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Extraction of collagen from *Pangasius bocourti* skin and its antioxidant activities

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Abstract. Fish skin discharged from the fish processing industry is considered an abundant source of raw materials for collagen production. The objective of this work was to optimize the experimental conditions for collagen extraction from *Pangasius bocourti* skin. The highest yield was obtained under the optimal conditions as follows: NaOH 0.1 M, 0.05 M citric acid for demineralization and extraction process, and 200 mL H_2O_2 5 %. Characterization of collagen was indicated in the IR spectrum and SEM. The antioxidant activities of collagen were evaluated *in vitro*. The collagen from *Pangasius bocourti* skin demonstrated appreciable antioxidant potential on total antioxidant activity, ABTS radical scavenging activity, and DPPH radical scavenging activity.

Keywords: Collagen, antioxidant activity, *Pangasius bocourti* skin, SEM

1 Introduction

The growing consumer demand for healthy fish products has resulted in a thriving fish processing industry worldwide. During the course of fish processing (which includes scraping, slicing, filleting, cooking, salting, and canning), a large portion of the fish (flesh, head, bones, fins, skin, tail, and offal) is left behind. This waste is often discharged into landfills or into the sea, leading to environmental problems that emphasize the need for rational use of fish waste to recover valuable products [1, 2]. Fish waste can be used as an ingredient in animal feeds and fertilizers, or used to recover valuable biomolecules such as fatty acids, omega, and collagen [2].

Besides, the demand for health and beauty enhancement products is concerned, especially products derived from nature. These include collagen, polysaccharides, flavonoid, steroid, and essential oils. Collagen is one of the most commonly used biomaterials thanks to its ability to absorb water, form gels, and form and stabilizes emulsions [3, 4]. In the biomedical and pharmaceutical fields, collagen is applied as part of drugs, and replacement proteins for human skin, blood vessels, and ligaments. Some studies also show that collagen has biologically active such as antibacterial, antioxidant, anticancer, and antihypertensive properties [5-9].

Extraction of collagen is an important process for their application or further research and development [10, 11]. Anil Kumar Anal *et al.* studied the effects of extraction parameters (such as extraction time, extraction temperature, and the ratio of solution to raw material) on the yield of collagen from chicken feet by papain hydrolysis [12]. Besides, Jin-Wook Woo and co-workers indicated NaOH concentration and NaOH treatment time affect the content of collagen from

yellowfin tuna (Thunnus albacares) dorsal skin [13]. In addition, Damodar Dhakal *et al.* used a Box-Behnken design in optimization for collagen extraction from sole fish skin [14]. Zhuang et al investigated extraction optimization of collagen with antioxidant activities [15], but none was on the optimization of process parameters for collagen extraction from growing *Pangasius bocourti* in Vietnam.

The objective of this work was to design experiments to optimize the yield of collagen extraction from the *Pangasius bocourti.* The structural characterization of collagen was investigated by a combination of infrared (IR) spectra and scanning electron microscopes (SEM). Besides, *in vitro* antioxidant activities of collagen such as total antioxidant activity and ABTS and DPPH radical scavenging activity were evaluated.

