Preparation of water-soluble melanin from squid ink using ultrasound-assisted degradation and its anti-oxidant activity

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Abstract Water-soluble squid melanin fractions were firstly prepared using ultrasound-assistant degradation method under alkaline condition, which is optimized by response surface methodology. The processing melanin fractions were divided into different molecular weight (Mw) fractions by membrane separation (below 10 kDa, among 10–50 kDa and over 50 kDa). The AFM image and particle-size analysis showed monomer units of the melanin were destroyed, and huge polymers were degraded into smaller soluble particles after ultrasound. While, UV, IR and solid ^{13}C NMR spectra indicated that the basic structure of melanin fraction was still retained after ultrasound process. Further analysis showed soluble melanin fractions obtained in 0.5 and 1 M NaOH, with Mw above 10 kDa exhibited much higher in vitro antioxidant potency. The IC_{50} of these fractions (IC_{50} among 19–80 μg) on scavenging O_2^- is more efficient than carnosine ($\text{IC}_{50}=355 \mu\text{g/ml}$), a commercialized antioxidant. They (IC_{50} among 115–180 $\mu\text{g/ml}$) are as efficient as carnosine ($\text{IC}_{50}=110 \mu\text{g/ml}$) on scavenging OH. Our research has reported a novel method for preparation of water-soluble melanin fractions from squid ink, which could be a promising free radical scavenger from nature resource.

Keywords Squid ink melanin · Water-soluble · Ultrasound · Structure, anti-oxidation

Practical application

We developed a novel method using ultrasound processing to obtain water-soluble squid ink melanin under alkaline condition. Our results showed that soluble squid ink melanin fraction as a natural pigment, is a new free radical scavenger with a promising prospect. The excellent solubility can increase absorption and enhance utilization rate in vivo, providing a potential prospect for squid ink melanin products as a nature antioxidant.

Introduction

Squid belongs to the invertebrate mollusk, like other cephalopods, relies on ejection of dark ink for defense. The squid ink has been reported to have anti-radiation activity, anti-tumor activity, immunomodulatory activity, procoagulant function and so on (Takaya 2000). Several bioactive molecules, including a glycosaminoglycan like polysaccharides (Chen et al. 2008), a tyrosinase (Naraoka et al. 2003) and an angiotensin-converting enzyme inhibitor (Kim et al. 2003) have been identified from squid ink. Among them, the melanin has attracted most research interests.

Natural eumelanin is a heterogeneous biopolymer widely distributed in many living organisms. It is commonly divided into three types: the brown-black eumelanin, the yellow-reddish pheomelanin and the dark brown neuromelanin (Liu and Simon 2003a, b). The eumelanin is a macromolecular polymer developed through the enzymatic oxidation of tyrosine (Riley 1997). The 5, 6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) are postulated to be the main monomeric building blocks of eumelanin (Meredith and Sarna 2006). Natural melanin has been reported to have defense activity, protection function (Kollias et al. 1991; Casadevall et al. 2000) and metal

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chelating ability (Liu et al. 2004). It could participate in physiological and pathological activities in human body (Riley 1997) and even become one of the treatment of Acquired Immune Deficiency Syndrome (AIDS) (Montefiori and Zhou 1991). Although the mechanisms of melanin in presenting these activities are complex, they are almost all related to free radical scavenging reactions (Rózanowska et al. 1999).

Melanin originates from different nature sources, such as *Osmanthus fragrans* seeds (Wang et al. 2006), *Pleurotus cystidiosus* (Selvakumar et al. 2008), and silky fowl (Tu et al. 2009), and has been reported to have excellent free radicals scavenge activity. Squid ink melanin is the most typical melanin usually used as melanin standard with strong free radical protection activities (Menon and Haberman 1977). However, squid melanin is insoluble in both water and organic solvents, such as ethanol, hexane, acetone, benzene, and chloroform (Harki et al. 1997), its insolubility greatly limits its utilization as a natural antioxidant.

Several technologies have been used to obtain soluble melanin from squid ink, but its monomer units can hardly split by either chemical method or enzymatic hydrolysis, because of its insolubility over a broad range of pH (Liu and Simon 2003a, b). Ultrasound-assisted degradation (UAD) has been broadly applied to degrade biopolymers, such as chitosan, starch, pectin and dextran (Lorimer et al. 1995; Czechowska-Biskupa et al. 2005; Liu et al. 2006; Kou and Wang 2010). The increasing of degradation efficiency by ultrasound is attributed to the propagation of ultrasound pressure waves, and resulting cavitation phenomenon (Vilkhu et al. 2008). Moreover, some supplementary means for the ultrasound degradation process, such as combine the use of ultrasound and enzyme, or ultrasound under hydrogen peroxide and acid conditions, were used to accelerate the degradation of the polymers (Entezari and Pétrier 2004; Kou and Wang 2010). However, there was no report on degradation of water-insoluble squid ink melanin using ultrasound technology.

