# Physicochemical characteristics and antioxidant activity of the intracellular melanin from *Lachnum sp.* YM-236

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**Abstract.** Melanin LEM236 was extracted and purified from *Lachnum* YM-236. Scanning electron microscopy showed LEM236 had a blocky, amorphous crystal structure with an irregular surface. Elemental analysis, infrared spectroscopy and $^1$H-NMR measurements indicated that the melanin had the structure model of a novel eumelanin-like pigment. The total antioxidant capacity of 1g/L the melanin was equivalent to that of 53.28±0.18 mmol/L α-tocopherol. $IC_{50}$ values of LEM236 for scavenging activity of $ABTS\cdot^+$, ·OH and $NO_2^-$ were 0.680 g/L, 0.759 g/L and 0.839 g/L, respectively.

## Introduction

Melanin is a polyphenolic polymer which has such functions as oxidation resistance, free radical scavenging and anti-aging [1]. To produce melanin via microbial fermentation has the advantages of being easily controlled, needing mild reaction conditions and not being impacted by geographical and climatic conditions. *Lachnum* is a category of saprophytic fungi that distributed throughout the world, and had been discovered could produce a great amount of melanin under submerged culture conditions. This melanin is stable to temperature, light, UV, $Na_2SO_3$ and sodium benzoate, having the structure model of both eumelanin and pheomelanin [2].

The aim of the present work was to prepare LEM236 from *Lachnum* YM-236, reveal its physicochemical characteristics, and assess its antioxidant activity in vitro.

## Materials and methods

### Materials

Sporocarps of *Lachnum* YM-236 was separated and preserved by the Laboratory of Microbial Resources and Application of Hefei University of Technology.

### Extraction and purification of LEM236

The method of Wang *et al* [3] with minor improvements was used. The dried mycelium was mixed with 0.5 mol/L $NH_3\cdot H_2O$ at the proportion of 1:40 (w/v), extracted in water bath at 80 ℃ for 5 h, The water extracts were centrifuged and the supernatants were pooled and acidified with 6 M HCl to pH 2.0. The precipitate was successively extracted with chloroform, ethyl acetate and absolute ethanol after hydrolyzed by 6 mol/L HCl solution at 100 ℃ for 4 h. The purified melanin was obtained after vacuum freeze-drying.

### Physicochemical characteristics of LEM236

The method of Ye *et al* [4] was used for scanning electron microscopy, infrared spectroscopy, $^1$H-NMR spectroscopy and elemental analysis of LEM236. The morphological features of the sample were imaged by a KYKY2800 scanning electron microscope (SEM) (KYKY Technology Development Ltd, China) at working voltage 20 kV/25 kV and working distance 6.0 mm. The percentages of C, H and N in the intracellular melanin were determined using an elemental analyzer (Elementar Vario EL elemental analyzer, Elementaranalysen systeme, Hanau, Germany). 5700 FT-IR spectrometer (ThermoElectron, Madison, WI, USA) was used for infrared spectroscopy

between 4000-400 $cm^{-1}$. And the $^1$H-NMR spectra of the sample was recorded at physiological temperature of 298 K on a Bruker Avance AV-400 spectrometer operating at 500.13 MHz.

**Antioxidant activity of LEM236**

Total antioxidant capacity (TAC) assay of LEM236. Measurement of the total phenolic content was assessed by using the method of Turkoglu *et al* [5]. The total antioxidant capacity was expressed by the α-tocopherol equivalent: A=0.011C+0.0049 ($R^2$=0.987), where A was the absorbance value at 695 nm, and C was the equivalent concentration of α-tocopherol (mmol/L).

Effect of scavenging $ABTS^{\cdot+}$ of LEM236. The ability of LEM236 to scavenge $ABTS^{\cdot+}$ free radical was determined by the method of Zhang *et al* [6]. When the reaction was completed, the absorbance was measured at 560 nm. The distilled water was used instead of the sample as control. $ABTS^{\cdot+}$ scavenging rate (%) = $(1-A_{sample}/A_{control}) \times 100\%$.

·OH scavenging assay of LEM236. ·OH scavenging activity was assessed by using the method of Ye *et al* [7]. The undamaged tubes were not added with $H_2O_2$, melanin solution or Vc solution. ·OH scavenging rate (%) = $[(A_{sampled}-A_{damaged}) / (A_{undamaged}-A_{damaged})]\times 100$.