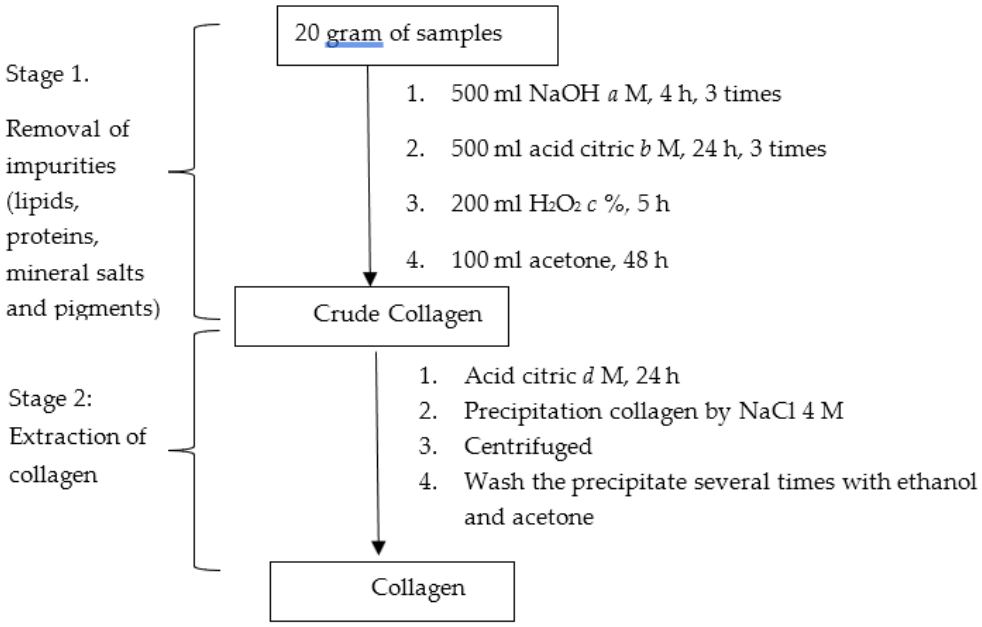
2 Materials and methods

2.1 Material samples and chemicals

Pangasius bocourti skin is brought from Multinational Development and Investment Joint Stock Company (IDI) in Vietnam. The chemicals listed below were purchased from Xilong Chemical Co. (China): sodium hydroxide, citric acid, hydroperoxide, sulfuric acid, chloramine-T, 4- dimetylaminobenzadehyde, L- hydroxyproline. All other reagents and solvents used were of analytical grade, that were purchased from local suppliers.

2.2 Extraction of collagen from *Pangasius bocourti* skin

The process of extraction of collagen is carried out through two main stages (Scheme1): stage 1 is to remove the impurities to obtain raw collagen (treatment with NaOH to remove lipid, with citric acid to eliminate mineral, with H_2O_2 to remove pigment). Stage 2 is to extract and refine collagen from the *Pangasius bocourti* skin [16]. In this study, we applied single-factor experiment to achieve optimal extraction condition. The variables considered in the experimental design are NaOH concentration, citric acid concentration, and H_2O_2 concentration.



Scheme 1. Flowchart for extraction collagen from *Pangasius bocourti* skin

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Stage 1: Removal of impurities: the *Pangasius bocourti* skin was cut 2 cm x 2 cm. The samples (20 g) were defatted and deproteinated with 500 mL of NaOH (0.025 M, 0.05 M, 0.10 M, 0.15 M, and 0.20 M) three times for 4 h each time. Afterward, the residue was treated with citric acid (0.0125 M, 0.025 M, 0.05 M, 0.075 M and 0.10 M), three times for 24 h each time to eliminate minerals. Then, the pigment were removed by 200 mL H_2O_2 (5 %, 10 %, 15 %, and 20 %).

Stage 2: Extraction of collagen: different citric acid concentrations were invested to extract the collagen (0.025 M and 0.05 M), then collagen was precipitated completely by NaCl 4 M. The resulting precipitate was collected by centrifugation and then washed sequentially with cold ethanol and acetone. Finally, the product was vacuum-dried at 30 °C to yield crude collagen.

2.3 Determine of collagen content by hydroxyproline assay

The collagen quantitative method of collagen is based on AOAC Official Method 990.26 and Ricky *et al.* [17]. Briefly, collagen samples (1 gram) were hydrolyzed at 105 °C for 16 h with 7.5 ml H_2SO_4 3.5 M and diluted with distilled water. Standard HyPro solutions ranging from 0.6 to 3 μg/mL were prepared. Distilled water was used as blank. Samples, standard solutions, and blanks were mixed with the chloramine-T reagent for 20 min. After Ehrlich's reagent was added, immersing the mixture in a water bath at 60 °C for 15 min. The mixture was then cooled down to 25 °C, and the absorbance was measured at a wavelength of 560 nm. The total collagen content was extrapolated by multiplying the total hydroxyproline content in each sample by a factor of 6.94.

2.4 Determine characterization of collagen

The characterization of collagen was evaluated by IRPrestige-21 infrared spectrophotometer (Shimadzu, Japan). Morphology was characterized by scanning electron microscope *(JSM-6010PLUS – JEOL, JEOL company, Japan).*

2.5 Antioxidant activities

Total antioxidant activity (TAC)

The antioxidative capacity was determined by reduction of Mo(VI) to Mo(V) at a low pH. An amount of 0.3 mL sample was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Then, the mixture was incubated at 95 °C for 90 min. The mixture was then cooled down to 25 °C, and the absorbance was measured at a wavelength of 695 nm against a blank that contains 3 mL of the reagent solution without the sample [18]. The total antioxidant activity is expressed as the number of equivalents of gallic acid (GA) [19] and ascorbic acid (AS) [20].