In the present study, we developed an efficient and economical degradation method for preparing the water-soluble melanin fraction from squid ink by ultrasound treatment in alkali solution. Two major conditions for degradation process, including temperature and the ultrasonic power were optimized. Atomic force microscope (AFM) and laser diffraction particle size analyzer were used for observing the morphology and size change of the melanin particle thus verify the degradation effects. The UV and IR spectra were analyzed to compare the effect of different alkali concentration on structure change during the UAD process. Solid NMR spectra further confirmed the effect of the ultrasound process on the structure. Furthermore, antioxidant activities of different soluble melanin fractions were compared, including superoxide anion radical and hydroxyl radical scavenging activities.

Materials and methods

Materials

The ink was obtained from squid sac (*Ommastrephes bartrami*) from Xing-Ye Fishery Company (Zhoushan, Zhejiang, China) and stored at -70°C before use. Nanopure water ($>18.2\text{ MX}$) obtained from a Simplicity TM system (Millipore, Billerica, MA, USA) was used for washing and preparing solutions. Ultra filtration membrane (MWCO 10 and 50 kDa) was from Hefei Shijie Membrane Engineering Co.; Scientz-IID ultrasonic cell crushing device (Ningbo Xinzhi, power 0-950 W, Ningbo, Zhejiang, China); low-temperature water bath, DC-1006 (Safe Corporation, Ningbo, Zhejiang, China); Atomic force microscope (Veeco Instruments, Santa Barbara, CA); Beckman Coulter LS 13 320 laser diffraction particle size analyzer (Beckman Coulter, Miami, FL, USA), UV-visible spectrophotometer, UV-2550 (Shimadzu, Kyoto, Japan); Nexus 470 FTIR Spectrometer (Nicolet Instruments, Madison, WI, USA); Bruker AvanceIII 400 spectrometer (Bruker BioSpin, Rheinstetten, Germany).

Preparation of intact squid ink melanin

The isolation and purification procedures were performed according to previously reported procedures (Liu and Simon 2003a, b). Briefly, ink from the fresh ink sacs was drained and mixed with 20-fold nanopure water. Then the mixture was centrifuged (5000 g, 15 min, 4°C). The precipitate was mixed with 20-fold nanopure water and centrifuged under the same conditions again. Such process was repeated six times to remove the water-soluble impurities in the ink. The final melanin pellet was lyophilized to get the intact pure sepia ink melanin. The sample thus obtained was examined by SEM, which showed high purity without contamination of any cellular fractions (Chen et al. 2009).

Optimization of ultrasound process by response surface methodology

Ultrasound process was carried out with an ultrasonic processor (Scientz-IID, Ningbo scientz biotechnology Co., Ningbo, Zhejiang, China) equipped with a 10 mm microtip. 2 g natural squid ink melanin was dissolved in 0.5 M NaOH solvent and immersed in a low-temperature water bath (DC-1006, Safe Corporation, Ningbo, Zhejiang, China) to maintain the stable temperature during the entire ultrasound process. The probe was submerged in the suspension 1 cm from the top surface of the suspension and treated for 1 h.

The condition for maximum production of soluble melanin fraction has been optimized by response surface methodology (RSM) using Central Composite Design (CCD). As two of the most important factors, the levels of temperature

(X) and ultrasonic power(Y) were chosen for this study (the effect of alkali concentration on the degradation process was studied separately in 2.4.). Each factor in the design was studied at three different levels (−1, 0, +1). A set of 13 experiments were carried out. The minimum and maximum ranges of variables were investigated and the full experimental plan with respect to their values in actual and coded form is listed in Table 1. The quadratic model for predicting the optimal point was expressed according to $Z=a+bX+cY+dX^2+eY^2+fXY$, where “Z” is the response calculated by the model; “X” represents temperature (°C); “Y” represents ultrasonic power (w); “a” is the intercept; “b”, “c” are linear coefficients; “d”, “e” are squared coefficients; “f” is the interaction coefficients. “Minitab” software was used for regression analyses of the data obtained.

Ultrasound processing of the squid ink melanin in alkali

In order to investigate the effect of different alkali concentration on degradation process. 2 g natural squid ink melanin was mixed with 100 ml NaOH solvent (0.1 M, 0.5 M and 1 M, respectively). The ultrasound process was the same as described in 2.3. The temperature and ultrasound power was according to the result of optimized condition. The treated solutions were neutralized with HCl, and centrifuged (10000 g, 20 min, 4°C) at high-speed to separate the insoluble fraction. The supernatant was lyophilized. The collected fractions under 0.1 M, 0.5 M and 1 M NaOH treatment were named as fraction A, B and C respectively.

