

EXTRACTION AND PURIFICATION OF COLLAGEN FROM THE SKINS OF BASA FISH (*PANGASIUH HYPHOPHTHALMUS*)

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ABSTRACT

Acid-solubilized collagen (ASC) was extracted from basa fish skin (*Pangasius hypophthalmus*) by acetic acid and purified by dialysis, lyophilization and supercritical carbon dioxide (SC-CO₂) extraction techniques. After 24 h under stirring condition, more than 93 % of salt was removed by dialysis. The efficiency of lipid extraction process reached 91.6 % at the following conditions: pressure of 200 bar, CO₂ flow rate of 10 g/min, temperature of 38 °C, concentration of ethanol as co-solvent of 10 % (v/v) and the retention time of 60 minutes. The obtained collagen has low impurities of lipid (0.33 %), and ashes (0.29 %). Moreover, extracted collagen has no smell, no color, high molecular weight, and is appropriate for cosmetic purposes, pharmaceutical and food applications.

Keywords: basa fish, super critical CO₂, *Pangasius hypophthalmus*.

1. INTRODUCTION

Collagen is the most abundant animal protein representing nearly 30% of total protein in animal. It is the main structural element of bones, cartilage, skin, tendons, ligaments, blood vessels, teeth, cornea and all other organs of vertebrates. The molecules of collagen contain three polypeptide chains wound together in a tight triple helix. Each polypeptide chain consists of repeated sequence of triplet, (Gly-X-Y)_n, where X and Y are often proline (Pro) and hydroxyproline (Hyp). Since collagen is an important biomaterial, many different industries are using it widely. Collagen is highly used in pharmaceutical area with some identical applications such as the production of wound dressings, vitreous implants and as carriers for drug delivery. Collagen is also used for the production of cosmetics because of a good moisturizing property of its [1]. In addition, collagen has been utilized to produce edible casings, which are needed in the meat processing industries (sausages, snack sticks, etc.). Heat denatured collagen is called gelatin, which is important in food manufacturing.

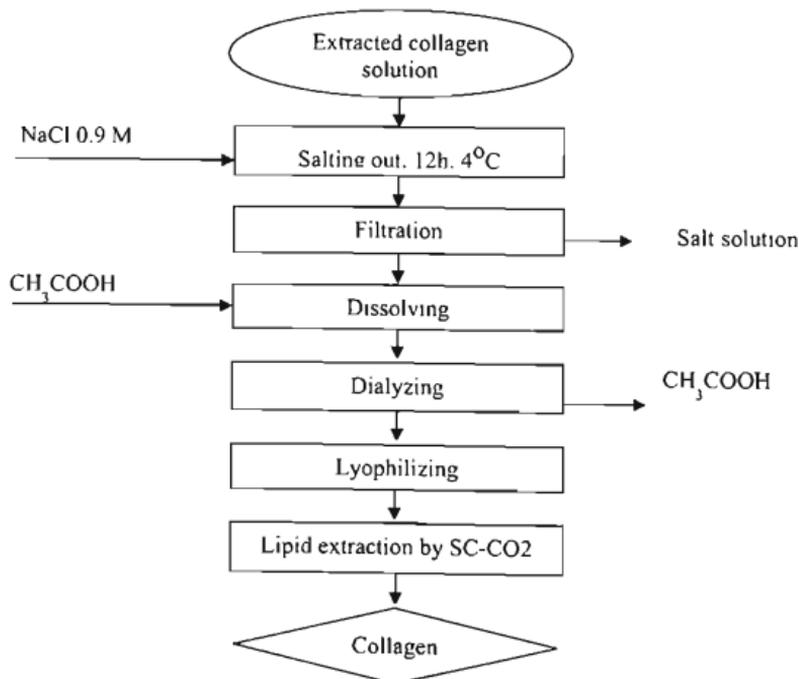
Up till now, most of the collagen in use have been produced from cattle. Nevertheless, due to the occurrence of the BSE (Bovine Spongiform Encephalopathy), TSE (Transmissible Spongiform Encephalopathy) and FMD (Food and Mouth Disease) diseases in swine and cattle, cattle-based collagen has worried consumers. Therefore, fish-originated collagen is currently

under investigation since such collagen has better characteristics for cosmetic and pharmaceutical applications. Collagen produced from fish wastes also helps to overcome religious barriers [2]. While cattle collagen is slowly absorbed on human skin, fish collagen could be totally absorbed. Due to the variety of water temperatures and pressures in which fishes live, fish collagen is resistant to physical and chemical damage [3]. Previous researches mainly focused on collagen extraction from fishes living in cold zones [4, 5, 6]; but few were found to work on fish of tropical regions. There is, so far, only one study of Kittiphattanabawon *et al* [7] which focused on the characteristics of collagen extracted from skins and bones of big-eye snapper using acetic acid. So far, no study has been conducted to purify and to identify the characteristics of collagen extracted from the wastes of basa fish processing.

