

Physicochemical Properties of Collagen from the Bone of *Harpadon nehereus* and Its Protective Effects against Angiotensin II-Induced Injury in Human Umbilical Vein Endothelial Cells

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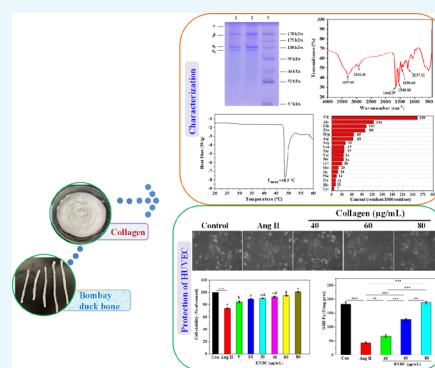
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ABSTRACT: The physicochemical characterization of a new collagen from the bone of *Harpadon nehereus* (HNBC) and its protective effects against Ang II-induced injury of human umbilical vein endothelial cells (HUVEC) were investigated. The triple helix of HNBC analyzed by SDS-PAGE was formed as $(\alpha_1)_2\alpha_2$, belonging to type I collagen. UV spectra showed that HNBC had a maximum absorbance at 230 nm. FTIR spectra indicated the triple helical structure and activity of HNBC. The high solubility of HNBC was observed in the low pH ranges (≤ 4) and NaCl concentrations ($\leq 2\%$, w/v). The maximum transition (T_m) of HNBC was determined to be 48.5 °C. The amino acid composition analysis showed that glycine, glutamic acid, proline, and alanine were the abundant amino acids available in HNBC. HNBC showed free radical (DPPH and OH \cdot) scavenging activities in the tested concentrations (0.5–6 mg/mL). In addition, HNBC could effectively protect against Ang II-induced injury of HUVEC by increasing the activities of antioxidant enzymes, such as CAT, SOD, and GSH-Px. Overall, collagen from the bone of *H. nehereus* has promising prospects in functional food and biomedical industries.



1. INTRODUCTION

Hypertension is the most common chronic disease and a major risk factor for cardiovascular diseases. At present, the complex mechanism of hypertension remains unclear. However, research has indicated that oxidative stress caused by reactive oxygen species (ROS) plays a crucial role in the development of vascular dysfunction.^{1–3} Angiotensin II (AngII), an important component of the renin–angiotensin system, can promote vasoconstriction and induce oxidative stress, resulting in vascular dysfunction.^{4,5} The antioxidant enzymes, such as catalase (CAT), superoxidase dismutase (SOD), heme oxygenase 1 (HO-1), and glutathione peroxidase (GSH-Px), play a key role in oxidative stress modulation.⁶ Recently, it was reported that some natural compounds could protect against Ang II-induced hypertensive injury by increasing the antioxidant enzyme activities.⁷ The oligopeptide LSGYGP could effectively protect human umbilical vein endothelial cells (HUVECs) against Ang II-induced injury through increasing SOD expression.⁸ Coenzyme Q10 upregulated the expression of SOD and GSH-Px, thereby improving angiotensin II-induced oxidative stress and endothelial dysfunction.⁹ Therefore, effective inhibition of AngII-induced oxidative stress is the main focus of hypertension prevention and treatment.

Collagen is the most abundant protein in the connective tissue of animal skin and bone. Because of its excellent biocompatibility, antioxidant activity, and immunomodulatory activity, collagen has been widely used in food, biomedicine,

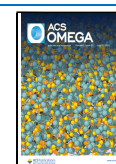
pharmacy, and other fields. Their applications include immune enhancement, tissue engineering, wound healing, and skin regeneration.¹⁰ Many reports have confirmed that collagen has good antioxidant activity and can effectively reduce the level of intracellular oxidative stress.^{11–13} The collagens from *Lophius litulon* skin and *Nibeia japonica* swim bladders showed good free radical scavenging activities and positive effects on wound healing.^{12,14} In addition, collagen peptides, the hydrolyzed products of collagen, also have a wide range of biological activities, such as antioxidant, antimicrobial, antitumor, and antihypertensive activity.^{15–17} The collagen peptides from skate (*Raja kenoei*) could enhance insulin sensitivity via attenuation of oxidative stress and inflammation in the liver.¹⁷ Therefore, the shortage of collagen resources has become increasingly prominent with its comprehensive application.

Traditionally, most of the collagen is derived from terrestrial mammals such as cow and pig skins. However, the possibility of mammalian diseases being transmitted to human beings has caused restrictions on the use of mammalian collagen. Consequently, much attention has been paid to the alternative

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sources of collagen, especially from marine organisms, because of its high availability, no risk of disease transmission, and high biological value. Collagen can be produced from the byproducts of seafood processing, such as the skin, bone, scales, swim bladder, and fins. Collagens from several marine organisms such as the spines and skulls of skipjack tuna,¹⁸ the skin of the giant croaker,¹⁹ the swim bladders of miyu croakers,²⁰ whale shark cartilage,²¹ and body walls of sea cucumbers^{22–25} have been extracted and characterized.