DPPH radical-scavenging activity

The DPPH free radical scavenging activity is used to perform the screening effects of antioxidation of the testing substances. Two mL of sample (in dimethyl sulfoxide (DMSO) with different concentrations (25 – 150 μg/mL)) were added to 1 mL of 100 μM DPPH (in methanol) for 30 min at room temperature. The absorbance is measured by UV–Vis method at 517 nm. The antioxidative activity was shown by the color reduction. Ascorbic acid were used as reference substances. DMSO was used as a control. The radical scavenging activity was evaluated using the IC_{50} value [21].

ABTH·+ radical-scavenging activity

The free radical scavenging capacity of samples was tested using ABTS radical decolorization

assay. ABTS radical was produced by the reaction of ABTS stock solution (7 mM) with 2.45 mM potassium persulphate, resulted in $ABTS^{+\cdot}$ solution. The mixture was kept in the darkroom temperature for 16 h before use. An amount of 0.1 mL sample with different concentrations (from 25 mg/mL to 150 mg/mL) were mixed with 3.9 mL of $ABTS^{+\cdot}$ solution. The absorbance was then measured at 734 nm [22]. Ascorbic acid was used as a positive control. The scavenging capability on $ABTS^{+\cdot}$ was calculated by the following formula:

Scavenging rate (%) = [1 − A1/A0] × 100

where A0 is the absorbance of the blank, and A1 is the absorbance of the sample.

The radical scavenging activity was evaluated using the IC_{50} value.

2.6 Statistical analysis

Unless otherwise stated, all the experiments were performed three times (n = 3). The results are presented as means value ± Standard deviation (SD) or Standard Error of the Mean (SEM). The statistical analysis system software involved the use of both Origin 8.0 and Microsoft Excel (2010).

3 Results and discussion

3.1 The optimal conditions of collagen extraction from *Pangasius bocourti* skin

The effect of NaOH concentration on removing lipid, protein, and the yield of collagen extraction

When treating fish skin with NaOH, impurities including lipids and non-collagen proteins are removed due to the saponification reaction, breaking of side chains, and formation of ionic bond bridges.

In this study, to examine the influence of NaOH concentration on removing lipids, proteins, and the yield of collagen extraction, different NaOH concentrations were studied, including 0.025 M, 0.05 M, 0.1 M, 0.15 M, and 0.2 M. Other extraction conditions were as follows: 0.05 M citric acid for demineralization and extraction process, 200 mL H_2O_2 5% for pigment removal.

Table 1 showed the yields of collagen extraction at various NaOH concentrations, in which, the yields were decreasing from 1.78 to 0.5 g parallel with the increasing of NaOH concentrations.

Table 1. The effect of NaOH concentration to the collagen obtained

NaOH concentration (mol/L)	0.025	0.05	0.1	0.15	0.2
Collagen content (gram)	1.78	1.77	1.74	0.78	0.50

In the range of NaOH concentration from 0.025 M to 0.1 M, the collagen extraction process exhibited a high yield with insignificant changes. However, as NaOH concentration continued to increase, the respective collagen produced strongly decreased. In addition, the lipid contents quantified in the removed extracts of 0.025 M and 0.050 M NaOH solution was lower than those of NaOH 0.1 M. As a result, impurity removal ability of 0.100 M NaOH was better than the other concentrations, that was selected for further experiments.

The effect of citric acid concentration on eliminating minerals and the yield of collagen extraction

Different citric acid concentrations, including 0.0125 M, 0.025 M, 0.05 M, 0.075 M, and 0.1 M, were tested to identify the suitable solution for the removal of minerals. Other conditions were as follows: NaOH 0.1 M, citric acid 0.05 M for extraction process, 200 mL H_2O_2 5 %.

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Table 2. The effect of citric acid concentration to the collagen obtained.

Citric acid concentration (mol/L)	0.0125	0.025	0.05	0.075	0.1
Collagen content (gram)	2.84	3.21	5.70	1.85	2.10

The results indicated (Table 2) that citric acid concentration was proportional to the content of the collagen when citric acid concentration was between 0.0125 M and 0.05 M. The content of the collagen was 5.70 g when the citric acid concentration was 0.05 M. After this point, the content of the collagen started to decrease with increasing the citric acid concentration. In our opinion, when the citric acid concentration is increased to 0.0750 M and 0.1000 M, hydrolysis and cleavage of collagen chains might occur. However, when treating citric acid at low concentrations, it can be close to the isoelectric point, causing collagen to precipitate.