$NO_2^-$ (nitrite) scavenging assay of LEM236. $NO_2^-$ scavenging assay was determined using a method of Jiao *et al* [8]. Sodium nitrite scavenging rate Sa (%)=$[A_s-(A_p-A_c)]/A_s\times 100$. Where $A_c$ was the absorbance of the control group , $A_s$ was the absorbance of the standard sample.

## Results and discussion

**Physicochemical characteristics of LEM236**

As shown in Fig.1, LEM236 had a blocky, amorphous crystal structure with an irregular surface, which is different from the spherical sepia melanin [9]. The percentages of C, H and N in LEM236 were 54.33±0.24 %, 4.168±0.054 % and 5.286±0.0335 %, respectively. Compared with the Dopa melanin (eumelanin) and brown melanin (Table 1) reported by Ito and Fujita, LEM236 contained less N and had a higher C: N ratio, indicating that LEM236 might contain aliphatic groups [10].

Infrared spectroscopy is the spectroscopy frequently used to examine the organic structure. It does not destroy the sample structure and can reveal the detailed information about functional groups of the sample. Fig.2 gives the infrared spectra of LEM236, which shows the following structural information of the melanin: N-H stretching vibration in the indole ring (3412 $cm^{-1}$), $CH_3$ asymmetric stretching vibration (2932$cm^{-1}$), stretching vibrations of COOH, C=O or C=C (1627 $cm^{-1}$), C-N stretching vibration (1398 $cm^{-1}$), COC asymmetric stretching vibration (1128 $cm^{-1}$). These results indicated that LEM236 contains benzoquinone structure and indole structure.

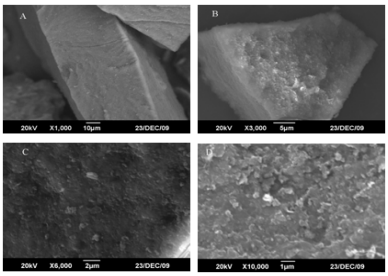

Fig.1 SEM image of LEM236

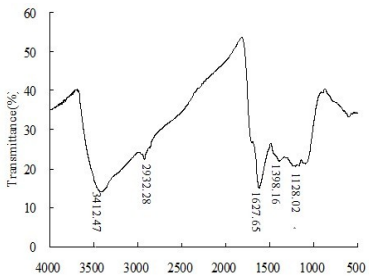


Fig.2 Infrared spectrum of LEM236

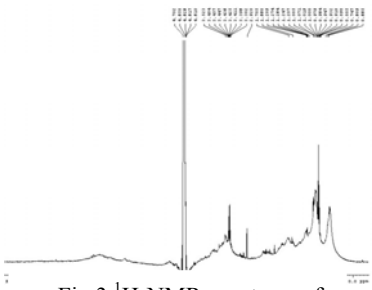

Fig.3 $^1$H-NMR spectrum of LEM236

$^1$H-NMR spectrum of LEM236 presented a series of broad peaks，which was similar with melanin from *Gliocephalotrichum simplex* and *Lachnum singerianum* [1, 4]. Signals in the 2.2-2.5 ppm and 2.5-3.5 ppm region were assigned to $CH_3$ group and NH group respectively that are connected to the indole groups. The resonance at 3.5-4.0 ppm was assigned to CH group that is connected to NH group. Absorption peak in the 7.0-7.5 ppm region may correspond to the indole or other aromatic heterocyclic ring of the melanin polymer chain.

Table 1 Elemental analysis for different melanins

| | Content (%) | | |
|---|---|---|---|
| | C | H | N |
| LEM236 | 54.33 | 4.168 | 5.286 |
| Dopa melanin (eumelanin)* | 56.45 | 3.15 | 8.49 |
| Brown melanin* | 46.24 | 4.46 | 9.36 |

*Its data is from Ito and Fujita' report (1985)

## Antioxidant activity of LEM236

The total phenolic content (TPC) and total antioxidant capacity (TAC) of LEM236 both increased with the increase of the LEM236 concentration, exhibiting a good dose-effect relationship (y=120.52x -7.8523, $R^2$=0.9812; y=43.846x+10.803, $R^2$=0.9615 ) (Fig.4-A and Fig. 4-B). The TAC of 1 g/L LEM236 was equivalent to 53.28±0.18 mmol/L α-tocopherol, and the TAC of 1 g/L BHA was equivalent to 176.3mmol/L α-tocopherol. As shown in Fig.4-C, there was a good linear correlation between the phenolic content and the antioxidant activity of LEM236 (y=2.6182x-32.961, $R^2$ = 0.9256), which was consistent with the result of Kumaran *et al* [11].

