TREATMENT OF TRA FISH *(PANGASIUS HYPOPHTHALMUS)* SKIN FOR COLLAGEN EXTRACTION

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Abstract

To produce highly qualified collagen from fishery wastes, non-collagen accompanied residues should be well removed. In this study, tra fish skin has been pretreated with NaOH, LASNa, Ethanol and H_2O_2 to remove lipid, minerals, colorants, and odorants. Treatment efficiency has been evaluated based on the hydroxylproline, amino acid, ash, and electrophoresis analysis, and apprears to be the following order: LASNa > NaOH > Ethanol. Results of physico-chemical analysis of treated tra fish skin as raw collagen by LASNa 0.5%, H_2O_2 1% showed that: collagen of the tra fish skin had the denaturalizing temperature Td of about 39^{o}C. Electrophoresis abalysis showed that collagen of tra skin was type I, which consists of two chains of α1, α2 with molecular weight of about 130kDa and one β chain with molecular weight of about 250kDa. It has been pointed out that with sodium laurylsulphate, higher collagen extraction efficiency could be attained.

1. Introduction

Collagen has been used widely in the production of pharmaceutical and cosmetic products (Bailey & Light, 1989; Cavallaro, Kemp & Kraus, 1994; Hood, 1987; Hassan & Sherief, 1994). Up till now, most of the collagen in use has been produced from cattles. Nevertheless, due to the presence of the BSE (Bovine Spongiform Encephalopathy), TSE (Tranmissible Spongiform Encephalopathy) and FMD (Foot and Mouth Disease) in swine and cattles, cattle-based collagen has worried the consumers. Therefore, fishery-originated collagen is currently under investigation since such collagen has better characteristics for cosmetic and pharmaceutical application. Collagen produced from fishery waste also helps to overcome religious barriers (Sadowska, Kolodziejska & Niecikowska, 2003). While cattle collagen is slowly adsorbed on human skin, fish collagen could be totally adsorbed. Fish lives in a wide range of temperature, depth, and pressure, so that; its collagen specially stands for chemical and physical disintegration (Gomez–Guillen et al., 2002). Previous researches mainly focused on collagen extraction from fishes living in cold zones such as Bechir et al. (2008), Bae et al. (2007), and Senaratne et al. (2005); but few was found to work on fish of tropical regions. There is, so far, only one study of Kittiphattanabawon *et al.*(2004), which "identify the characteristics of collagen extracted from skins and bones of bigeye 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 tra fish processing.

In this study, we used tra fish skins to be the raw materials and focused on the purification process to remove non-collagen substances such as lipid, minerals, non-collagen protein, colorants, odorants etc out of the skins. The purpose of this process was to receive clean skins whose main component is collagen. The purified skin was the source of collagen extraction. In the process of producing collagen, the impurities removal is very important since it influences the efficiency of collagen collection, structure and physico-chemical properties of the finished collagen

2. Materials and Methods

Materials

Tra fish skin was supplied by the Viet An Company in An Giang province in Vietnam. Pre-treatment method was based on Le *et al.* (2010) with some minor modifications to be suited to the practical applications. After removing remained meat, skin was washed by cold water, packed in PE bag and kept storage under (-20) ^{o}C.

Methods

Collagen amount was relatively quantified via the content of hydroxyproline, an amino acid occupied approximately 14% of collagen. This content of hydoryproline, in turn, was determined by the method of Switzer (1991). The remained fat content after washing was determined using the TCVN 4331:2001 norm. TCVN 4326:2001 was used for moisture measurement. AOAC 923-03 norm was used for ash determination.

Treatment of fish skin with NaOH, LASNa and Ethanol solutions

Fish skin was defrosted, washed, drained and dipped in NaOH or LASNa solutions of 0.5%, 1%, and 1.5% or Ethanol of 5%, 10% , 15%. The ratio of fish skin/solution was of 1:10 (w/v). The temperature was maintained at 4^{o}C. Sampling was made every 2, 4, 6, and 8 hours for the NaOH, LASNa solutions, and every 4,8,12, 16 and 24 hours for Ethanol, to determine the amount of lipid, hydroxyproline, organic substances, moisture and ash.

