# Comparation of structure and properties of sea cucumber melanin before and after degradation

Xinjia Li, Jianbo Wang, Hengxu Liu, Lihong Chen, Aili Jiang*

Department of Biochemical Engineering, Yantai University, Yantai, Shandong Province 264005, China

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**Sea cucumber is rich in active substance such as triterpene glycosides, polysaccharide collagens, and chondroitin sulfates. Melanin shows protective and defensive functions in animals including anti-oxidation and free radical removal, but very few studies were on melanin in sea cucumber. Although most of the products sold in the market were dried sea cucumber, current researches were mainly focused on fresh sea cucumber. In this study, crude melanin was extracted from dried sea cucumber, and the structural properties of melanin was studied. The sea cucumber body wall was digested with trypsin, pancreatin, and pepsin, respectively. Then, sea cucumber melanin in the enzymatic hydrolysate was purified by alkali-soluble acid precipitation method and degraded by $H_2O_2$ to investigate its anti-oxidation before and after degradation. The structural differences were analyzed by ultraviolet-infrared spectroscopy. The results showed that the three types of melanin belonged to eumelanin, which had obvious indole structure, and the structure of degradation products did not show much difference. The total antioxidant capacity and superoxide radical scavenging ability of melanin produced by trypsin treatment were strong. The melanin extracted by pancreatin had better ability to scavenge superoxide anion. The melanin extracted by pepsin showed stronger scavenging ability to 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals. The scavenging ability of hydroxyl radical of the degraded products of melanin were higher than that of melanin itself. The other degradation products of oxidative activity *in vitro* were weaker than that of melanin before degradation.**

**Keywords:** sea cucumber; melanin; degradation; antioxidant activity; structure.

***Corresponding author:** Aili Jiang, Department of Biochemical Engineering, Yantai University. No. 32, Qingquan Road, Laishan District, Yantai Shandong, China. Phone: +86 0535 688 1942. Email: jal9035@163.com

## Introduction

Sea cucumber contains many functional ingredients and has been used as tonic food in China for thousands of years. In recent years, the nutritional value and health functions of sea cucumber have attracted extensive attention of researchers. Many compounds have been studied including polysaccharides, peptides, phospholipids and glycolipids, glycosides (encephalosides), polyunsaturated fatty acids, phenols, triterpene glycosides, lectins and melanins [1-8].

Melanin is one of the main pigments responsible for colors in living cells, which is a ubiquitous heterogeneous polymer pigment widely distributed in various life forms and usually shows dark brown or black [9]. Melanin can be divided into indole-type animal melanin and catechol-type plant melanin. In addition, animal melanin can be divided into eumelanin and phaeomelanin. Eumelanin is black or brown pigment formed by oxidative polymerization of tyrosine, dihydroxyphenylalanine, dopamine, and tyramine, while phaeomelanin is slightly lighter than eumelanin and follows the same

synthetic pathway as eumelanin, which involves cysteine or glutathione [10].

Melanin could be used as healthy and multifunctional additive due to its natural color and antioxidant activity. Multiple antioxidant activity of melanin [11, 12] have been found as well as radiation protection [13], anti-tumor activity [14], anti-HIV activity [15], immune stimulation [16], and anti-inflammatory activity [17]. Melanin could protect microorganisms, such as bacteria and fungi, from the damage caused by heat, chemical substances such as heavy metals and oxidants, and biochemical stresses such as host defense against invasive microorganisms [18]. There is also a link between the form, morphology, and physiological characteristics of melanin and the reproductive behavior of animals [19].

At present, studies on melanin in China and abroad mostly focused on plants such as black sesame, black bean, and black rice, while researches on animal melanin mainly included squid ink, black bone chicken, and loach. Many studies have found that the ultraviolet absorption spectra of melanin extracted from different animals or plants are different [20, 21]. Moreover, the structure and physicochemical properties of melanin extracted by different methods may be slightly different. Little attention had been paid to sea cucumber melanin, as well as reliable method for qualitative analysis. Current research was focused on fresh sea cucumbers while most of the products sold in the market were dried sea cucumber.