AFM image

The natural squid ink melanin and soluble melanin fraction B in water suspension were dropped on freshly cleaved mica,

and then air-dried in the dark. AFM images (height, amplitude and phase) were collected using atomic force microscope (Nanoscope IIIa Bioscope, Digital Instruments/Veeco, Santa Barbara, CA) in an atmospheric condition of 50 % humidity level. All images were obtained in tapping mode. The scan area is 20×20 μm.

Particle size measurements

The particle size distribution of melanin samples was measured using a laser diffraction particle size analyzer (Beckman-Coulter, LS-230, Miami, FL, USA). The natural squid ink melanin and soluble melanin fraction B were dispersed in deionized water. The particle size distribution (volume percent) was produced by the computer-controlled Coulter LS 230 instrument via Beckman-Coulter LS 13 320 software program according to the principle of light scattering.

UV and IR scanning

The natural squid ink melanin and soluble melanin fraction A, B and C were dissolved in deionized water with a final concentration of 0.5 μg/mL. The samples were scanning by the UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

IR samples were prepared by mixing dried sample (0.5 mg) with KBr and pressed into tablets, using the Nexus 470 FTIR Spectrometer to get the IR spectra (Nicolet Instruments, Madison, WI, USA).

Solid-state NMR parameters

For solid state CP/MAS NMR measurements, the samples were pressed into 4 mm zirconia rotors and spun at 14 kHz. The measurements were conducted on a Bruker Avance III

Table 1 Experimental design and results

	Code values temperature		Real values ultrasonic power		Yield (g)	Rate of production(%)
	x	y	X(°C)	Y(w)		
1	1	−1	40	500	0.613	30.65
2	0	−1	27.5	500	0.634	31.70
3	−1	1	15	800	0.599	29.95
4	1	1	40	800	0.658	32.90
5	0	0	27.5	650	0.647	32.35
6	0	0	27.5	650	0.653	32.65
7	−1	0	15	650	0.568	28.40
8	0	0	27.5	650	0.658	32.90
9	0	0	27.5	650	0.650	32.50
10	0	0	27.5	650	0.659	32.95
11	0	1	27.5	800	0.690	34.50
12	1	0	40	650	0.629	31.45
13	−1	−1	15	500	0.528	26.40

X: temperature(°C); Y: ultrasonic power(w)

400 spectrometer (Bruker BioSpin, Rheinstetten, Germany) with a resonance frequency of 100.62 MHz for ^{13}C . The signal-to-noise ratio was enhanced by applying cross polarization. For natural squid ink melanin, the contact time was 3000 us, the relaxation delay was 2 s. For soluble melanin fraction B, the contact time was 1000 us, the relaxation delay was 0.8 s. A ramp-contact and spinal 64 decoupling pulse program was employed in the CP/MAS experiments. All ^{13}C NMR spectra were calibrated using the methine carbon atoms of adamantane as an external standard ($\delta=29.47$ ppm).

Fraction of degraded squid ink melanin fraction

Soluble melanin fractions A, B and C were dissolved in 1000 ml water. The solution was initially processed in a 10 kDa membrane, the permeate was lyophilized and denoted as soluble-melanin ultrafiltered through 10 kDa membrane ($M_w < 10$ kDa). The retentate was diluted to achieve the concentration suitable for further ultrafiltration under conditions similar to those employed in the first ultrafiltration stage, and processed through the 50 kDa membrane. The permeate was lyophilized to yield a fraction denoted as soluble-melanin permeated through the 50 kDa membrane ($10 \text{ kDa} < M_w < 50 \text{ kDa}$). The retentate from the 50 kDa membrane was lyophilized, denoted as soluble melanin fraction over 50 kDa ($M_w > 50 \text{ kDa}$).

The collected fractions under 0.1 M NaOH treatment were named as A-1 for $M_w < 10$ kDa, A-2 for $10 \text{ kDa} < M_w < 50 \text{ kDa}$, A-3 for those with $M_w > 50 \text{ kDa}$; the fractions under 0.5 M NaOH treatment were named as B-1 for $M_w < 10$ kDa, B-2 for $10 \text{ kDa} < M_w < 50 \text{ kDa}$, B-3 for those with $M_w > 50 \text{ kDa}$; and the fractions under 1.0 M NaOH treatment were named as C-1 for $M_w < 10$ kDa, C-2 for $10 \text{ kDa} < M_w < 50 \text{ kDa}$, C-3 for those with $M_w > 50 \text{ kDa}$, respectively.