We used basa fish skin treated by LASNa 0.5 %, and H_2O_2 1 % in NaOH solution [8] for the extraction of collagen by acetic acid, followed by filtration to collect acid-solubilized collagen (ASC). In this study, we focused on the purification of ASC in order to collect refined collagen. The purification process consists of two steps, namely dialysis and lipid extraction by supercritical carbon dioxide (SC-CO_2).

2. EXPERIMENTAL METHODS

2.1. Purification process



Schema 1. Extraction of acetic acid- solubilized collagen (ASC).

Basa fish skins were supplied by the Viet An Company in An Giang province in Vietnam. Pre-treatment method was adopted from Le Thi Thu Huong *et al.* [8]. After removing remained flesh, the skins were washed in cold water, packed in PE bags and kept storage under -20°C .

The fish skins were first defrosted, washed, drained and dipped in LASNa 0.5 % in 6 hours. Then they were dipped in H_2O_2 1% in NaOH 0.05N solution in 2 hours to remove lipid, minerals, colorants, and odorants. After that, they were cut into smaller pieces using scissors. The small pieces of the skins were extracted by 0.75 M acetic acid at the ratio of solid/liquid = 1/50 at 3°C for 24 h. The extracted solution was filtered to collect acid-solubilized collagen (ASC). ASC was then salted out by adding NaCl so that the final concentration was 0.9 M.

2.2. Dialysis of the collagen precipitate in a Regenerated Cellulose Dialysis Membrane Tubing

The collagen precipitate was dissolved in a 0.5 M acetic acid solution with a solid/liquid ratio of 1/10 (w/v); then the received collagen solution was poured into a dialysis tubing, its two ends tied. The membrane tubing was then soaked with 30 volumes (v/w) of 0.1 M acetic acid solution at 4°C for 12 h, 24 h, 36 h under static and stirring conditions, alternatively. The dialysis buffer was changed every 8 hours. Once the buffer is changed, the movement of salt particles from high (inside the membrane) to low (outside the membrane) concentration will resume until a new equilibrium is again established. With each change of dialysis buffer, collagen inside the membrane is further purified by a factor equal to the volume difference of the two compartments.

2.3. Extraction of lipid by SC- CO_2

Lipid contaminant of freeze-dried crude collagen was removed by SC- CO_2 with CO_2 flow rates of 5, 10, 15 g/min; pressures of 100, 200, 300 bar; temperatures of 34, 38, 42°C and ethanol contents of 5, 10, 15 % for 30 minutes. After that, the effect of extraction time (30 -120 minutes) on the lipid removing yield and the characteristic of collagen product were investigated. The lipid content in collagen was determined before and after extraction. The extraction of lipid was performed in a SC- CO_2 equipment of Thar Technology, *Thar SFC SN.11419 model*. The supercritical fluid extraction system consists of two pumps for pumping carbon dioxide and co-solvent (ethanol); one heat exchanger; one extractor; one collection vessel and one automated control system. At first liquid CO_2 was pumped from a tank, pressurized to reach a supercritical condition. Supercritical CO_2 and co-solvent were heated before being entered the extractor where occurred the contact with collagen sample (solid particles). Afterward, the solute, CO_2 and co-solvent left the extractor and lipid was precipitated in collector, where CO_2 became gaseous. Collagen was removed from extractor.

2.4. Gel SDS-polyacrylamide (SDS-PAGE) electrophoresis

SDS-PAGE gel electrophoresis was performed [9] using the buffer system of 0.1 % SDS, 0.025 M Tris and 0.192 M glycine, a mini- PROTEAN Tetra cell manufactured by BIORAD. The resolving gel was 7 % and stacking gel was 5 %. After electrophoresis, gel was dyed by 0.05 % (w/v) Coomassive blue R-250 in 15 % methanol and 5 % (v/v) acetic acid. Then the gel was dipped in the solution of 30 % (v/v) methanol and 10 % (v/v) acetic acid to remove the

color. The molecular mass of collagen protein was determined using a standard protein scale, ranging from 75 to 250 kDa.