Degradation of melanin into a soluble substance is the key to studying the functional activity and chemical composition of melanin. At present, the common degradation methods of melanin mainly include chemical oxidation degradation method, thermal pyrolysis gas phase mass spectrometry, etc. The chemical oxidation degradation method includes potassium permanganate oxidative degradation method and alkaline hydrogen peroxide decomposition method. In this paper, the continuous introduction of hydrogen peroxide into the reaction device was used to degrade sea cucumber melanin, which showed the advantages of easy control and non-foaming in the reaction process.

At present, there are few studies on the extraction of melanin from dried sea cucumber. Our previous studies found that melanin may bind to proteins in sea cucumber, and several proteases including trypsin, pancreatin, and pepsin were used to hydrolyze the body wall of sea cucumber, then the melanin was extracted according to the characteristics of alkali-soluble and acid precipitation. The structure of sea cucumber melanin was preliminarily identified by infrared spectroscopy (IR) and ultraviolet-visible spectroscopy (UV), and the antioxidant activities were compared between melanin and degraded melanin. The results should provide theoretical basis for the comprehensive utilization of sea cucumber as well as the follow-up research of melanin in sea cucumber.

## Materials and Methods

### Preparation of melanin

Dried sea cucumber was purchased from local market in Yantai, Shandong Province, China, and was soaked in the conventional way. 20 g of soaked sea cucumber was homogenized with deionized water in 1:10 (W/V) ratio. The pH was adjusted to the optimum of the protease. Three different types of proteases, Trypsin, pancreatin, and pepsin (Hongrunbaoshun Technology, Beijing, China), were added at 360 U/mL, respectively. After enzymatically hydrolyzed at the optimum temperature for 10 h, the hydrolysis solution was centrifuged at 8,000 rpm for 15 min, and the precipitate was dissolved with 0.1 g/L NaOH. The pH of supernatant was adjusted to about 2.0 to completely precipitate the melanin for half an hour. The crude melanin was obtained through centrifuging and vacuum drying. The alkali-soluble acid precipitation step was repeated three times to obtain refined sea cucumber melanin.

**Degradation of Melanin**

0.1 g refined sea cucumber melanin was mixed with 200 mL of 0.1 M NaOH solution in flask. 3.4 mL of 30% $H_2O_2$ was pumped into the solution at a rate of 0.18 mL/min, then stirred for 12 h at 90°C. When the reaction finished, the flask was immediately cooled to room temperature with running water. After centrifuged at 4,000 rpm for 5 min, the supernatant was poured into a glass crucible and dried to constant weight at 100℃ to obtain the degraded melanin product.

**Structural Analysis**

**(1) Infrared Spectrum (IR):**

2 mg refined melanin and degraded melanin were grinded with 50 mg potassium bromide powder under infrared lamp for 1-2 min, respectively. The samples were pressed into transparent sheets by 10 T pressure for 5 min. The sheets were transferred to 100N Fourier Transform Near Infrared Spectrometer (Suzhou Shimadzu Instrument, Suzhou, Jiangsu Province, China), scanned from 400 to 4,000 nm to obtain the infrared spectrum of sea cucumber melanin.

**(2) Ultraviolet spectrum (UV):**

The refined melanin and degraded melanin were fully dissolved in dimethyl sulfoxide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), respectively, and prepared into 0.2 g/L melanin solution. The melanin solution was transferred to TU-1901 Dual Beam Ultraviolet-Visible Spectrophotometer (Beijing General Instruments, Beijing, China), and scanned from 190 to 900 nm to obtain the ultraviolet spectrum of melanin.

**Antioxidant activity assay**

The sea cucumber melanin and their degradation product were sufficiently dissolved in dimethyl sulfoxide to prepare a melanin solution with mass concentrations of 0.20, 0.40, 0.60, and 0.80 g/L, respectively.