Free radical scavenging activity of water-soluble squid ink melanin

Superoxide anion radical ($\text{O}_2^{\cdot -}$)

The reaction procedure was the same as previously described (Lin 2003). Briefly, every sample was mixed with PBS (0.05 M, pH 7.8) to prepare concentration on 0, 50, 100, 150, 200, 300, 350, 400 $\mu\text{g} / \text{mL}$ solution, and add 400 μL in a small test tube. Then add 50 μL pyrogallol (0.625 μM) and 550 μL luminol (0.1 mM), pull cover and measuring the value of luminol chemiluminescence C. Three parallel experiments in each group. Inhibition rate (%) = $(C_0 - C) / C_0 \times 100\%$ (C_0 is empty antioxidants light value; C is the luminous value of antioxidants). IC_{50} , which is the concentration of each sample when inhibition rate is 50 %, was obtained according to the relationship between concentration and inhibition rate curve.

Hydroxyl radical ($\cdot\text{OH}$)

The reaction procedure was the same as previously described (Szpoganicz et al. 2002), with minor modification. Every sample was mixed with PBS to prepare different concentration of solution as 2.8.1. described, and add 550 μL in small test tube respectively. Then add 0.2 mL ascorbic acid (2 mM), 0.4 mL CuSO_4 (2 mM), 50 μL of luminol (0.1 mM), 0.2 mL of yeast suspension solution (75 mg / mL). Mixed and incubated for 30 mins. After that, add 0.6 mL H_2O_2 (6.8 mM) to start reaction. Pull cover and began measuring luminol chemiluminescence value C. Calculate the inhibit rate (%) and IC_{50} .

Results and discussion

Optimization by response surface methodology

Response surface methodology is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data and has been adopted as a tool to obtain best process conditions. Our previous studies showed temperature, ultrasonic power and alkali concentration are three major factors in degradation process. Note that the alkali concentration has a great influence on the ant oxidation activity of the products, we study this factor separately in the following part. Here, we optimized the condition of temperature and ultrasound power to make preparations for the following study.

The experimental design for the various treatments was based on preliminary work, which established that an optimum could be found within the range of parameters studied. Regression analysis was performed to fit the response function with the experimental data (Table 1). The model expressed by equation: $Z = 10.1106 + 1.28015X + 7.7 \times 10^{-5}Y - 0.0187421X^2 + 1.1 \times 10^{-5}Y^2 - 1.7 \times 10^{-4}XY$, where the variables take their coded values, represents rate of production (Z) as a function of temperature (X) and ultrasonic power (Y).

For testing the goodness of fit of the regression equation, the multiple correlation coefficient R and the determination coefficient R^2 were evaluated. The R^2 was calculated to be 0.9918 for rate of production. This implied that the sample variation of 99.18 % for rate of production was attributed to the independent variables and only 0.82 % of the total variation was not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data.

The statistical significance of the second order model equation was evaluated by the F -test analysis of variance (ANOVA) which revealed that this regression is statistically significant ($P < 0.0001$) of confidence level (Table 2). The Model F -value 169.12 for rate of production implied that

Table 2 ANOVA for response surface quadratic model

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P
Model	5	56.3852	56.3852	11.2770	169.12	0.000
Residual	7	0.4668	0.4668	0.0667		
Lack of fit	3	0.2038	0.2038	0.0679	1.03	0.468
Pure error	4	0.2630	0.2630	0.0657		
Total	12	56.8519				

$R^2=0.9918$, DF degree of freedom; $Seq\ SS$ sequential sum of squares; $Adj\ SS$ adjusted sums of squares; $Adj\ MS$ adjusted mean square

the model is significant. Values of 'ProbF' less than 0.05 indicated that the model terms are highly significant. X and XY are significant model terms ($P<0.05$); the intercept and X^2 are highly significant model terms ($P<0.01$; X: temperature; Y: ultrasonic power). The 'Lack of Fit F-value' of 0.468 implied the lack of fit is significant.

It was concluded that the temperature of 30.4040 °C and the ultrasonic power of 800w should be selected since they supported maximum production rate of 34.56 % and yield of 0.6912 g. To confirm these results, ultrasound degradation was performed under optimized conditions. In this assay, yield of soluble melanin fraction is 0.695 g. These results clearly showed that the model fitted the experimental data well and therefore, described the region studied well.

Ultrasound processing of the squid ink melanin in alkali

Given that the alkali concentration on the degradation process would make an enormous impact on the antioxidant activity of the products, we further investigate this factor independently based on the optimized temperature and ultrasonic power.