Treatment of fish skin by H_2O_2 solution

Fish skin, after revoming fat by LASNa, was washed, drained and dipped in H_2O_2 solution of 0.5%, 1%, and 1.5% to be decolored. The ratio of fish skin/solution was of 1:10 (w/v). The temperature was maintained at 4^{o}C. Sampling was made every 0.5, 1, 1.5, 2, and 3 hours, to assess the color of the skin using sensory evaluation.

Analysis of amino acids

After defrosting, the skin was hydrolyzed with the 0.2% (v/v) solution of 4M methanesulfonic acid in 3-2 (2-aminoethyl) indole at 115^{o}C in 24 hours. The extract was neutralized by the solution of 3.5M NaOH and then diluted up to pH 2.2 by a buffer solution of 0.2M citrate. Amino acid content was analysed by MLC-703 method (Phanat *et al.*, 2005).

Gel SDS-polyacrylamide (SDS-PAGE) electrophoresis

SDS-PAGE gel electrophoresis was performed (Laemmli, 1970) using Buffer System, 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 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 range from 75 – 250kDa.

Denaturalizing temperature of collagen

The denaturalizing temperature of collagen was identified using the method of Kimura *et al.* (1988). The viscometer Ostwald was filled up with the 0.3% (w/v) solution of collagen in 0.1M acetic acid. After that, the viscometer was dipped in a temperature equilibrium sink. The temperature of the collagen solution was increased from 33.5 to 48.5^{o}C with the gradient of 1^{o}C/time. Each specific temperature was kept in 10 mins for the temperature of collagen equal with the temperature of water in the sink. The relative viscosity was calculated by the following formula:

Relative viscosity =(maximum viscosity – measured viscosity)/(maximum viscosity - minimum viscosity).

The denaturalizing temperature is the temperature at which the relative viscosity is equal 0.5.

3. Results and discussions

Chemical compounds of tra fish skin

Table 1 shows that the moisture of the skin was 61.35%. This was less than the moisture of the skin of brown backed toadfish (Senaratne et al., 2006) as well as less than bigeye snapper's skin (Kittiphattanabawon, 2004). The amount of raw protein, raw lipid, and ash of tra fish skin were 75.3%, 15.33% and 1.11%, respectively. Compared to skin of brown backed toadfish, tra fish skin was less in protein and ashes, but 10 times higher in lipid amount. Compared to skin of bigeye snapper (Kittiphattanabawon, 2004), tra fish skin has the same amount of protein, but less ashes and 7 times higher in lipid. Therefore, to purify collagen from tra fish skin, removing fat was the main concern.

Amino acid components

Amino acid components in tra fish skin are shown in table 2. Compared to skin of skate Raja kenoji (Hwang et al., 2005), tra fish skin has similar amino acids, except that the amino acid Cys in tra fish skin was 120, much higher than that in skate Raja kenoji. Hydroxyproline in tra fish skin (57/1000 amino acid) was less than that in skate Raja kenoji (72/1000 amino acid). In tra fish skin, the components Met, Tyr, His, Ile, Hyl and Phe were low in content, while Gly possessed high ratio of above 23% as similar with collagen of other fishes (Bae et al., 2007).

Table 1: Chemical compound of tra fish skin

Compound	Amount - wet weight(*) (%)	Amount- dry weight(*) (%)
Protein	31.1 ± 0.27	75.3 ± 0.94
Lipid	6.5± 0.41	15.33 ± 0.74
Moisture	61.35 ± 0.24	-
Ash	0.436 ± 0.19	1.11 ± 0.38
Other	0.614 ± 0.11	8.26 ± 0.33

(*) Mean ± SD calculated from a pool of 3 samples at the same condition

Table 2: Amino acid components of tra fish skin (Pangasius hypophthalmus)

Acid amin	**Component**	**Acid amin**	**Component**
Asp	51	Ile	9
Hyp	57	Leu	27
Thr	25	Tyr	5
Ser	32	Phe	13
Glu	96	Hyl	5
Pro	102	Lys	32
Gly	235	His	2
Ala	94	Arg	68
Val	25	Cys	120
Met	2		

Results of treating fish skin by NaOH

Figure 1 (1a, 1b, 1c, and 1d) shows that the more the concentration of NaOH solution increased (0.5%, 1.0%, 1.5%), the more the impurities and HP decreased, except for lipid.