**(1) Total antioxidant capacity:**

According to Pilar *et al*. [22], 1 mL melanin solution of different concentrations was mixed with 1 mL of 3M sulfuric acid solution, 1 mL of 28 mM trisodium phosphate solution, 1 mL of 4 mM ammonium molybdate solution, and 1 mL of distilled water. The solution was shaken at 95°C for 30 mins. The reaction liquid was then cooled to room temperature in cold water bath, and the absorbance was measured at 695 nm. Distilled water was used instead of the sea cucumber melanin solution as a blank reference.

**(2) Superoxide radical scavenging ability:**

The superoxide radical scavenging ability of melanin was determined by using pyrogallol method [23]. 5.4 mL of 50 mM Tris-HCL buffer (pH 8.2) was mixed with 0.2 mL of melanin solution and kept at 25°C for 10 minutes. Then 0.3 mL of pyrogallol solution (preheated at 25°C) was added immediately. After reacted for 3 minutes, 0.1 mL of 8 M hydrochloric acid solution was added to terminate the reaction. Melanin solution was replaced by distilled water in control group and pyrogallol solution was substituted by distilled water in reference group. The absorbance (A) was measured at 420 nm using distilled water as blank. The scavenging activity (SA) of radical was calculated by the following equation:

$$SA\ (\%) = [(A_{sample} - A_{reference}) / A_{control}] \times 100\%$$

Where $A_{sample}$ is the absorbance of the sample group; $A_{reference}$ is the absorbance of the reference group; $A_{control}$ is the absorbance of the control group.

**(3) Hydroxyl radical scavenging ability:**

The hydroxyl radical scavenging ability of melanin was determined by using salicylic acid method [24]. 1 mL of sea cucumber melanin solution of different concentrations were mixed with 1 mL of 4 mM $FeSO_4$ solution and 1 mL of 0.02% $H_2O_2$ solution. The mixtures were allowed to react for 10 minutes before adding 1 mL of 6 mM salicylic acid solution and reacted for 30 minutes. The absorbance (A) was measured at 510 nm by using distilled water as blank. Sea cucumber melanin solution was replaced by dimethyl sulfoxide in reference group, and the $H_2O_2$ solution was substituted by dimethyl sulfoxide in control

group. The scavenging activity (SA) of hydroxyl radical was calculated by the following equation:

$$SA\ (\%) = [1 - (A_{sample} - A_{control}) / A_{reference}] \times 100$$

Where $A_{sample}$ is the absorbance of the sample group; $A_{reference}$ is the absorbance of the reference group; $A_{control}$ is the absorbance of the control group.

**(4) DPPH radical scavenging ability:**

According to Chen *et al*. [25], 0.5 mL of sea cucumber melanin solution was mixed with 3 mL of 0.1 mM 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Nanjing Odofoni Biotechnology, Nanjing, Jiangsu Province, China) solution and 1 mL of distilled water. In reference group, DPPH solution was substituted by absolute ethanol, and sea cucumber melanin solution was replaced by distilled water. DPPH solution was substituted by absolute ethanol in control group. The reaction was carried out in dark for 30 minutes. The absorbance (A) was measured at 517 nm using anhydrous ethanol as blank. The scavenging activity (SA) of radical was calculated by the following equation:

$$SA\ (\%) = [1 - (A_{sample} - A_{control}) / A_{reference}] \times 100$$

Where $A_{sample}$ is the absorbance of the sample group; $A_{reference}$ is the absorbance of the reference group; $A_{control}$ is the absorbance of the control group.

**Statistical Analysis**

All the experiments were carried out in triplicate, and the results were shown as mean ± standard deviation. Statistical analysis was conducted using one-way ANOVA followed by LSD multiple-range test by using IBM SPSS Statistics software (version 19.0 for Windows).