The weight of each soluble melanin fractions from every 2 g natural squid ink melanin was showed in Table 3. 0.645, 0.695 and 0.793 g of soluble melanin fractions were obtained by treating with 0.1 M, 0.5 M, and 1.0 M NaOH, respectively. The yields of soluble melanin fraction increased as the alkaline concentration increased, indicating higher alkaline concentration could lead to more severe degradation of the melanin, which could increase the solubility of the natural

Table 3 The weight of each fractions treated with different concentration of NaOH

Alkali concentration (M)	Weight of fraction			
	<10 kDa (g)	10–50 kDa (g)	>50 kDa (g)	Total (g)
0.1	0.438	0.159	0.048	0.645
0.5	0.371	0.238	0.086	0.695
1.0	0.296	0.376	0.121	0.793

melanin, thus provide a better reaction environment and lead to a more sufficient interaction through ultrasound process.

The melanin fractions thus obtained showed excellent solubility in water (solubility >0.5 g/ml). The soluble melanin fractions are still dark black but a little lighter than natural melanin.

The morphology and size change of the melanin particle

The morphology and spatial structure of natural melanin and water soluble melanin fractions (use the fraction B prepared under 0.5 M NaOH as a typical sample) were investigated by laser diffraction particle size analyzer and AFM to investigate the structure change of the melanin during UAD.

Particle-size analyzed

The particle size distribution of soluble fraction B and natural melanin was analyzed and presented in Fig. 1 and Table 4. As reflected in Fig. 1 is the size distribution of the two fractions. The natural melanin granules exhibit a broad range of distribution from 1.26 μm to 121 μm , with the mean diameter of 17.34 μm . However, after UAD process, the particle size appeared as a bimodal, with two peaks around 0.04–0.08 μm and 2–3.86 μm . Its mean diameter is 1.467 μm , which is much smaller than natural melanin. The D10, D25, D50, D75 and D90 corresponding to the particles with sizes below 10 %, 25 %, 50 %, 75 % and 90 % of the total volume of particles analyzed, respectively (Table 4). The volume statistics size of D50 of soluble fraction B was 2.307 μm , which was very close to the original melanin (2.5 μm). However, D90 of the fraction B (3.065 μm) was much smaller compared to the original melanin (58.72 μm). That is means 50 % of the particles for both sample are smaller than 2.5 μm , while 90 % of the particles are below 3.065 μm and 58.72 μm

Fig. 1 Particle size distributions of natural melanin and soluble melanin fraction B treated under 0.5 M NaOH solution. Samples were dispersed in deionized water. The particle size distribution (volume percent) of the suspension melanin was produced according to the principle of light scattering.

Table 4 The percentage of particle size distribution measured by a laser diffraction particle size analyzer

%≤	D10	D25	D50	D75	D90	Mean
UAD melanin(μm)	0.048	0.054	2.307	2.831	3.065	1.467
natural melanin (μm)	1.482	2.017	2.842	31.75	58.72	17.34

*D10, D25, D50, D75 and D90 corresponding to the particle sizes below 10 %, 25 %, 50 %, 75 % and 90 % of the total volume of particles analyzed

for fraction B and natural melanin respectively, indicating that these original melanin with large particle size was break down through ultrasound treatment and leads to a much more narrow size distribution (Fig. 1).

AFM image

Melanin granules can be imaged by tapping mode AFM, the variation of the height of the cantilever across the scanning area (height image) gives real three-dimensional topology information and the variation of the phase of the cantilever vibration across the scanning area (phase image) can provide information concerning whether there are boundaries between regions of differing hardness. Thus, it directly reflects a real morphology of the melanin polymer.

As observed in Fig. 2, the natural squid ink melanin (left side) showed significant multi-μm-sized aggregation morphology with rough surface. Further observation revealed that

these μm-sized granules were found to be comprised of tightly packed multi-nm-sized particles. However, after UAD procedure, the aggregation morphology disappeared and the polymer's spatial structure was destroyed (right side). Most multi-nm-sized particles were liberated from the μm-sized polymers and no aggregates similar to natural melanin were observed. There have been a variety of spectroscopic and imaging studies on synthetic eumelanins arguing that the pigment is an aggregated system of small oligomeric molecules (Zajac et al. 1994). Our results suggested that the μm-sized insoluble natural melanin polymers might be dissociated into nm-sized particles using UAD process.

Particle size observation, which was consistent with the result of the AFM, reflected that the superpolymers were break down into smaller micromolecule after ultrasound processing and lead to a narrow size distribution, thus increased its solubility. It may be inferred that some bonds in natural melanin were broken and the huge macromolecule was fractured into soluble melanin particles.