Bombay duck (*Harpadon nehereus*) is widely distributed in the southern Yellow Sea, the East China Sea, and the estuary waters of the South China Sea. It is the main edible marine fish in the coastal areas of China because of its tender meat, large output, and low price. Bombay duck has no scales and swim bladder, so bones are the main byproducts of its food processing industry. Bombay duck bones contain collagen, but its properties have not been studied. Therefore, this study was undertaken to isolate pepsin-soluble collagen from the bone of bombay duck (HNBC) and examine its physicochemical properties. In addition, we evaluated the protective effect against Ang II-induced injury of HUVEC for the first time. This study is expected to lay a foundation for the application of bone collagen in the prevention and treatment of hypertension and provide new ideas for its application in biomedicine.

2. MATERIAL AND METHODS

2.1. Materials. Bombay duck (*Harpadon nehereus*) was purchased from the seafood market of Zhoushan city. The protein marker, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Dulbecco's modified eagle's medium (DMEM) were purchased from Sigma-Aldrich (Shanghai, China). The assay kits including malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), heme oxygenase 1 (HO-1), and glutathione peroxidase (GSH-Px) were from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). HUVEC was purchased from Bena Culture Collection Co., Ltd. (Shanghai, China). All other reagents were analytical grade.

2.2. Extraction of Collagen from the Bone of *Harpadon nehereus*. The bone of *Harpadon nehereus* was treated and cut into pieces. The samples were soaked for 48 h with 0.1 M NaOH solution at a ratio of 1:20 (w/v) to remove the noncollagen substances. The fish bones were washed until neutral and then soaked in 20 times the volume of 0.5 M EDTA for 48 h to remove metal ions. Then, the samples were stirred in 15% isopropanol solution for 48 h to remove the fat. Finally, the precipitate after centrifugation was used to extract the fishbone collagen in a 30-fold volume of 0.5 M acetic acid (containing 0.5% pepsin) for 24 h. After centrifugation for 10 min at 4 °C and 12 000 r/min, NaCl was added into the supernatant with the final concentration of 0.9 M. After 6 h, the samples were centrifuged for 10 min at 12 000 r/min, and the precipitate was dissolved in 0.5 M acetic acid. The sample solution was then dialyzed to neutrality with deionized water, and the collagen from the bone of *Harpadon nehereus* (HNBC) was obtained after freeze-drying.

2.3. SDS-PAGE. An amount of 0.5 mg of HNBC was dissolved in 1 mL of 0.1 M acetic acid solution. The sample solution was mixed with the loading buffer at a ratio of 4:1 (v/v) and boiled in a water bath for 5 min. After centrifuging at 12 000 r/min for 5 min, 10 μ L of supernatant was taken and mixed with the gel composed of 8% separating gel and 5% stacking gel for separation. Electrophoresis was conducted at 80 and 120 V in sequence. After the separation, the gel was

soaked in the staining solution for 40 min for staining and then decolorized with eluent (30% methanol and 10% acetic acid).

2.4. Collagen Ultrastructure. The freeze-dried collagen was taken and sputter coated with gold using a sputter coater. The morphology of the collagen was observed at $\times 100$, $\times 200$, $\times 500$, and $\times 1000$ by scanning electron microscopy (SEM) using Hitachi TM-100.

2.5. UV-vis and FTIR Spectra. An amount of 5 mg of collagen samples was dissolved in 10 mL of 0.5 M acetic acid. UV-vis spectra analysis was implemented using a spectrophotometer UV-1800 (Mapada Instruments Co., Ltd., Shanghai, China) at a wavelength of 220–400 nm. The collagen samples were mixed and ground with KBr at a ratio of 1:50 (w/w) under dry conditions. FTIR spectra were collected from 4000 to 500 cm^{-1} at a resolution of 0.482 cm^{-1} by the spectrophotometer Nicolet 6700 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.6. Solubility. An amount of 5 mL of 0.5 M acetic acid was adjusted with either 6 M HCl or NaOH to obtain the final pH ranging from 1 to 10. An amount of 4 mL of the adjusted solution was added to 10 mg of HNBC and stirred at 4 °C for 60 min. The supernatant was used to measure the protein content after centrifugation at 4 °C. The relative solubility is the ratio of the absorbance of each sample to the highest absorbance.

HNBC was dissolved in 0.5 M acetic acid to prepare a collagen solution with a final concentration of 4 mg/mL. NaCl was added into the collagen solution to obtain the final concentration of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, and 8.0% (w/v). After being stirred at 4 °C for 30 min, the mixture was centrifuged at 12 000 r/min for 15 min. The supernatant was collected and used to measure the protein content. The relative solubility is the ratio of the absorbance of each sample to the highest absorbance.