The effect of H2O2 concentration on removing pigment and the yield of collagen extraction

The H_2O_2 concentration was respectively set at 5 %, 10 %, 15 %, and 20 % to examine the influence of pigment removal and the content of collagen. The other conditions were as follows: NaOH 0.1 M, 0.05 M citric acid for demineralization and extraction process. Table 3 showed the content of collagen at various H_2O_2 concentrations. The content of collagen was decreasing from from 4.76 g to 1.73 with a decreasing H_2O_2 concentration. The highest content of collagen was 4.76 g obtained at H_2O_2 5 %.

Table 3. The effect of H_2O_2 concentration to the collagen obtained.

H_2O_2 concentration (mol/L)	5	10	15	20
Collagen content (gram)	4.76	3.50	2.14	1.73

The effect of citric acid concentration on collagen extraction process

In this experiment, citric acid concentration on extraction progress collagen was in turn set at 0.025 M and 0.05 M when other conditions were as follows: NaOH 0.1M, citric acid 0.05 M for demineralization, 200 mL H_2O_2 5 % for pigment removal. The effect of citric acid concentration on the content of the collagen was investigated. The results indicated (Table 4) that citric acid concentration was proportional to the content of the collagen. The content of the collagen was 7.08 g when citric acid concentration was 0.05 M. Therefore from the results, we concluded that a high extraction yield could be obtained with a citric acid concentration of 0.05 M.

Table 4. The effect of citric acid concentration on extraction progress collagen.

Citric acid concentration (mol/L)	0.025	0.05
Collagen content (gam)	5.02	7.08

3.2 Characterization of collagen extracted from *Pangasius bocourti* skin

Figure 1 exhibits the scanning electron microscope (SEM) images of collagen extracted. It can be seen that collagen sample exhibited a homogeneous surface and have filamentous with a diameter of about 0.6-2.6 μm.

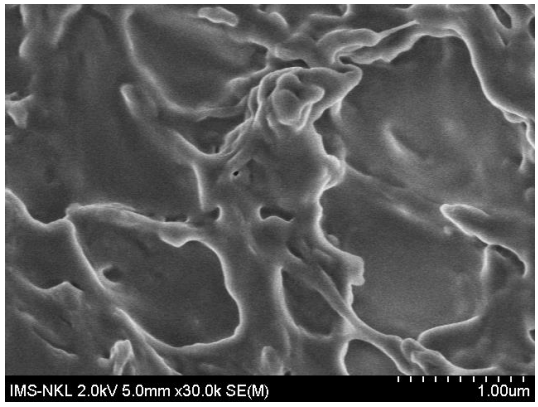

Fig. 1. SEM images of collagen extracted from *Pangasius bocourti* skin

Fourier transforms infrared (FTIR) spectroscopy was employed to explore the bonding characteristics of the collagen, as shown in Figure 2. The collagen sample reveals an unambiguous peak in the 3318 cm-1 regions that characterize the valence vibrations of the bond of N-H. The strong peaks centered at 1743 cm^{-1}, 1466 cm^{-1} and 1171 cm^{-1} could be assigned to amide I, II, and III bands, respectively, which are the typical characterizations of collagen [23, 24].