Figure 1a shows that treatment by NaOH 1.0% yielded the best result, which was that lipid amount decreased from 15.4% to 2.75% after 6 hours and to 2.4% after 8 hours. Figure 1b shows that the amount of hydroxyproline in the skin decreased rapidly in coresponding with the increase of NaOH concentration. However, the goals of the purification were to minimize the loss of hydroxyproline, as well as to maximize the removal of lipid, impurities and ashes. To achieve these goals, we chose the NaOH treatment as following: NaOH concentration of 1%, skin/solution of 1/10, and duration of 6 hours. At this condition, the contents of components in the skin after treatment were: HP: 62.8 mg/g, organic substances: 33.09%, lipid: 2.75% and ashes: 0.17%.

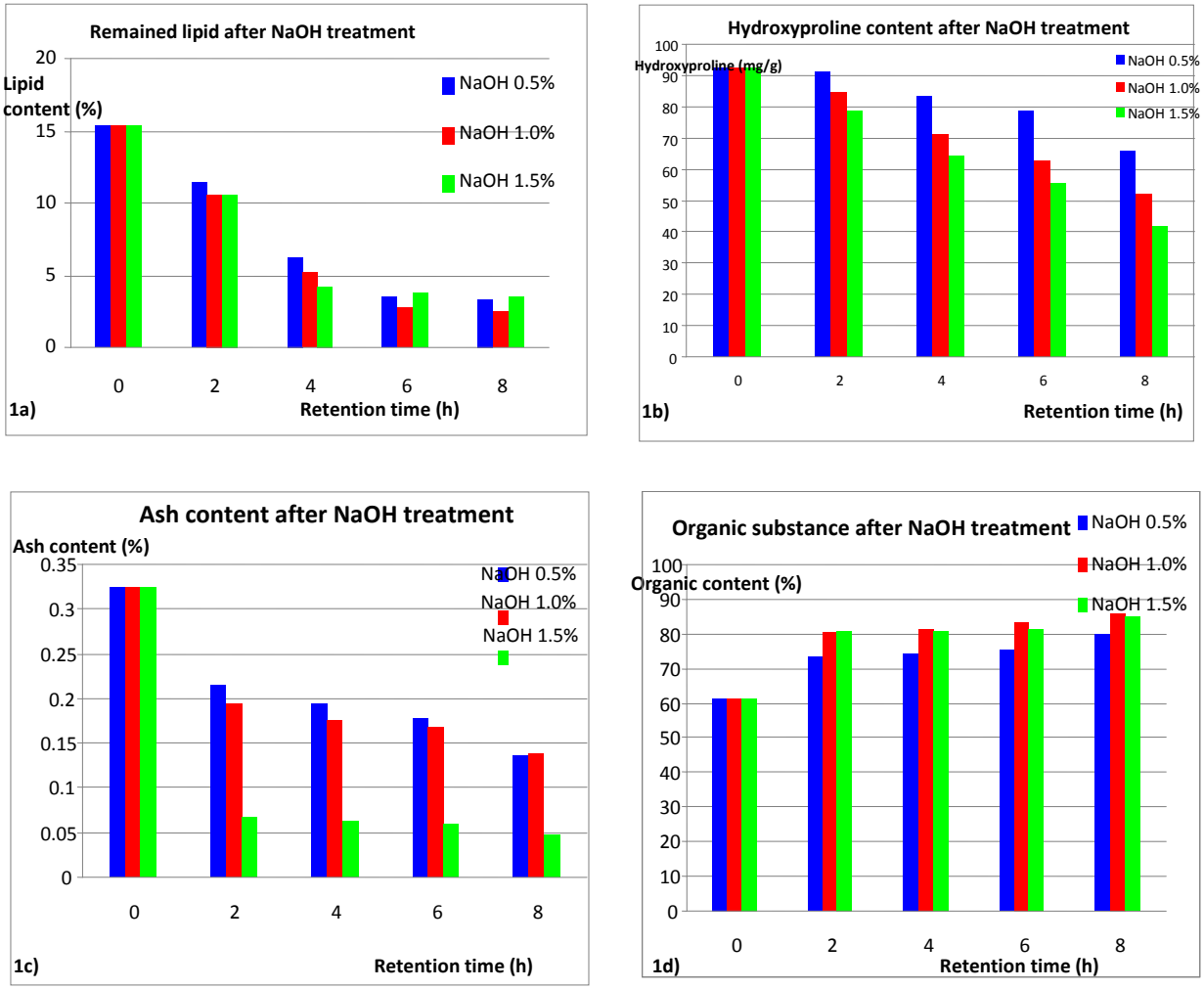

Figure 1: Influence of NaOH concentration by time on the amounts of lipid (1a), hydroxyproline (1b), ashes (1c) and organic substances (1d) in tra fish skin (Pangasius hypophthalmus)

Results of treating tra fish skin by LASNa

Figure 2 (2a, 2b, 2c, 2d) shows the influence of LASNa on the purification process and the loss of hydroxyproline (HP) by time. Figure (2a) shows the influence of LASNa on

the lipid removal. The findings were that treatment by LASNa 0.5% yielded the best efficiency, which was that the lipid amount decreased from 14.6% to 2.34% after 6 hours and to 2.12% after 8 hours. Figure (2b) - influence of LASNa on hydroxyproline loss by time – shows that the hydroxyproline content in the skin strongly decreased by the increase of the NaOH concentration. However, the goals of the purification were to minimize the hydroxyproline loss, and maximize the separation of impurities, lipid and ashes. To achieve these goals, we chose the condition for LASNa treatment as following: LASNa concentration was of 0.5%; ratio of skin/solution was 1/10, treatment length was 6 hours. At this condition, the amounts of the componnets in tra fish skin after treatment by LASNa 0.5% were: HP: 71.93 mg/g, organic substances: 19.33%, lipid: 2.34% and ashes: 0.14%.

