 shows LSM has a strong O_2^-· scavenging activity. With the increase of the LSM concentration, the O_2^-· scavenging activity of LSM increased gradually. The scavenging rate of LSM on O_2^-· was 43.48% at the concentration 200 mg·L^{-1}, whereas the scavenging rate of Vc was 46.24% at the same concentration. IC_{50} values of LSM and Vc were 275.27 mg·L^{-1} and 266.27 mg·L^{-1}, respectively. Consequently, IC_{50} values of LSM was significantly lower than that of melanin from the muscles of Taihe Black-bone silky fowl (1879±90.9 mg·L^{-1}) [2].

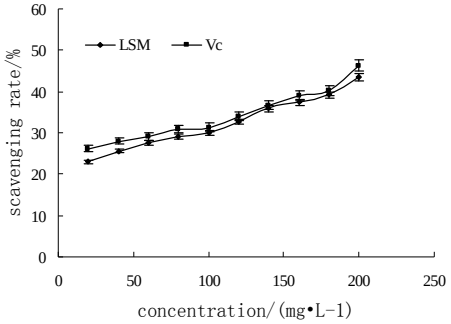

Fig.4. The scavenging activity of LSM on O_2^-·

Ⅳ. Conclusions

In this study，the intracellular melanin was extracted from the mycelium of *Lachnum* YM-292 for the first time, and the purified LSM of 8.85 g was obtained from the mycelium of 100 g, indicating that *Lachnum singerianum* YM-292 is a good species to produce melanin.

The melanin was only soluble in alkaline solutions (NaOH solution and ammonia water), and insoluble in water, acid solvents and common organic solvents (ethyl acetate, chloroform, ethanol, acetone and ethyl ether, etc.). This melanin was not only very stable to light, temperature and reducing agents, but also stable to sucrose and sodium citrate (not including citric acid and Vc), which showed that it has a physico-chemical characteristics of typical melanin. Besides, this melanin had a higher scavenging rate of ·OH, DPPH· and O_2^-·, which indicated it has a strong antioxidant activity. This melanin can be used as a new antioxidant component in medicine or health products. However, further studies were required to clarify the relationship between biological activities and structure of this melanin.

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V. REFERENCES

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