## Results

**Infrared spectrum of melanin**

**(1) Melanin before degradation:**

The infrared spectra of melanin extracted by three different enzymes were shown in Figure 1. The absorption peak near the wavelength of 2,920/cm was caused by the stretching vibration of C-H, that was, the resonance absorption peaks of -CH and -$CH_2$. The absorption near 1,460/cm was the bending vibration of aliphatic C-H, which proved that there were alkanes in sea cucumber melanin. The strong absorption peak near 1,650/cm was most likely the stretching vibration absorption of C=C or COO- in aromatic ring, as well as the stretching vibration of C=O in amide band I. The olefins in near 3,070/cm C-H stretching vibration melanin skeleton indicated the existence of olefins. It showed that indole ring occupied a large proportion in the structure of melanin [26]. The absorption intensities of amide structure and cyclic olefin structure in infrared spectra were large, which indicated that these two structures were the main structures of melanin. Moreover, a wide peak of strong resonance absorption appeared in region 3,600-3,100/cm, which was generated by the stretching vibration of N-H in O-H and amide. The absorption of C-O and C-N in the 1,300-1,000/cm region at 1,030/cm and the absorption of C-H stretching and O-H deformation vibration in the 1,232-1,274/cm region at 1,250/cm proved that the melanin skeleton structure contained hydroxyl groups. The characteristic absorption peaks in the range of 1,030-1,050/cm might be due to C-O stretching vibration. The characteristic absorption peaks of sea cucumber melanin obtained by pancreatin and trypsin were very close to each other, but different in absorption intensity. Compared to the infrared spectra of sea cucumber melanin obtained by pepsin, the shape of peak spectra was slightly different.

**(2) Melanin after degradation:**

Figure 2 was the infrared spectrum of degradation products of melanin extracted after the enzymatic hydrolysis of three different enzymes. Compared to the spectrum of melanin, there were many similarities and some structural characteristics differences. In the range of 4,000-3,300/cm, the absorption peaks of degradation