2.7. Thermal Stability. HNBC was dissolved in 0.05 M acetic acid at 1:40 (w/v) and stirred at 4 °C for 48 h. The sample solution was weighed on aluminum pans, sealed, and measured for the maximum transition temperature (T_m). The enthalpy of HNBC was recorded from 20 to 60 °C using 0.05 M acetic acid as a reference by DSC Q2000 (Mettler Toledo Technology Co., Ltd., Shanghai, China).

2.8. Analysis of Amino Acid Composition. An amount of 0.5 mg of HNBC was added into 6 mL of 6 mol/L of HCl and hydrolyzed at 110 °C for 24 h. The amino acid composition in the hydrolyzed solution was determined on an automated HITACHI L8900 amino acid analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.9. Antioxidant Activity. The DPPH and OH \bullet scavenging activity of HNBC was performed according to methods in our previous report.²⁶

2.10. Cell Culture. HUVEC was seeded into DMEM and incubated in 5% CO₂ at 37 °C. DMEM contains 10% fetal bovine serum and 1% penicillin–streptomycin.

2.11. Cell Viability Assay. HNBC was dissolved in DMEM containing 0.1% DMSO. The HUVECs were seeded in 96-well plates (1×10^4 cells/well) and treated with HNBC (5, 10, 20, 40, 60, and 80 μ g/mL) for 4 h. Subsequently, Ang II (1 μ M) was added to the culture medium. After incubation for 24 h at 37 °C, the cell culture was removed, and MTT solution (1 mg/mL, 200 μ L) was added to each well for 4 h. After removing the supernatants, 200 μ L of DMSO was added to measure the absorbance at 540 nm.

2.12. Cell Morphology Observation. The cells were inoculated in 6-well plates (1×10^5 /well), pretreated with HNBC (40, 60, and 80 $\mu\text{g}/\text{mL}$) for 4 h, and then incubated with Ang II (1 μM) for 24 h. The cell morphology was observed and photographed under an inverted light microscope immediately.

2.13. Determination of MDA, CAT, SOD, and GSH-Px. The cells were cultured and collected as described above. After being washed three times with PBS, the cells were lysed with cell lysates. The supernatant fractions collected by centrifugation were used to determine the activities of antioxidant enzymes (SOD, GSH-PX, and CAT) and the content of MDA according to the kit instructions.

2.14. Statistical Analysis. All analyses were carried out on triplicate samples. Data were analyzed using Microsoft Excel 2010 (Redmond, WA, USA) and shown as mean \pm standard deviation.

3. RESULTS AND DISCUSSION

3.1. SDS-PAGE of HNBC. The SDS-PAGE pattern of extracted collagen from the bone of *Harpadon nehereus* (HNBC) and collagen type I from calf skin was shown in Figure 1. HNBC consisted of two α -chains (α_1 and α_2) at a

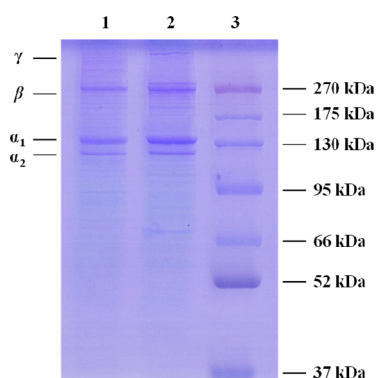


Figure 1. SDS-PAGE pattern of HNBC. Lane 1: HNBC. Lane 2: type I collagen from calf skin. Lane 3: protein marker.

ratio of approximately 2:1, which was similar to the standard collagen type I isolated from calf skin with the molecular structure of $(\alpha_1)_2\alpha_2$. The pattern suggested that the collagen from the bone of *Harpadon nehereus* was characterized as type I collagen. HNBC also contained high molecular weight (MW) components, including β -components, but not γ -components. According to reports, γ -components were observed in acid-soluble collagen (ASC) but did not appear in pepsin-soluble collagen (PSC) due to degradation of it by pepsin.^{27,28} The collagen from the bone of *Harpadon nehereus* was consistent with those of bone collagens from the leather jacket fish (*Odonus niger*),²⁹ bighead carp,³⁰ Spanish mackerel (*Scomberomorus niphonius*),²⁷ and mackerel (*Scomber japonicus*)³¹ but significantly different from that in the cartilage of Amur sturgeon (*Acipenser schrenckii*)³² and shark.³³

3.2. Ultrastructure of HNBC. The scanning electron micrograph (Figure 2) of HNBC was obtained at $\times 100$, $\times 200$, $\times 500$, and $\times 1000$. A soft white sponge with a tight, uniform, and regular reticulated pore structure was observed. HNBC exhibits dense and sheet-like films under SEM at $\times 100$ and $\times 200$ (Figure 2A and 2B), the surface of which is partially curled and wrinkled. As the multiple increases, the fibrillar