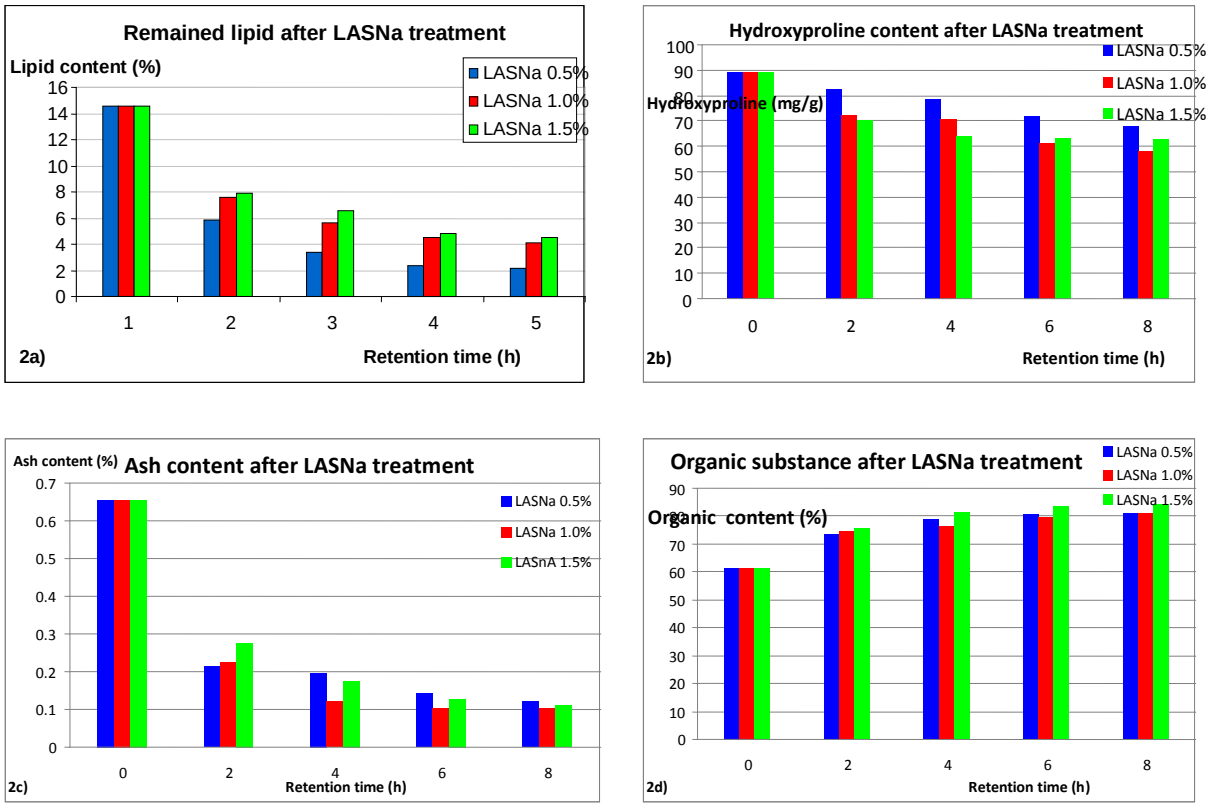

Figure 2: Influence of LASNa concentration by time on the amount of lipid (2a), hydroxyproline (2b), ashes (2c) and organic compounds (2d) in tra fish skin (Pangasius hypophthalmus)

Results of Ethanol Treatment

Figure 3 (3a, 3b, 3c, 3d) – influence of ethanol on the purification process and hydroxyproline (HP) loss by time- shows that: increase in the concentration of ethanol (5%, 10%, 15%) decreased the amount of orgaic compound and HP, except for lipid and ashes. Figure (3a) – influence of Ethanol on lipid removal by time – shows that: treatment by Ethanol 10% was the best method, which was that lipid decreased from 16.04% to 3.43%

after 24 hours and down to 3.16% after 48 hours. Figure (3c) demonstrated the changes of ashes amount by time treatment with ethanol at concentrations of 5%, 10% and 15%. The findings showed that when treating the skin by ethanol 5%, the ashes decreased very little. But when treating the skin by ethanol 10%, ashes amount decreased rapidly starting from 16-24 hours. After 24 hours, the decrease was not significant. When treating by ethanol 15%, ash amount decreased evenly by time, but not significantly. Figure (3d) – the influence of Ethanol on the hydroxyproline loss by time- shows that: the hydroxyproline amount in tra fish skin decreased evenly by the increase of concentration of NaOH solution, and by time. The least decrease of HP was when the skin was treated by ethanol 5%, and the most decrease was when treated by ethanol 15%. Nonetheless, the goals of the purification were to minimize the hydroxyproline loss, and maximize the separation of impurities, lipid and ashes. To achieve these goals, we chose the condition for Ethanol treatment as following: Ethanol concentration of 10%, skin/ solution of 1/10, treatment duration 24 hours. At this condition, the contents of the chemical compounds in tra fish skin after treatment by Ethanol 10% were: HP: 62.74 mg/g, organic compounds: 25.84%, lipid: 3.43% and ashes: 0.13%.