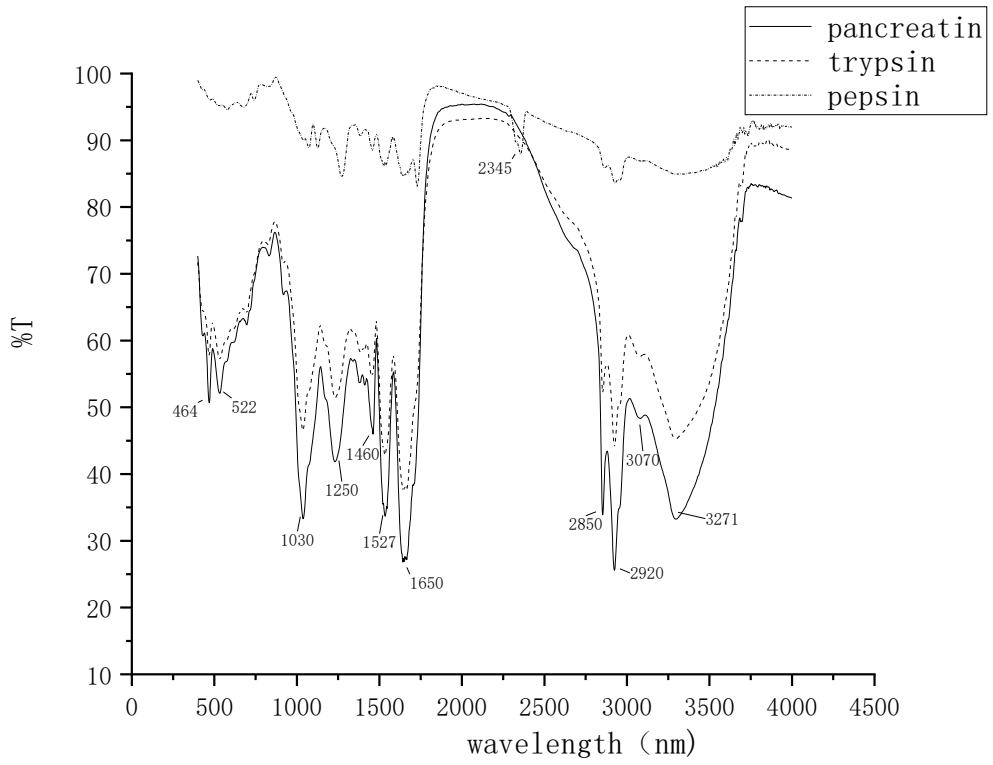


**Figure 1.** Infrared spectra of melanin extracted by three enzymes.

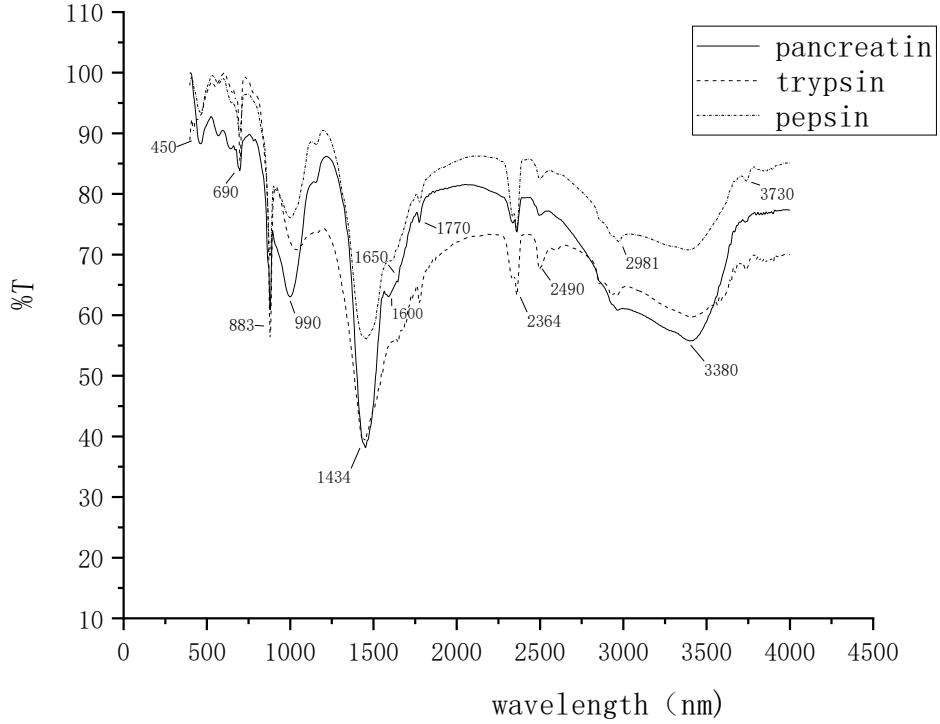


**Figure 2.** Infrared spectrum of melanin degradation products extracted by three enzymes.

products were wider and stronger than that of melanin, which might be the exposing of N-H and O-H groups in melanin caused by oxidative degradation and the forming of strong intermolecular hydrogen bonds. However, the structure of different degradation products was basically the same. Around 3,000-2,800/cm, symmetric and antisymmetric stretching vibration absorption peaks of methylene existed in sea cucumber melanin and its degradation products, which was speculated as the skeleton structure and structural fragments of melanin in this area. In the range of 2,300-1,800/cm, there was no absorption peak of sea cucumber melanin and its degradation products. The absorption peaks near 1,700-1,500/cm region at 1,600/cm and 1,650/cm could be considered as the stretching vibration of C=O in amide band I and the deformation vibration of N-H in amide band II, respectively. Both melanin and degradation products had peaks in this band, indicating that carbonyl-containing substances were produced during degradation. It was presumed that the degradation caused the main structure to be broken, and the -OH attached to the cyclic olefin was exposed. Below 1,000/cm, there were mainly the angular vibration beyond the plane of CH, NH, C=O, and other structures. The absorption of melanin degradation products in this interval was stronger than that of melanin, and there were more peaks, which indicated that the dense outer structure of sea cucumber melanin was interrupted after degradation, and organic compounds containing hydrocarbons, aldehydes, ketones, and amides were produced. In the degradation products, the absorption peak near 1,450/cm was the bending vibration of aliphatic C-H, while the intensity of absorption peak of olefin was weak. The differences in infrared spectra of the degradation products of sea cucumber melanin extracted after the enzymatic hydrolysis of three different protease were not significant, indicating that the structures of the three melanins were basically consistent after degradation.