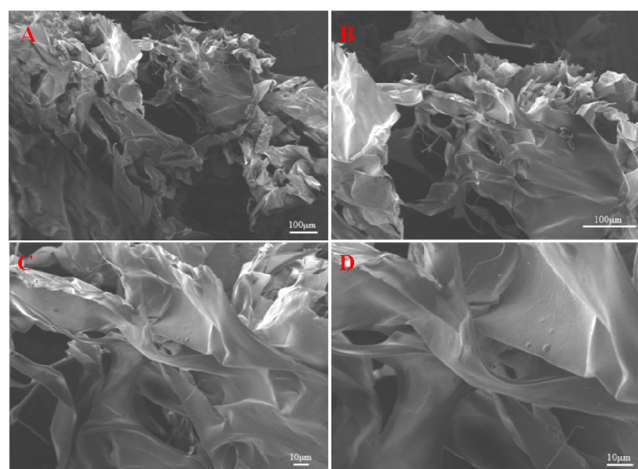


Figure 2. SEM images of HNBC from bones of *Harpadon nehereus*. A: $\times 100$. B: $\times 200$. C: $\times 500$. D: $\times 1000$.

structure of HNBC with interconnected network pore configurations can be observed at $\times 1000$, which was similar to that of collagen from the bone of Spanish mackerel.²⁷ It was reported that the interconnectivity of fibrous and sheet-like film structures of collagen played an important role in its use in wound healing, cell growth, and tissue formation. Therefore, HNBC from *Harpadon nehereus* has great potential for application in the field of biomedicine and biomaterials.

3.3. UV and FTIR Spectra. UV and FTIR are the important methods for identifying the secondary structure of collagen. As presented in Figure 3A, the maximum absorption peaks of HNBC appeared at 230 nm, which was similarly reported for ASC (231.3 nm) and PSC (231.8 nm) from sheep bone.³⁴ The strong absorption peak at 230 nm was due to the presence of C=O, -COOH, and CONH₂ in the polypeptide chains of HNBC. Theoretically, the amino acids such as tryptophan and tyrosine exhibit strong absorption at 280 nm. HNBC exhibited no distinct absorption peak at 280 nm, suggesting that there were very low amounts of these amino acids in HNBC.

The absorption peaks in the FTIR spectra of type I collagen commonly exhibited five amide bands: amide A, B, I, II, and III. As shown in Figure 3B, the absorption peak at 3297.68 cm^{-1} of HNBC was the amide A band, which is due to N-H stretching vibration and hydrogen bonds. According to the reports, when N-H participates in the formation of a hydrogen bond, the wavenumber of its stretching vibration could move from 3400 to 3440 cm^{-1} to $\sim 3300 \text{ cm}^{-1}$.^{20,35} The 2930.30 cm^{-1} of HNBC was the amide B band, which is related to the asymmetrical stretch of CH₂. The 1642.57 cm^{-1} band of HNBC belongs to the amide I band, which has strong absorbance in the 1600–1700 cm^{-1} range. The amide I band is related to the stretching vibrations of C=O groups, which participate in the formation and maintenance of the triple helical structure of collagen.³⁶ The amide II band is related to N-H bending and C-N stretching vibrations, which generally occur in the 1550–1600 cm^{-1} range. However, a shift to lower wavenumbers will be observed when it participates in the formation of hydrogen bonds. The amide II band of HNBC was observed at 1549.04 cm^{-1} , which is consistent with the theoretical results. Amide III (1220–1320 cm^{-1}) is related to C-N stretching vibration and N-H deformation.³¹ When the absorption ratio between amide III and the 1453–1455 cm^{-1}

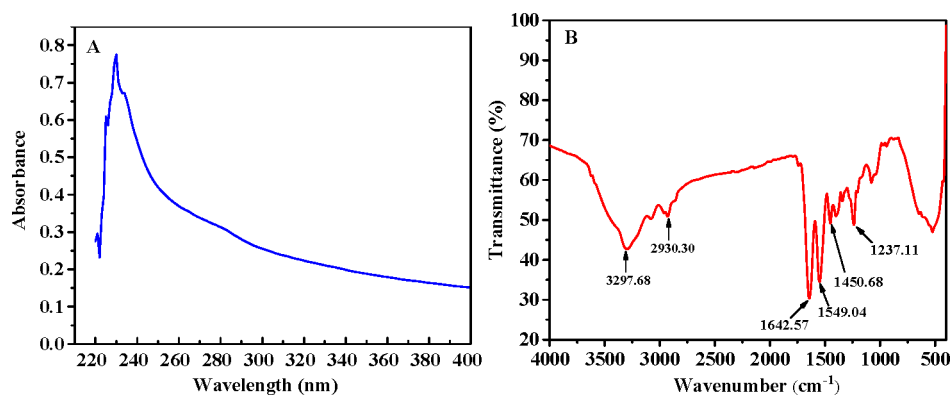


Figure 3. UV (A) and FTIR (B) spectra of HNBC isolated from *Harpadon nehereus* bone.