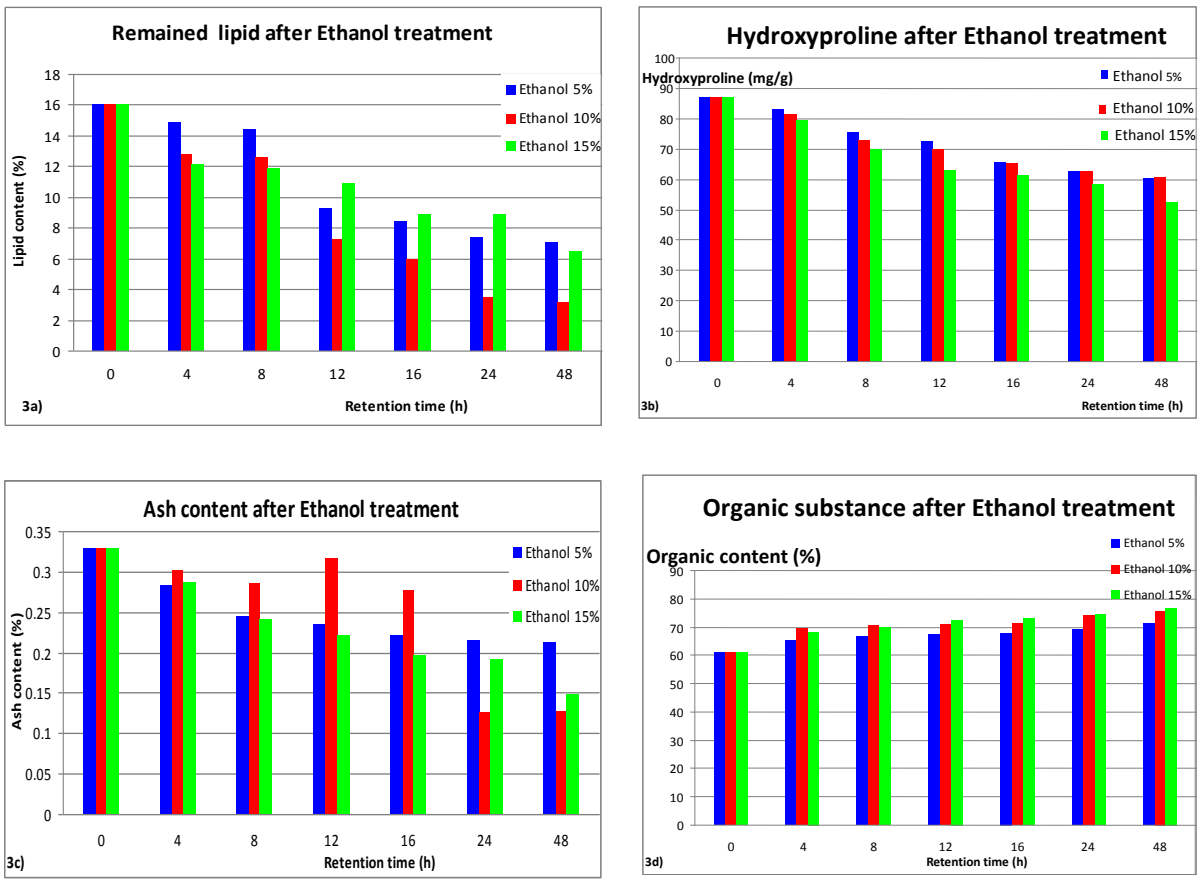

Figure 3: Influence of Ethanol concentration by time on the content of lipid (3a), hydroxyproline (3b), ashes (3c) and organic compound (3d) in tra fish skin (Pangasius hypophthalmus)

Results of H_2O_2 treatment

The skin originally was dark in the middle and lighter from the head to tail. In the collagen purification technology, ill color treatment will yield to products with grey color. Therefore, de-coloration is an important process in collagen purification. In previous studies, no evidence of the de-coloration was reported. In this study, we used H_2O_2 to de-color skin based on researches of extracting colorants from chicken feets and de-coloration of some seafoods. Acording to TCVN, H_2O_2 is used in food at the concentration below 3. H_2O_2 is a strong oxidizer, reduced quicky to yield water and oxy. Treating fish skin by H_2O_2 at the concentrations of 0.5%, 1.0% and 1.5% in NaOH 0.05N showed that at the concentration of 1.0%, after 2 hours, the skin was white, and much less fishy. Therefore, the de-coloration condition has been chosen as H_2O_2 1.0% reagent in 2 hours time.