**Ultraviolet spectrum of melanin**

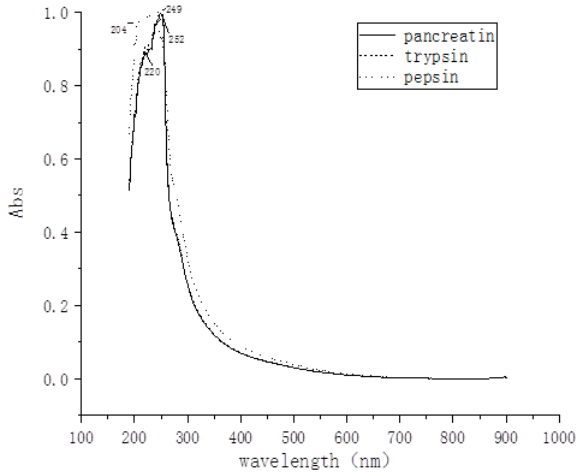


**Figure 3.** Ultraviolet spectra of melanin extracted by three different enzymes.

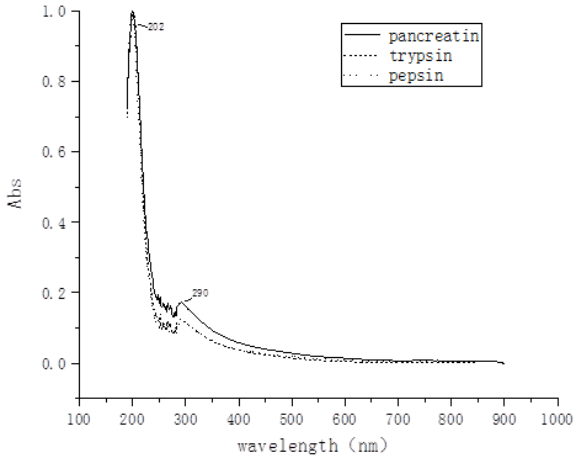


**Figure 4.** Ultraviolet spectra of melanin degradation products extracted by three different enzymes.

Figure 3 and Figure 4 showed the ultraviolet spectra of melanin and its degradation products extracted after the enzymatic hydrolysis of three different enzymes. The three types of sea cucumber melanin showed an absorption peak around 250 nm in the ultraviolet region, which might be due to the presence of aromatic rings in the melanin. There was no obvious absorption peak in the visible region. The absorption peak of the three types of melanin gradually decreased with the increase of wavelength, which was a typical ultraviolet absorption image of melanin [27]. The ultraviolet absorption peaks of the three degradation products were mainly

concentrated at 290 nm, which might be caused by the presence of olefins in the structure. However, there were also an absorption peaks at 250 nm, which might be caused by the incomplete degradation of the main structure of melanin. Three degradation products also had absorption peaks at 200 nm, which might be the absorption of aldehydes. The ultraviolet spectra of the three degradation products showed little difference, indicating that the structures of the three degradation products were basically the same.

**Antioxidant activity assay**

**(1) Total antioxidant capacity:**

The antioxidant activities were expressed in terms of absorbance at 695 nm. According to the research by Wang [28], melanin had good heat resistance and would not degrade under the conditions of this experiment. As showed in Figure 5, the total antioxidant capacity of melanin increased with the increase of concentration, while the degradation products extracted by pancreatin and pepsin were less affected by concentration and were significantly lower than that before degradation (P<0.05). The total antioxidant capacity of melanin extracted by pepsin showed no significant difference before and after degradation (P>0.05).