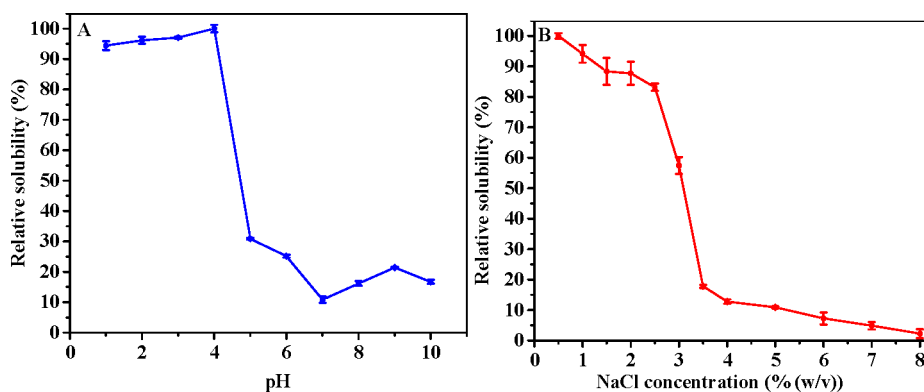


Figure 4. Solubilities of HNBC from bone of bombay duck (*Harpadon nehereus*) at different pH (A) and NaCl concentrations (B). All values were mean \pm SD with triplicate results.

band is 1, a complete triple helix structure is indicated.³⁷ HNBC from the bone of *Harpadon nehereus* showed amide III at 1237.11 and 1450.68 cm^{-1} bands with an absorption ratio of 0.993, suggesting that HNBC isolated from *Harpadon nehereus* bone maintained the triple-helical structure.

3.4. Effect of pH and NaCl Concentration on Collagen Solubility. As shown in Figure 4A, HNBC was easily solubilized in the low pH range from 1 to 4. However, when the pH was greater than 4, the solubility of HNBC decreased sharply with the increase of pH. The solubility of HNBC reached a minimum at pH 7.0 and then began to increase slowly as the pH continued to increase. Pepsin-soluble collagen (PSC) from the cartilages of brown-banded bamboo shark (*Chiloscyllium punctatum*)³³ and the bone of Spanish mackerel²⁷ showed similar results. However, the bone collagen from bighead carp (*Hypophthalmichthys nobilis*) exhibited maximum and minimum solubility at pH 6 and 9, respectively.³⁰ It is known that a protein has the lowest solubility in buffer at its isoelectric point (pI), and the solubility is increased when the pH is lower or higher than pI. Therefore, the differences in the solubility of different collagens by pH may be attributed to the differences in the pIs of collagens.

Figure 4B showed that the solubility of HNBC remained high (>88%) in the presence of NaCl lower than 2% (w/v) and then decreased sharply with the increase of NaCl concentration. The solubility of HNBC remained at a low level after NaCl concentration exceeded 4% (w/v). The solubilities of PSC from the bone of Spanish mackerel (*Scomberomorus niphonius*) and bighead carp (*Hypophthal-*

michthys nobilis) exhibited similar trends.²⁷ Theoretically, the increase of ion concentration can lead to the decrease of protein solubility by enhancing the hydrophobic–hydrophobic interaction and the competition of ionic salts for water.^{20,27} Therefore, the solubility of HNBC decreased with increasing NaCl concentration, which is consistent with the protein “salting-out” properties.

3.5. Thermal Behaviors. The maximum transition temperature (T_m) of fiber is commonly used to evaluate the thermal stability of collagen. The hydrogen bonds are gradually broken during the transformation of the α_1 chain into a random coil structure with increasing temperature.³⁸ Collagen denaturation occurs with half of the triple helix degradation when the temperature is raised to T_m .²⁵ T_m of HNBC was 48.5 °C (Figure 5), which was higher than that of collagens from the bighead carp bone (36.4 °C),³⁰ black drum bone (34.9 °C),²⁸ sheepshead bone (34.5 °C),²⁸ and the cartilages of *Chiloscyllium punctatum* (35.98 °C).³³ It was reported that the T_m value of collagen from different sources was related to the content of imino acids and normal habitat temperature.³⁹ Generally, collagens from organisms with a high habitat temperature exhibited better thermal stability due to the higher amounts of imino acids. Many fish collagens had lower T_m than mammalian collagens. However, the relatively high T_m of HNBC suggested the high heat resistance and good structural stability of the collagen from the bone of bombay duck, which may be beneficial for its use as potential substitutes for mammalian collagens.