2.5. Quantitative analysis methods

Collagen amount was relatively quantified via the content of hydroxyproline, an amino acid occupied approximately 14 % of collagen. This content of the hydroxyproline, in turn, was determined by the method of Ignat'eva, 2005 [10]. The remained lipid content after SC-CO₂ extraction was determined using the TCVN 4331:2001. TCVN 4326:2001 was used also for moisture measurement, while AOAC 923-03 was used for ash determination. The remained sodium chloride content in collagen before and after dialysis were determined using the Mohr's method.

3. RESULTS AND DISCUSSIONS

3.1. Dialysis

Figures 1 (1a, 1b) shows the change of salt content by time in static and dynamic conditions. In static condition, the salt content decreased from 3.74 % to 0.33 % after 24 hours and to 0.24 % after 36 hours (Fig. 1a). In dynamic condition, the salt content decreased from 3.74% to 0.25% after 24 hours and to 0.19 % after 36 hours (Fig. 1b). In brief, removing salt by dialysis in stirring condition was better than in static condition.

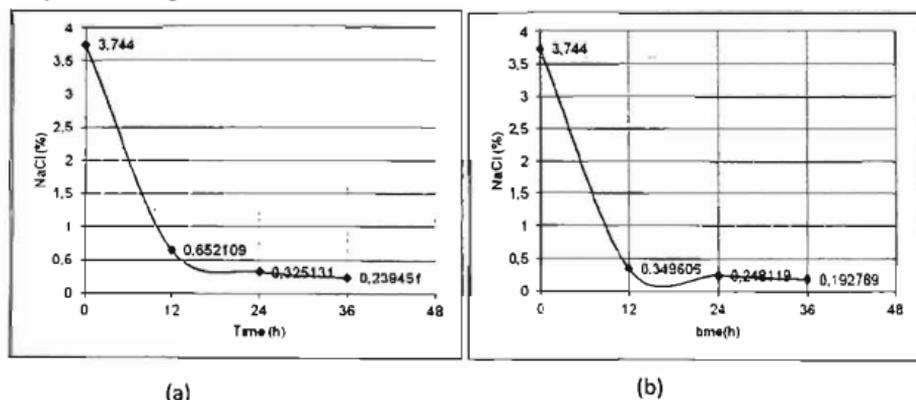


Figure 1. Salt removal by dialysis in static (a) and dynamic (b) conditions.

3.2. Lipid extraction by SC-CO₂

Figures 2 (a), (b), (c), (d) show that the lipid content decreased when pressure of CO₂, flow rate of CO₂, temperature and co-solvent- ethanol percentage were increased.

Figure 2(a) shows the influence of CO₂ pressure on lipid extraction (other conditions were set as follows: 10 g/min of flow rate; 38 °C of temperature; 30 min of retention time). When the pressure of CO₂ increased from 100 to 300 bar, the lipid content decreased from 4.07 % to 0.69

% The supercritical fluid pressure has close relation with its solvent ability for lipid. When the pressure increases, the solubility and thus the lipid extraction efficiency is increased [11, 12].

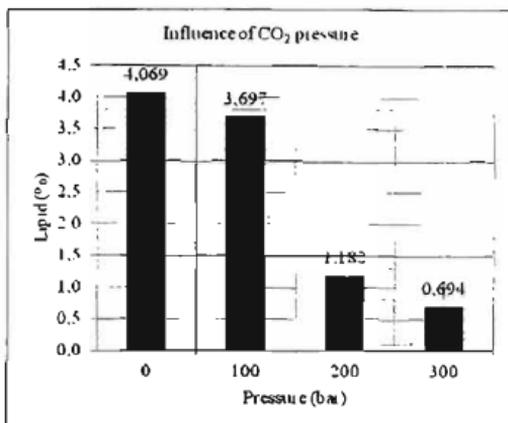


Figure 2(a). Influence of CO₂ pressure.

Figure 2(b) shows the influence of CO₂ flow rate on lipid extraction. When the flow rate increased from 0 to 10 g/min, the lipid content decreased rather quickly, from 4.07% to 1.17%, but when the flow rate changed from 10 to 15 g/min, the lipid content decreased more slowly, from 1.17% to 0.89%. The rapidly decrease of lipid content can be observed between 5 g/min and 10 g/min of CO₂ flowrate while its differences between 0 g/min and 5 g/min as well as 10 g/min and 15 g/min were inconsiderable. Therefore, the CO₂ flowrate of 10 g/min was chosen for further investigation.