Spectra analysis of soluble melanin fraction

Based on the spatial structure analysis of the melanin particles by AFM and particle size analyzer, we further investigated the preliminary chemical structure changes of the melanin during ultrasound treatment, especially those groups related to the antioxidant acidity of the melanin. The UV, IR and ¹³C CP/MAS NMR spectra of natural squid ink melanin and soluble melanin fractions were analyzed to

Fig. 2 AFM image of natural melanin (a) and soluble melanin fraction B treated under 0.5 M NaOH solution (b). A drop of melanin suspension were dropped on freshly cleaved mica, and then air-dried in dark. (1) Phase image; (2) amplitude image. Scan area is 20×20 μm. The black scale bars are 1 μm in the images

compare the effect of different alkali concentration on structure change during the degradation process.

Melanin UV spectrum

The ultraviolet–visible absorption spectrum of the melanin fractions were shown in Fig. 3. All fractions showed strong optical absorbance in a wide spectral range.

Peaks near 310 nm and 430 nm were still appeared in all the three soluble melanin fractions as the original melanin, indicating the presence of indole units (Das et al. 1978), which were known to be abundant in DHI and DHICA structure. This result indicated the main DHI and DHICA unit was still remained in the water soluble melanin fractions. However, the strength of the peaks for soluble melanin fractions (fraction A, B and C) were slightly weaker than natural melanin, which might represent that the DHI group suffered minor damage.

IR spectrum

IR spectra were also used to a more detailed comparison of fractions treated with different concentrations of alkali solvent. Since melanin is a biological macromolecule with complex structure, its IR spectrum is a series of broad and strong absorption peaks (Fig. 4). Each broad peak is formed by the vibration of a variety of functional groups, lacking characteristic. However, the minor changes of peaks can still help us understanding the structure change of the melanin during the degradation.

The IR spectra of natural melanin and fraction A, B, C were shown in Fig. 4. The absorption peaks were assigned according to the previously report on neuromelanin, dark-hair melanin and synthetic melanin (Stainsack et al. 2003; Liu et al. 2005; Centeno and Shamir 2008). All the three fractions showed similar spectra as the original melanin with characteristic absorption peak at follows: 3300–3400 cm^{-1} for -OH stretching ($\sim 3400 \text{ cm}^{-1}$) and -NH ($\sim 3300 \text{ cm}^{-1}$) in

Fig. 3 UV spectra of natural melanin and soluble melanin fraction A, B, C treated by 0.1 M, 0.5 M and 1 M NaOH respectively. All samples were dissolved in deionized water with a final concentration of $0.5 \mu\text{g}/\text{mL}$. Samples were scanning by the UV-Visible spectrophotometer

Fig. 4 IR spectra of natural melanin and soluble melanin fraction A, B, C treated by 0.1 M, 0.5 M and 1 M NaOH respectively. IR samples were prepared by mixing dried melanin (0.5 mg) with KBr and pressed into tablets, using the Nexus 470 FTIR Spectrometer to get the IR spectra

indole or pyrrole; 2850–2920 cm^{-1} for stretching vibration of aliphatic C-H groups from amino acid; 1610–1690 cm^{-1} , the aromatic ring C=C, C=N bending and C=O stretching (non-carboxylic acid group); 1580 cm^{-1} for the ionization of the COO^- and C=O; 1457 cm^{-1} for $-\text{CH}_2\text{CH}_3$ bending; 1410 cm^{-1} for pyrrole ring stretching; 1360–1380 cm^{-1} , C-N stretching from amino acids and 1050–1100 cm^{-1} , alcoholic O-H from amino acids. These results indicated after ultrasound process, the soluble melanin fractions still kept the backbone structure of the melanin, these chemical groups related to the antioxidant activity of the melanin was not severely destroyed.

However, there does some differences exist between natural melanin and soluble fractions. Compared to natural melanin, the soluble melanin fractions showed relatively weaker peaks: decreasing absorption of -OH and -NH stretching (3300–3400 cm^{-1}), which were assign to the indolic and pyrrolic systems (Magarelli et al. 2010), indicated a lower content of DHICA and DHI; reduced peaks around 1634 cm^{-1} indicated the some bond rupture occurred on the benzene ring (from the DHICA and DHI) after UAD process. Similarly, the stronger signals related to COOH groups (1580 cm^{-1} for the ionization of the COO^- and C=O) of fraction A, B and C showed a more severe degree of oxidation.

The above results indicated the DHICA and DHI structures of the melanin were destroyed during the ultrasound (although the effect was not significant), and the backbone structure of melanin is retained. The extent of destruction enhanced as the decrease of the alkaline concentration used. This might due to low alkaline concentration would decrease the solubility of the melanin, therefore diminish the interaction between the melanin and solvents, and impedes

the degradation reaction. Though the melanin was eventually degraded into soluble fraction in low alkaline concentration group, the damage of its structure is more severe.