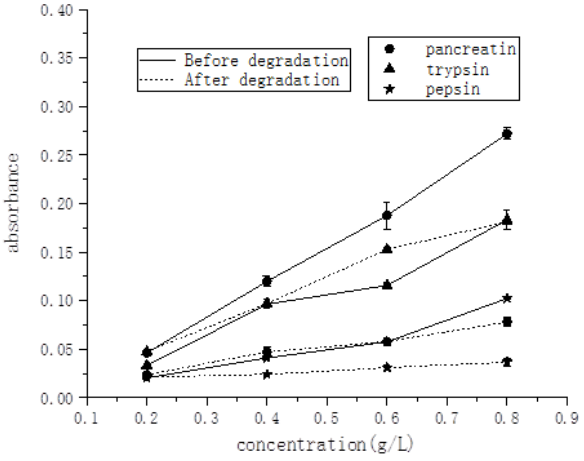


**Figure 5.** Comparison of total antioxidant capacity of melanin extracted by three different enzymes before and after degradation.

**(2) Superoxide radical scavenging ability:**

Figure 6 was the comparison of the scavenging ability of superoxide radical of melanin extracted by three different enzymes before and after degradation. The scavenging abilities of melanin were significantly higher than that of their degradation products (P<0.05). The scavenging abilities of melanin extracted by pepsin increased with concentration, while those of melanin extracted by pancreatin and trypsin showed no significant change with concentrations. The scavenging abilities of the degraded melanin extracted by pancreatin and pepsin did not change significantly with the concentration (P>0.05), while the scavenging ability of the degraded melanin extracted by trypsin was significantly higher than that of the other two (P<0.05), and increased with the increasing of concentration.

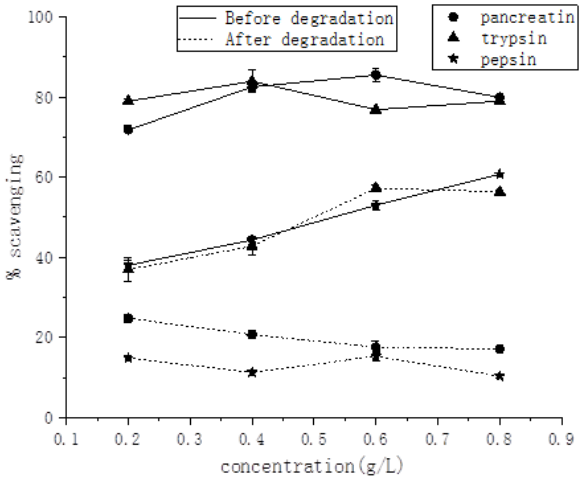


**Figure 6.** Comparison of superoxide radical scavenging activities of melanin extracted by three different enzymes before and after degradation.

**(3) DPPH radical scavenging ability:**

As showed in Figure 7, the scavenging abilities of the three degradation products changed little with the concentration (P>0.05), and the scavenging abilities were about 40%. The scavenging abilities of melanin extracted by pancreatin and pepsin increased with the increasing of concentration and reached the highest at 0.8 g/L as 60.23% and 70.49%, respectively.

**(4) Hydroxyl radical scavenging ability:**

As showed in Figure 8, the scavenging abilities of

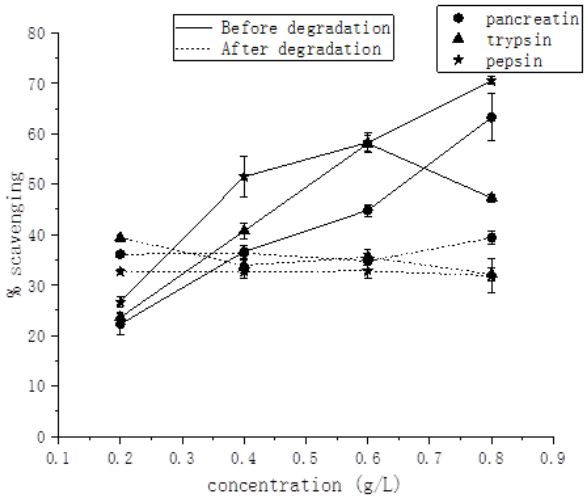


**Figure 7.** Comparison of DPPH radical scavenging abilities of melanin extracted by three different enzymes before and after degradation.