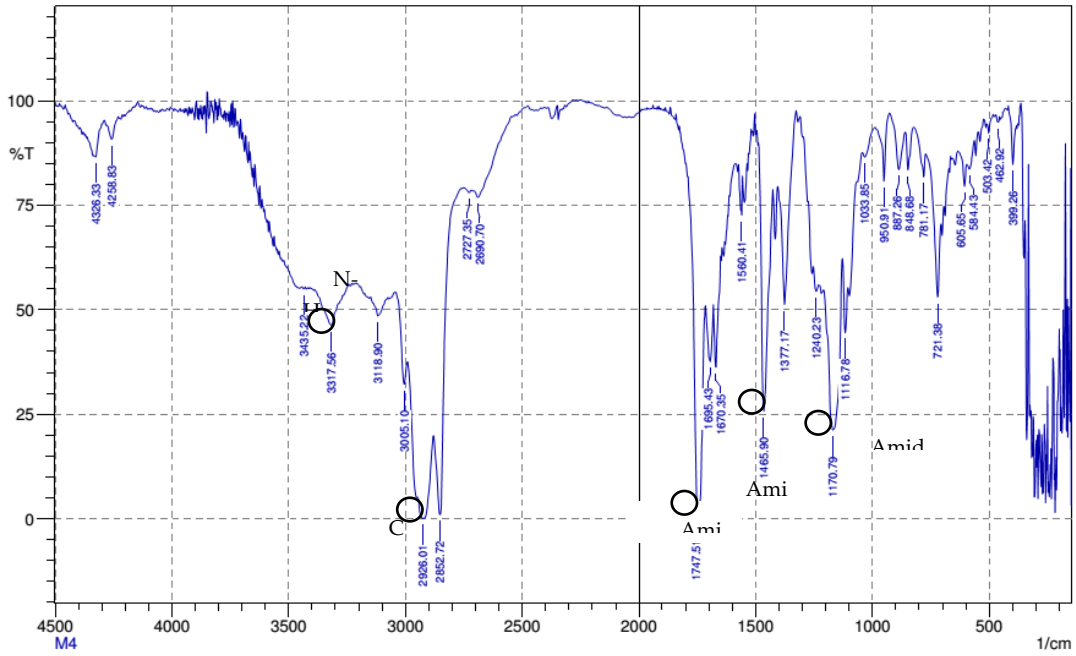

Fig. 2. The FTIR spectrum of collagen from *Pangasius bocourti* skin

3.3 *In vitro* antioxidant activity of collagen extracted from *Pangasius bocourti* skin

After optimizing the extraction process and the characterization of collagen obtained, *in vitro* antioxidant activities of collagen were evaluated. The total antioxidant capacity was determined by assessing the electron-donating capacity of the sample. The antioxidant capability was represented as the number of equivalents of gallic acid.

The linear regression equations of gallic acid was: Abs = 1.952 C_{GA} + 0.2372, R^2 = 0.9992, at concentration from 0.05 mg/mL to 0.5 mg/mL.

The study revealed that the antioxidant capacity of collagen was observed at the concentration of 1.5 mg/mL where the total antioxidant capacity of collagen from *Pangasius bocourti* skin contained 6.54 ± 0.13 mg GA/g.

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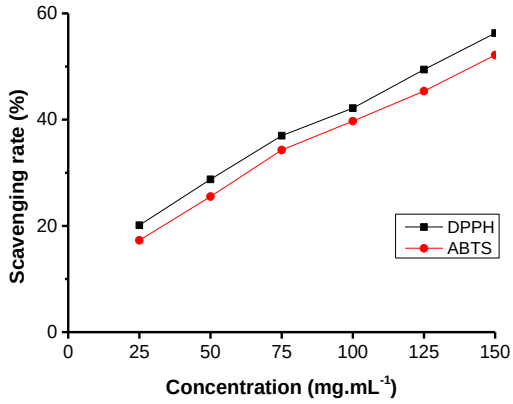

Fig. 3. Antioxidant activity of the collagen from *Pangasius bocourti* skin

The DPPH and ABTS radical scavenging activity determine the hydrogen transfer capability of the antioxidant compounds in the test samples. The scavenging activity of the collagen is presented in Figure 3.

The free radical scavenging capacity of the collagen sample increases with concentration. The antioxidant capacity of the collagen was relatively good, with low IC_{50} values (128.14 μg/mL for DPPH radical and 132.68 μg/mL for ABTS radical). At a concentration of 150 μg/mL, the DPPH and ABTS free radical scavenging capacity of the collagen was over 50%; however, the activity of the collagen was lower than that of ascorbic acid (IC_{50} values of ascorbic acid are 1.84 μg/mL for DPPH radical and 2.27 μg/mL for ABTS radical). However, the antioxidant activity of collagen obtained was higher than compared to some previous reports [25, 26]. It can be seen that the DPPH and ABTS radical scavenging activity of collagen was higher than that of collagen from Yellowfin Tuna (*Thunnus albacares*) Skin (IC_{50} values: 560.51 μg/mL for DPPH radical and 313.29 μg/mL for ABTS radical) [26] and collagen of jumbo squid (*Dosidicus gigas*) [25].

4 Conclusions

On the basis of a single-factor experiment for the yield of the collagen extraction from *Pangasius bocourti* skin, the appropriate ranges of NaOH concentration, citric acid concentration, and H_2O_2 concentration were preliminarily determined. The extraction parameters were NaOH 0.1 M, 0.05 M citric acid for demineralization and extraction process, and 200 mL H_2O_2 5 % for pigment removal. Characterization of collagen was indicated in IR spectrum and SEM. The collagen from *Pangasius bocourti* skin exhibits antioxidants with low IC_{50} values (128.14 μg/mL for DPPH radical and 132.68 μg/mL for ABTS radical) and the total antioxidant capacity was 6.54 ± 0.13 mg GA/g. The experimental results may provide theoretical basis for further system research, development and extraction of the collagen from *Pangasius bocourti* skin. The results showed that the collagen from *Pangasius bocourti* skin had appreciable antioxidant activity *in vitro*.

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Conflicts of Interest

The authors declare no conflict of interest

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