¹³C NMR spectrum

Based on the results from the UV and IR spectra, we further confirmed the specific structure changes of the melanin using ¹³C CP/MAS NMR spectra. As three fraction of the soluble melanin showed very similar spectra, we used the fraction B suffering moderate degradation as a preventive.

Figure 5 showed the ¹³C CP/MAS NMR spectra for natural squid ink melanin and soluble fraction B. Both of the two samples showed similar peaks among three characteristic spectral regions as described in the previously literature for the NMR of the melanin (Adhyaru et al. 2003; Tian et al. 2003; Jalmi et al. 2012): (1) δ10–95 ppm was assigned to aliphatic carbons, which was due to proteinaceous material; (2) δ95–165 ppm was assigned to aromatic carbons, including indole or pyrrole type carbons within the polymer; (3) δ165–200 ppm, carbonyl carbon atoms in amides, carboxylates and quinones, which came from the carbonyl in the oxidized quinone form of DHI and DHICA. More specifically, the signals near ~δ30 ppm and ~δ50 ppm were assigned to aliphatic groups which come from dihydro-derivatives of DHICA, DHI (Knicker et al. 1995; Adhyaru et al. 2003; Tian et al. 2003; Jalmi et al. 2012); the signals near ~δ103 ppm was suggestive of C-3 of indole nucleus, which was from the anomeric carbon of carbohydrates; the peak near ~δ130 ppm was assigned to aromatic group; the signal near ~δ174 ppm was due to carbonyl group present as carboxylic acid group, which was derived from DHICA, and the degradation products of DHI and DHICA. The appearance of these groups in both the natural melanin and degraded melanin fraction indicated that the backbone

Fig. 5 Solid-state ¹³C CP/MAS NMR spectra for natural squid ink melanin and soluble melanin fraction B treated under 0.5 M NaOH solution. The samples were pressed into 4 mm zirconia rotors and spun at 14 kHz. The measurements were conducted with a resonance frequency of 100.62 MHz for ¹³C. All ¹³C NMR spectra were calibrated using the methine carbon atoms of adamantane as an external standard (δ=29.47 ppm)

structure of the melanin was retained after UAD, this was consistent with the results from the UV and IR spectra.

Apart from the similarity, there were some tiny differences existing between two samples. In comparison with the fraction B, natural melanin exhibited higher intensity of signals in the range δ95–165 ppm. This was presumably attributable to the greater percentage of protonated carbon atoms (δ95–130 ppm) and quaternary carbon atoms in the melanin backbone (δ130–145 ppm) (Adhyaru et al. 2003), which indicated that aromatic structure was broken down in some extent through the degradation process.

Also, an interesting phenomenon was noticed that during the NMR scanning, for natural melanin, the contact time was 3000 μs and the relaxation delay was 2 s, while for fraction B, the contact time was 1000 μs with the relaxation delay of 0.8 s. These parameters were optimized according to ¹H NMR spectrum (not shown). We speculated that this was caused by the change of crystal structure. During the UAD process, the spatial structure of huge melanin polymers was destroyed, the aggregation morphology disappeared and the soluble fractions were transformed to amorphous structure. This was consistent with our results from particle size analyzer and AFM image.

Based on all the above structure analysis, we can conclude that although the melanin particles were degraded to smaller size and spatial structure was fractured after ultrasound process, the preliminary structure of the melanin, such as DHI and DHICA structure was only slightly destroyed as the increase of the alkaline concentration. The mechanism of ultrasound on squid melanin may include three aspects by referring from the previously literature (Basedow and Ebert 1977): firstly, the high temperature, high pressure and the mechanical shear stress, as a result of the collapse of cavitation bubbles during the ultrasound process, which breaks the structure of the melanin molecules; secondly, the oxidation-reduction reaction which degrades the polymer into smaller molecules; thirdly, higher concentration of NaOH treatment of melanin may lead to more intense breaking of covalent bond and hydrogen bond, thus exacerbating the degradation process.

Free radical scavenging activity of degraded melanin fractions

According to the free radical theory, senescence and its related degenerative disease can be largely attributed to cell components and connective tissues which are attacked by free radicals, in which superoxide anion ($O_2^{\cdot-}$) and hydroxy radical ($\cdot OH$) played an important role. The capability of water-soluble melanin fractions on scavenging these two radicals was investigated. IC_{50} on superoxide anion radical and hydroxy radical of soluble melanin fractions degraded by different concentration of alkali were shown in Table 5.