Results of SDS-polyacrylamide (SDS-PAGE) gel electrophoresis

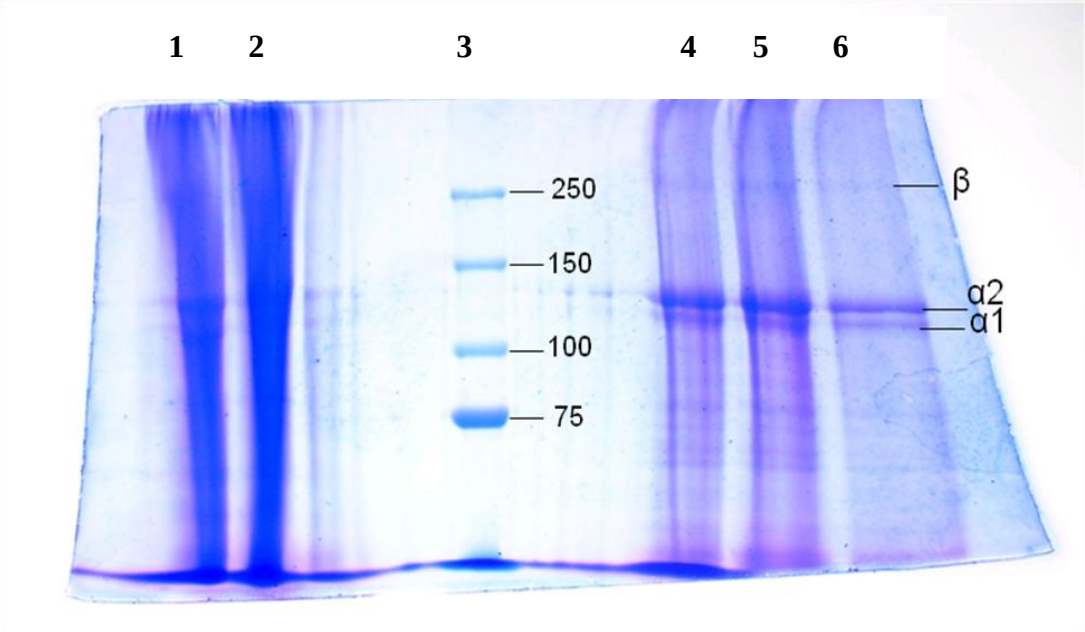

Figure 4: Column 1,2: Collagen before removing residue components; Column 3: protein standard; Column 4, 5, 6: Collagen after removing residue components.

Figure 4 shows that a collagen molecular purified from tra fish skin consists of two chains of α1 and α2, whose molecular mass are about 130kDa and an β chain whose molecular mass is about 250kDa. The electrophoresis result shows that collagen from tra fish skin was similar to collagen type I from skate *Raja kenojei* (Hwang et al., 2007).

Identifying the denaturalizing temperature of collagen

The denaturalizing temperature (T_d) of collagen extracted from tra fish skin was about 39 ^oC, determined based on the denaturalizing temperature curve in figure 5. Compared with the denaturalizing temperature of collagen from skin of brown backed toadfish, which is T_d = 28^oC (Senarate *et al.*, 2005), T_d of collagen from tra fish skin was about 11^oC higher. This

was because T_d of collagen relates to the body temperature of the fish and the temperature of its habitat environment (Rigby, 1968). Tra fish fish lives in fresh water with temperature of about 28-30^oC, then Td = 39^oC; while brown backed toadfish lives in sea water with temperature of about 20^oC then Td = 28^oC.