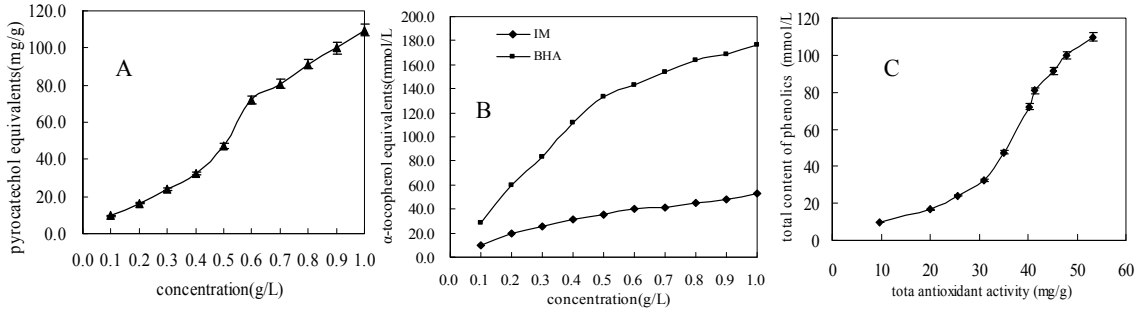


Fig.4 Total phenolic contents (A), total antioxidant capacity (B) and its correlation (C) of LEM236

The scavenging effects of $ABTS^{\cdot+}$ and ·OH were shown in Fig.5-A and Fig.5-B. Both of them had good linear correlations between the content and the antioxidant activity of LEM236 (y=80.886x-4.9679, $R^2$=0.9814; y=83.681x-13.521, $R^2$=0.9388). Compared with the control BHT, LEM236 had a lower $ABTS^{\cdot+}$ scavenging activity, with the $IC_{50}$ value being 0.680 g/L. And the hydroxyl radical scavenging activity of LEM236 was lower than that of Vc or BHA. $IC_{50}$ value of LEM236 was 0.759g/L, whereas $IC_{50}$ values of Vc and BHA were 0.677 g/L and 0.570 g/L, respectively. Fig.5-C shows that, with the increase of the LEM236 concentration, the $NO_2^-$ scavenging ability of LEM236 gradually increased, having a lower scavenging activity than Vc or BHA of the control groups at the same concentration. $IC_{50}$ value of LEM236 for $NO_2^-$ was 0.839g/L, and $IC_{50}$ values of BHA and Vc were 0.145 g/L and 0.184 g/L, respectively.

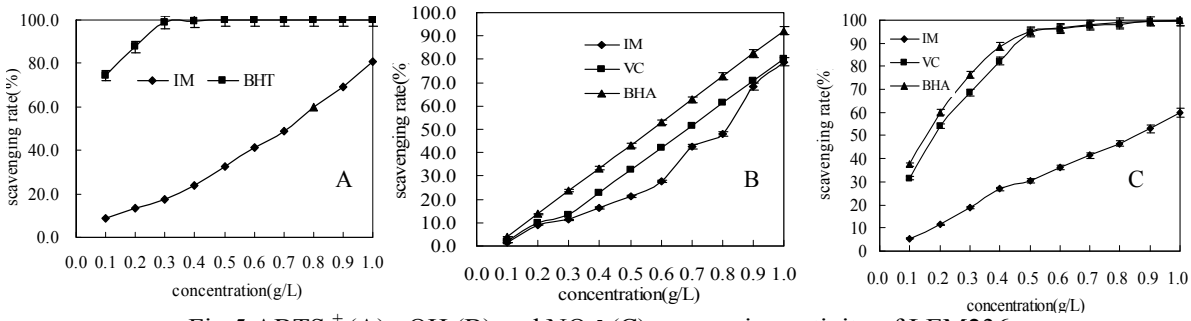


Fig.5 $ABTS^{\cdot+}$ (A), ·OH (B) and $NO_2^-$ (C) scavenging activity of LEM236

## Conclusions

It was concluded that, based on the analysis of elemental analysis, infrared spectroscopy and $^1$H-NMR spectroscopy, LEM236 had the structure model of a novel eumelanin-like pigment. Scanning electron microscopy showed LEM236 had a blocky, amorphous crystal structure with an irregular surface. It had strong scavenging activity against $ABTS^{\cdot+}$, ·OH and $NO_2^-$, indicating that the melanin had a high antioxidant activity, and can be expected to be used as a new antioxidant in such industries as food and medicine.

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