3.6. Amino Acid Composition. The amino acid composition of HNBC was measured and expressed as

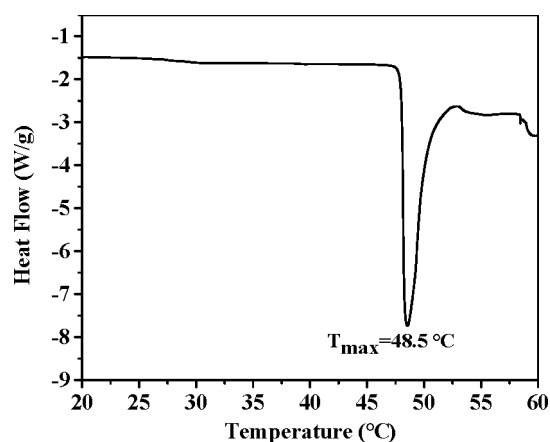


Figure 5. Typical DSC thermograms of HNBC from the bone of *Harpadon nehereus*.

residues/1000 total amino acid residues. As shown in Figure 6, HNBC was rich in glycine (Gly, 253 residues), alanine (Ala,

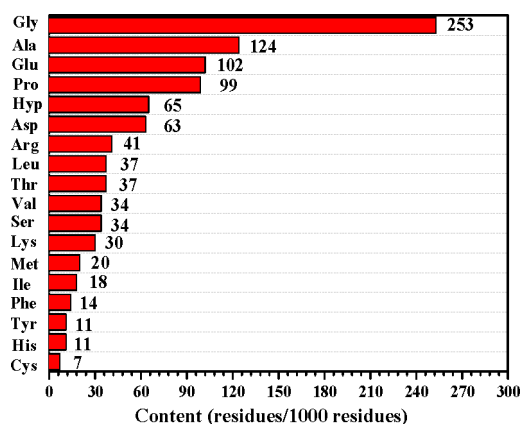


Figure 6. Amino acid compositions (residues/1000 total amino acid residues) of HNBC from the bone of *Harpadon nehereus*.

124 residues), and proline (Pro, 99 residues), which was similar to amino acid compositions of many other bone collagens.^{32,34,40,41} Generally, Gly is the major amino acid and occurs as every third residue in collagen. The Gly content in HNBC was 253 residues/1000 residues, which was lower than those of many bone collagens (320–340 residues) but higher than that of channel catfish head bone gelatins (only more than

20%)⁴² and similar to Kacang goat bone gelatin.⁴³ In addition, the content of glutamic acid (Glu) with 102 residues/1000 residues in HNBC was significantly higher than that in collagens from the carp bone (76 residues), black drum bone (68.7 residues),²⁸ sheepshead bone (65.8 residues),²⁸ deep-sea redfish bone,⁴⁴ and leather jacket bone (35.8 residues).²⁹ Pro- and hydroxyproline (Hyp) were the unique amino acids in collagen, and their content is greatly related to the thermal stability of collagen. The total content of imino acid (Pro and Hyp) of HNBC was 164 residues/1000 residues, which were significantly lower than those of bone collagens from Spanish mackerel (184.3 residues),²⁷ leather jacket (190.3 residues),²⁹ black drum (191.6 residues),²⁸ and *Cyprinus carpio* (192 residues)⁴⁵ but similar to that of bone collagen from deep-sea redfish (163 residues).⁴⁴ Similar to other bone collagens, the contents of cysteine (Cys), tyrosine (Tyr), and histidine (His) of HNBC from the bone of bombay duck were very low.^{30,31,34,41}

3.7. Antioxidant Activity of HNBC. Collagen from marine organisms has attracted extensive attention in the field of functional food and biomedicine due to its excellent antioxidant activity. It was reported that collagen exerted the antioxidant effect possibly by reducing hydrogen peroxide content, inactivating reactive oxygen species, and scavenging free radicals.⁴⁶ Therefore, the scavenging rate of free radicals is regarded as an important indicator for evaluating antioxidant capability. As shown in Figure 7, the DPPH and OH• scavenging rates of HNBC and positive control (V_c) were measured. The collagen from the bone of bombay duck showed antioxidant activity against DPPH and OH•. The scavenging rates of both DPPH and OH• were proportional to the concentrations of HNBC. The scavenging activities of DPPH and OH• increased from 19.3% to 37.3% and from 9.0% to 40.4% in the range of 1–6 mg/mL of HNBC, respectively. The pepsin-soluble collagen from carp scales revealed 6–20% of DPPH scavenging activity at concentrations of 0.2–1.0 mg/mL.⁴⁷ The DPPH scavenging activity of collagen from *A. molpadioides* was from 14.6% to 66.5% at concentrations ranging from 0.5 to 10 mg/mL.¹³ The collagen from miuy croaker showed 10%–50% OH• scavenging activity in the concentration range of 0.5–3 mg/mL.²⁰

3.8. Cytoprotective Effect of HNBC on Ang II-Stimulated HUVEC. Recently, a large number of studies have suggested that chronic vascular diseases such as hypertension may be greatly associated with endothelial dysfunction caused by oxidative stress.^{8,48} The level of