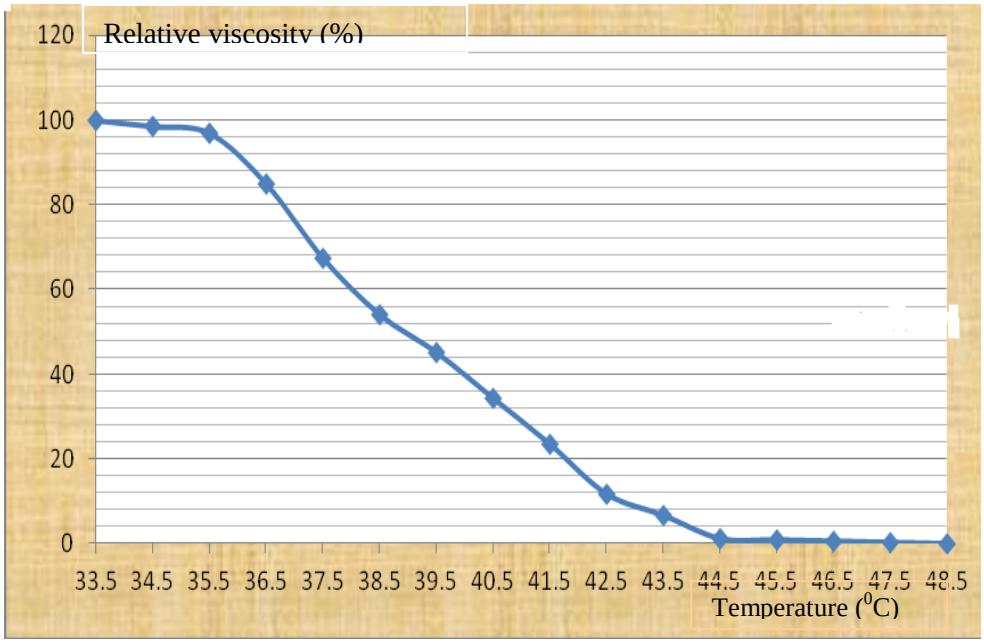

Figure 5: Denaturalizing temperature of collagen extracted from basa fish skin

Conclusions

After the comparison of three skin treatment methods by NaOH, LASNa, and Ethanol, the following conclusion could be figured out:

For economic aspect, based on the efficency of collagen yield (by the amount of hydroxyproline) and purification, we found that skin treatment with LASNa 0.5% in 6 hours was the best method. The components of the skin after treatment were: HP: 71.93 mg/g, organic compounds: 19.33%, lipid: 2.34% and ashes: 0.14%.

De-coloration of fish skin by H_2O_2 1% in NaOH 0.05N in 2 hours was appropriate. The skin after decoloration was white, and less fishy. The skin was a bit swollen and not denaturalized.

The results of purification of skin by LASNa 0.5%, H_2O_2 1% showed that the skin after purification (raw collagen) had the denaturalizing temperature T_d of 39^oC. The results of gel electrophoresis showed that collagen from tra fish skin was of the type I, composed of two chains of α1, α2 with the molecular mass of 130kDa and one β chain with molecular mass of 250kDa.

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TÁCH TẠP CHẤT TỪ DA CÁ TRA *(PANGASIUS HYPOPHTHALMUS)* ĐỂ THU NHẬN COLLAGEN

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Tóm tắt

Để tách các tạp chất: lipid, chất khoáng, chất màu, chất mùi từ da cá tra (*Pangasius hypophthalmus)*, các loại dung môi sau NaOH, LASNa, Ethanol và H_2O_2 đã lần lượt được sử dụng. Hiệu quả tách tạp chất xếp theo thứ tự sau: LASNa > NaOH > Ethanol. Kết quả xác định một số tính chất hóa lý của da cá tra sau khi tách tạp chất (collagen thô) bằng LASNa 0,5%, H_2O_2 1% cho thấy : collagen của da cá tra có nhiệt độ biến tính T_d khoảng 39^oC ; kết quả điện di cho thấy collagen của da cá tra là collagen kiểu I, thành phần cấu tạo gồm hai chuỗi α_1, α_2 có phân tử lượng khoảng 130kDa và chuỗi β có phân tử lượng khoảng 250kDa.