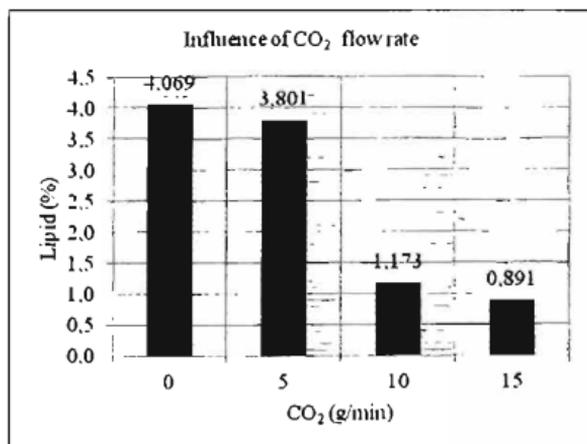


Figure 2 (b). Influence of CO₂ flow rate.

Figure 2(c) shows the influence of temperature on lipid extraction. If the temperature of SC-CO₂ increased (above the critical point) from 34 to 42 °C with 200 bar, and the flow rate of CO₂ (10 g/min) was held constantly, the lipid content decreased from 4.07 % to 1.27 %. The result was consistent with the conclusion of [12] which stated that above the supercritical point, the extraction efficiency was increasingly proportional to the temperature of CO₂ flow.

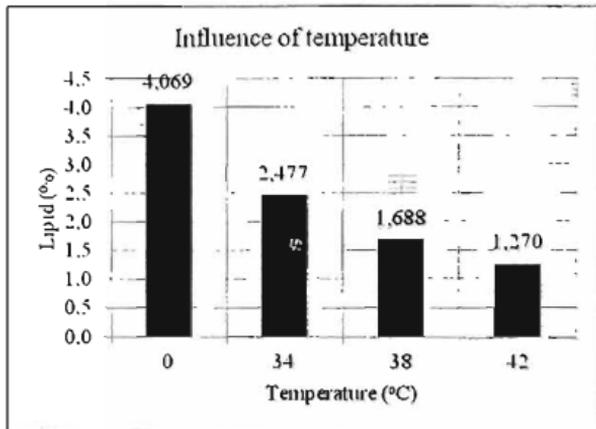


Figure 2 (c) Influence of temperature.

The influence of ethanol on lipid extraction is shown in Fig. 2(d). Ethanol plays a role as a polar co-solvent to enhance extraction efficiency. If the ethanol content increased from 0 % to 10 %, the lipid content decreased from 4.07 % to 0.4 %. The extraction efficiency was rapidly increased when CO₂ was mixed with ethanol in investigated concentrations. This is due to the fact that carbon dioxide can only extract non-polar lipid materials, whereas mixtures of ethanol and carbon dioxide can extract both polar and non-polar lipid materials [11, 12, 13, 14].

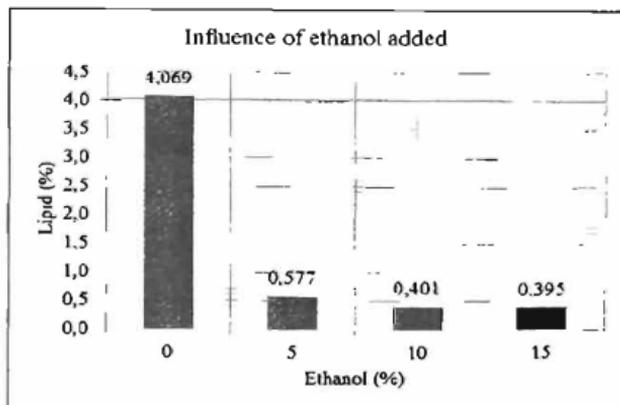


Figure 2 (d) - Influence of ethanol added.

Figure 3 shows the influence of retention time on lipid extraction. The extraction was performed at CO₂ pressure of 200 bar, CO₂ flow rate of 10g/min, temperature of 38 °C, ethanol concentration 10 % (v/v). After first 30 minutes, the extraction rate increased very fast and the lipid content decreased from 4.07 % to 0.4 %. In the next 30 minutes, the extraction rate almost didn't change, and the lipid content decreased only a little, from 0.4 % to 0.33 %.

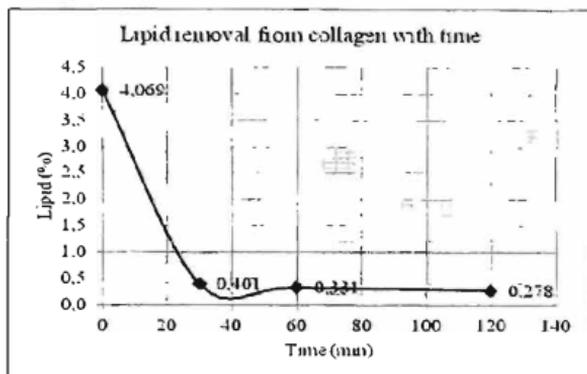


Figure 3. Lipid removal from collagen with time.