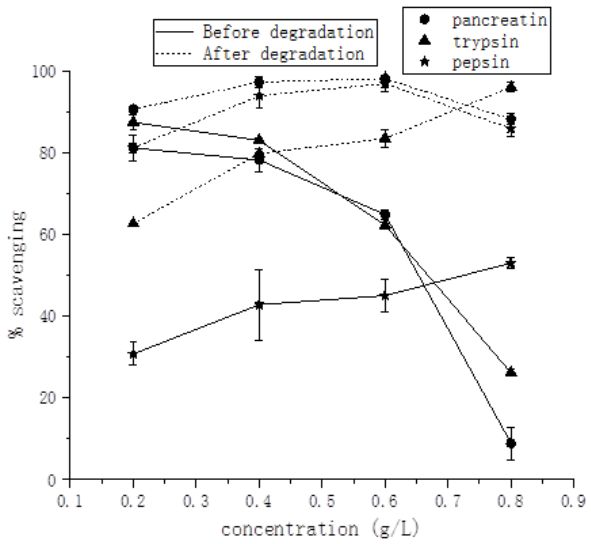


**Figure 8.** Comparison of hydroxyl radical scavenging abilities of melanin extracted by three different enzymes before and after degradation.

degraded melanins extracted by pancreatin and pepsin were higher than that before degradation with pepsin showed the significant difference ($P<0.05$), which could exceed 80% due to the breakdown of the main structure of melanin and the exposure of -OH on cyclic hydrocarbons during degradation. The scavenging abilities of melanins extracted by pancreatin and trypsin decreased with the increasing of concentration, the scavenging ability of melanin extracted by pepsin showed the opposite trend. The scavenging abilities of degraded melanin extracted by pancreatin and pepsin fluctuated slightly with concentration, and the scavenging ability of degraded melanin extracted by trypsin increased with the increasing of concentration.

## Discussion

Compared to the UV-visible absorption spectrum of melanin in processing waste liquor of sea cucumber [29], the melanin extracted from dried sea cucumber in this study had no obvious absorption peak at 310 nm before and after degradation. This might be related to repeated boiling and curing of the sea cucumber during the drying process, which destroyed the melanin precursor 5,6-dihydroxyindole (DHI). The results showed that the traditional dry method of sea cucumber had some effects on the structure and activity of melanin.

The similar infrared spectrum and UV-Vis spectra of the degraded melanin indicated that the structures of degradation products were not much different. Degradation caused some groups to be exposed such as N-H and O-H groups in melanin and formed strong intermolecular hydrogen bonds. After degradation, the dense outer structure of sea cucumber melanin was interrupted to produce in organic compounds containing hydrocarbons, aldehydes, ketones, and amides.

Infrared spectrum showed that C=O stretching vibration in COOH near 1,610/cm was stronger when enzymatically digested with pepsin than that of the other two enzymes, which might be due to the decarboxylation of melanin occurred during enzymatic hydrolysis at the optimum pH of pepsin (pH 2-3). This phenomenon was consistent with the decarboxylation of squid ink in strong acid in the literature [30].

The melanin extracted by the three different enzymes had little difference in the scavenging

ability of superoxide radical. It might be related to the monomeric DHI and DHICA (5,6-dihydroxydecanoic acid) in melanin, which could catalyze the conversion of $O^{2-}$ to $H_2O_2$ and $O_2$ [31]. The ability of melanin to scavenging hydroxyl radicals was weaker than that of its degradation products, which might be the reason for the degradation of more hydroxyl groups.

## Conclusion

This study reported the structure and properties of the melanin extracted from dried sea cucumber. Three types of melanins obtained with enzymatic hydrolysis of three different proteases in this study all had obvious indole structure and belonged to eumelanin. Three enzymes had affected the structure and functional groups of melanin. The degradation of $H_2O_2$ interrupted the carbon chain and main structure of melanin, and produced alkanes, hydroxyl groups, etc., thus improving the scavenging ability of degradation products to hydroxyl radicals. After the degradation of melanin, the scavenging DPPH radical ability of degradation products changed little with concentration ($P>0.05$). The results showed the sea cucumber melanin had obvious free radical scavenging activity and could be used as a UV absorber, as well as an antioxidant.

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