Table 5 IC₅₀ on superoxide anion radical and hydroxy radical of soluble melanin fractions degraded by different concentration alkali

Alkaline concentration(M)	IC ₅₀ on superoxide anion radical (μg/mL)			IC ₅₀ on hydroxy radical (μg/mL)		
	<10 kDa	10 to-kDa	> 50 kDa	<10 kDa	10-50 kDa	> 50 kDa
0.1	>400	240	165	>550	>550	300
0.5	330	35	27	540	145	130
1	235	80	19	>550	180	115

Figure 6(a) showed scavenging activity on superoxide anion radical of each fraction (A-1, A-2, A-3, B-1, B-2, B-3, C-1, C-2, C-3 as 2.4. mentioned), as well as the commercialized antioxidant carnosine. The results showed that those fractions prepared by UAD under 0.5 and 1 M alkaline, with molecular weight of 10-50 kDa and more (B2, B3, C2 and C3), showed higher antioxidant activity than other fractions. The IC₅₀ values were between 19 μg/mL and 80 μg/mL, even lower than the commercial antioxidant carnosine (IC₅₀ is 355 μg/mL). On one hand, the O₂⁻ scavenging ability increased as the alkali concentration rose. This might due to the higher concentration of alkali would aggravate the degradation degree thus explored more reactive groups for reaction. On the other hand, higher MW fractions exhibit stronger O₂⁻ scavenging activity at the same concentration of alkaline; we speculate that the structural damage of low MW fractions is more severe and high MW fractions might retain more reactive groups, such as DHI and DHICA unit, which can react with free radicals. This speculation is consistent with our result of spectrum. Hence, these fractions with MW over 10 kDa and treated by NaOH which concentration is over 0.5 M, exhibited a significant superoxide anion radical scavenging activity.

The hydroxyl radical scavenging activity of each fraction was also detected by Luminol chemiluminescence system, the results were presented in Fig. 6(b). It showed the similar tendency as the effect on scavenging superoxide radical (Fig. 6(a)). The fractions degraded by 0.5 and 1 M NaOH, with Mw above 10 kDa, also showed relatively low IC₅₀ value of OH between 115 μg/mL and 180 μg/mL, which were similar to that of carnosine (IC₅₀ 110 μg/mL).

The relevant functional groups in eumelanins have been identified to be DHI and DHICA (Mosca et al. 1998). When DHI and DHICA convert between the structure of hydroquinone and benzoquinone, two electrons will lose during the redox reaction. DHI and DHICA played a similar role of superoxide dismutase in this process that catalyzes the O₂⁻ into H₂O₂ and O₂. Experimental results show if melanin content is high in normal pigment cell, it is helpful to inhibit production of extracellular H₂O₂ (Mosca et al. 1998). The significant radical scavenging activity of fractions with MW over 10 kDa under 0.5 and 1 M NaOH was attributed to the more DHI and DHICA were retained in these fractions, as we have identified in IR spectra.

Fig. 6 Antioxidant activities of water soluble melanin fractions prepared by different concentration of alkali. **a** Scavenging activity on superoxide anion radical of water-soluble melanin fractions; **b** Scavenging activity on hydroxy radical of water-soluble melanin fractions. The scavenging activities were detected by Luminol chemiluminescence system. Three parallel experiments in each group

Conclusion

There has been a growing interest in the development of natural pigment used in food industry. Squid ink melanin is the most typical melanin with strong free radical protection activities. However, such melanin is water insoluble particle, which limits its absorption by human as well as its application. In the present study, we developed a novel method using ultrasound degradation under alkaline condition for preparing water-soluble squid ink melanin fractions. The optimization by response surface methodology showed the temperature of 30°C and the ultrasonic power of 800w is the optimal conditions for UAD of melanin. AFM image and particle-size analysis confirmed the fact that insoluble melanin polymer was degraded into small soluble particles after ultrasound. UV, IR and NMR spectra compared the structure change of soluble melanin fractions treated with different solvent. The result indicated that during the UAD process, despite some groups were slight damaged, the basic structure of melanin is retained. The structure of soluble melanin fraction treated by 0.5 M NaOH suffered minimum change among the fractions. These results reflected that low concentration of alkali treatment lead to more severe degree of oxidation. The active groups, including DHI and DHICA unit, were oxidized. Due to this reason, in free radical scavenge assay, the fractions treated by higher concentration of NaOH with higher MW, which had greater characteristic absorption peak of -OH and -NH structure showed remarkable free radical scavenging activity.

In conclusion, our results showed that soluble squid melanin fractions as a natural pigment is a new free radical scavenger with a promising prospect. The excellent solubility can increase absorption and enhance utilization rate *in vivo*, providing a potential prospect for squid melanin products as a nature antioxidant.

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