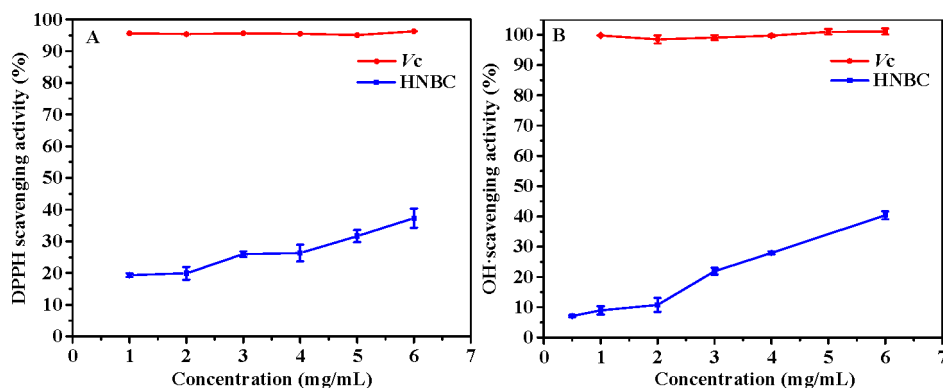


Figure 7. Scavenging activities of DPPH (A) and OH• (B) at different concentrations of HNBC. All values were mean \pm SD with triplicate results.

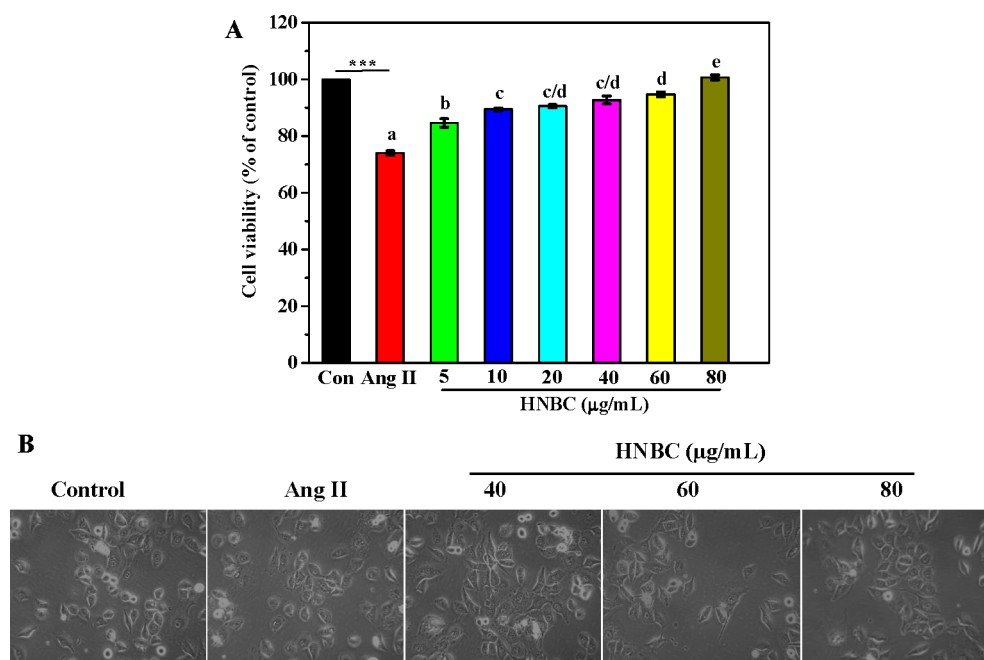


Figure 8. Effects of HNBC on cell viability (A) and morphology (B) of Ang II-induced HUVEC. *** $p < 0.001$, compared with the control group. (a–e) Values with different letters indicated significant differences between different groups after Ang II treatment ($p < 0.05$).

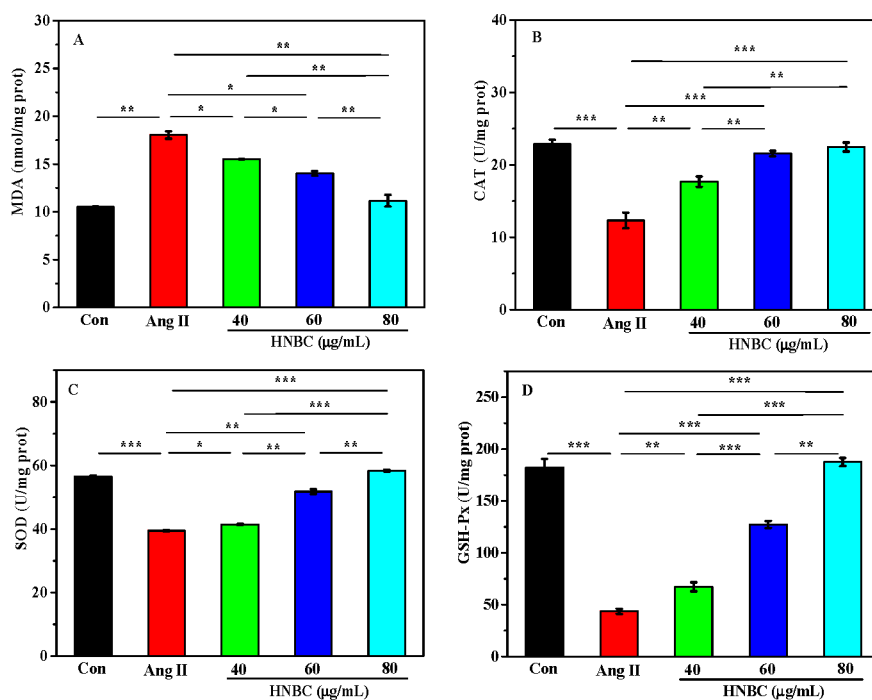


Figure 9. Effects of HNBC on levels of MDA (A), CAT (B), SOD (C), and GSH-Px (D) in HUVEC induced by Ang II. *, **, and *** represented $p < 0.05$, 0.01, and 0.001, respectively.