3.3. SDS-polyacrylamide (SDS-PAGE) gel electrophoresis

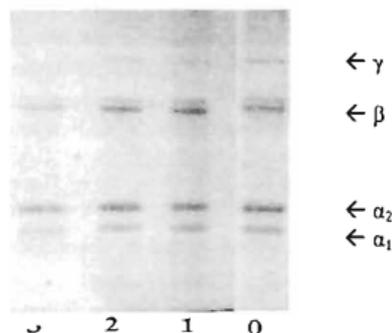


Figure 4. Molecular weights of different samples of collagen. 0- collagen sample before lipid extraction, 1- 3: collagen samples during extraction (1 – after 30 minutes, 2 - after 60 minutes, 3 - after 120 minutes).

Figure 4 shows the influence of SC-CO₂ extraction retention time on collagen molecular mass. Sample 0, sample 1 and sample 2 consisted of α_1 , α_2 , β and γ chain; only sample 3 consisted of α_1 , α_2 , β without γ chain. This proves that the longer the retention time, the higher the collagen degradation level. Therefore, the extraction duration of 60 minutes was chosen for ensuring acceptable high-enough molecular weights of the targeted collagen when the extraction yield of lipid reached 91.6 %.

3.4. Analytical result of collagen product

After removing lipid by SC-CO₂ at the conditions chosen above; the product collagen has been analysed for chemical components. The result is as follows:

Table 1. Composition of collagen product.

Composition	Content ⁽¹⁾
Hydroxyproline (mg/g)	93,72 ± 8,19
Lipid (%)	0,33 ± 0,02
Moisture (%)	15,25 ± 0,338
Ashes (%)	0,291 ± 0,074
Organic compounds (%)	84,02 ± 0,302
⁽¹⁾ ± SD - each result was repeated three times	

4. CONCLUSIONS

Collagen extracted from basa fish skin has been purified by dialysis, followed by SC-CO₂ extraction to remove as much as possible the accompanied salt and lipid.

The dialysis efficiency of salt removal reached 93.3 %, corresponding to the decrease of salt content in collagen from 3.74 % to 0.25 %. The result was obtained under the dialysis conditions as follows: collagen sample / buffer ratio of 1/30, temperature of 4 °C, with constantly mechanical stirring in 24 hours.

The efficiency of the lipid extraction by SC-CO₂ reached 91.6 %, corresponding to the decrease of lipid content in collagen from 4.07 % to 0.33 %. The extraction process was performed at CO₂ pressure of 200 bar, CO₂ flow rate of 10g/min, temperature of 38 °C, ethanol concentration as co-solvent of 10 % (v/v) and the retention time of 60 minutes.

Collagen consists of two chains of α_1 and α_2 about 130 kDa, one β chain about 250 kDa and one peptide chain about 80 kDa.

After precipitation, the product collagen has the following specification: 93.72 mg/g HP, 84.02 % organic compounds, 15.25 % moisture, 0.33% lipid and 0.29 % ash. The specification meets the requirements of collagen additive applied in cosmetics, pharmaceuticals and foods.

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TÓM TẮT

NGHIÊN CỨU QUÁ TRÌNH TÍNH CHẾ COLLAGEN TỪ DA CÁ TRA (*PANGASIVS HYPOPTHALMUS*)

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Collagen trích li từ da cá tra (*Pangasius hypophthalmus*) được hòa tan trong acid acetic 0,5 M (ASC). Dung dịch ASC có thành phần chính là collagen, ngoài ra còn chứa một số tạp chất như NaCl, lipid, chất màu, chất mùi. Quá trình tính chế collagen gồm hai công đoạn: thẩm tích để loại muối và sử dụng CO₂ ở trạng thái siêu tới hạn (SC-CO₂) để tách lipid. Việc thẩm tích được thực hiện ở hai chế độ: tĩnh và động. Kết quả cho thấy hiệu suất tách muối ở chế độ có khuấy tròn, sau 24 giờ đạt 93,3 %, cao hơn chế độ thẩm tích tĩnh (91,2 %). Hiệu suất tách lipid bằng SC-CO₂ đạt 91,6 % ở áp suất 200 bar, tốc độ dòng CO₂ là 10 g/min, nhiệt độ 38 °C, nồng độ ethanol 10 % (v/v) và thời gian lưu 60 phút. Collagen sau tính chế có hàm lượng lipid 0,33 %; độ tro 0,29 %; collagen có màu trắng và không còn mùi tanh.

Từ khóa: cá tra, SC- CO₂, *Pangasius hypophthalmus*.