angiotensin II (Ang II) in vascular endothelium is often increased during the beginning of hypertension.⁴⁹ High levels of Ang II can induce oxidative stress in vascular endothelial cells, resulting in cell apoptosis that leads to arterial failure and increased arterial wall tension, thereby exacerbating the progression of hypertension.⁵⁰ Our above experiments have demonstrated that HNBC has a certain antioxidant activity, which may reduce the level of oxidative stress in cells. Therefore, the protective effects of HNBC in Ang II-induced HUVEC were evaluated using an MTT assay. As shown in

Figure 8A, in the tested concentrations (5–80 μ g/mL), HNBC treatment showed a protective effect on Ang II-induced HUVEC in a dose-dependent manner. The morphology of HUVEC in Figure 8B was more regular and complete under the treatment of HNBC, also indicating that HNBC could effectively prevent cell damage caused by Ang II. These results suggested that the bone collagen from bombay duck may have certain preventive and relieving effects on the occurrence and development of hypertension.

3.9. Effects of HNBC on the Level of MDA and Activities of Antioxidant Enzymes in Cells. MDA is the final product of the peroxidation reaction between free radicals and lipids in the body. Therefore, the level of MDA indirectly reflects the level of intracellular oxidative stress. Antioxidant enzymes such as SOD, CAT, and GSH-Px, which can convert peroxides formed in the body into less toxic or harmless substances, can effectively inhibit cellular oxidation and prevent damage caused by peroxidation. It was shown that HNBC could effectively prevent cell damage caused by Ang II in previous research. In this section, the effects of HNBC on the level of MDA and activities of antioxidant enzymes in HUVEC were investigated to clarify whether the protective effect of HNBC against Ang II-induced damage was due to its antioxidant effect.

As shown in Figure 9A, the MDA content increased significantly after Ang II treatment, indicating that the degree of oxidative stress increased in cells. HNBC treatment significantly reduced the levels of MDA in a concentration-dependent manner. When the concentration of HNBC was 80 $\mu\text{g/mL}$, the level of MDA in HUVEC was comparable to that in the control group. Ang II treatment greatly inhibited the activity of CAT, SOD, and GSH-Px in cells, especially GSH-Px, which decreased by 76% compared with that in the control group. Similarly, the activity of CAT, SOD, and GSH-Px recovered to a level comparable to that of the control group at the HNBC concentration of 80 $\mu\text{g/mL}$.

As a bioactive substance widely used in functional food and biomedicine, collagen has been recognized for its antioxidant activity. For example, the collagen from sea cucumber *Acaudina molpadioides* played a protective role against H_2O_2 -induced injury of RAW264.7 cells by upregulating the levels of SOD and GSH-Px and downregulating the contents of MDA.¹³ In our studies, the collagen from bone of bombay duck (*Harpadon nehereus*) effectively attenuated Ang II-stimulated HUVEC injury due to its antioxidant, which is reflected in free radical (DPPH and $\text{OH}\bullet$) scavenging activities and higher antioxidant enzyme activities in HUVEC of HNBC treatment. It was reported that the antioxidant activity of collagen was mainly attributed to its high content of proline and hydroxyproline, and its hydrolysate (collagen peptides) tended to exhibit better antioxidant activity.⁵¹ HNBC and its hydrolysate (collagen peptides) in the body by oral administration of HNBC may reduce the level of oxidative stress caused by Ang II in the blood vessels, thereby preventing the oxidative stress-induced damage of vascular endothelial cells and slowing the process of hypertension. Therefore, HNBC may have potential application values in the prevention of hypertension.

4. CONCLUSION

In this study, the bone collagen from *Harpadon nehereus* (HNBC) was isolated, and its physicochemical properties were characterized. Similar to many bone collagens, HNBC was mainly composed of type I collagen. However, HNBC showed the higher T_m (48.5 $^\circ\text{C}$) and Glu content (102 residues/1000 residues) but lower content of Gly (253 residues/1000 residues) as compared to other bone collagens. In addition, it showed scavenging rates for both DPPH and $\text{OH}\bullet$ radicals in a dose-dependent manner. Furthermore, HNBC exhibited a protective effect against Ang II-stimulated HUVEC injury by reducing the MDA contents and increasing the antioxidant enzymes activities. This study is the first report that bone

collagen can protect HUVEC from Ang II-induced damage, indicating that HNBC has potential application in the prevention of hypertension.

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Notes

The authors declare no competing financial interest.

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