Green Technique for the Extraction of Acid Soluble Collagen from Basa Fish (*Pangasianodon hypothalmus*)

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***Abstract*- Basa fish (*Pangasianodon hypopthalmus*) is the top fifth most consumed fish in Thailand. The production of Basa fish generates a large number of by-products. Nowadays, most of these residues are recovered as animal feedstocks or fertilizer. However, it is interesting to explore another choice of waste management practice such as the recovery of valuable material. This study aimed to extract collagen from the skin and bone of Basa fish (*Pangasianodon hypopthalmus*) by using a green solvent. Two extraction methods were carried out. First, the conventional extraction was conducted by using acetic acid and compared with the physical extraction which employed water acidified with carbon dioxide. Conventional extraction was conducted using 0.75 M acetic acid at 4°C and solid-liquid ratio 1:40 (w/v). The physical extraction using water acidified by carbon dioxide was conducted at 37°C, 10 bars and solid-liquid ratio 1:40 (w/v). The extraction time of each technique was varied from 3 to 24 hours. The FTIR results confirmed the presence of amide A, amide B, amide I, amide II, and amide III of the extracted collagen obtained from these two techniques. When using fish skin and bone as raw material, the physical extraction technique increased the yield of dry weight collagen by 8 times and 7 times, respectively. Additionally, the physical extraction could shorten the processing time (from pretreatment until freeze-drying) by 56 hours.**

Keywords—Basa fish (Pangasianodon hypopthalmus); Collagen; Extraction; Green Technique

I. INTRODUCTION

Collagen is macromolecule which constitutes 30% of fibrous protein. It is widely used in various industrial applications such as the food industry, cosmetics, and medication [1-5]. It is estimated that the demand for collagen is approximately 326,000 tons each year [6]. Nowadays, 98% of collagen is from 2 major sources which are bovine and swine. However, the use of these two sources of raw material is threatened because of an occasional outbreak of some transmitted diseases such as Foot and Mouth Disease (FMD) and Bovine Spongiform Encephalopathy (BSE) [7]. Additionally, the utilization of swine related materials is prohibited by some religions [8]. Therefore, the use of alternative raw materials (such as fish) is required. It is noted that approximately 30% of the fish weight will be discarded as by-products and become worthless [9]. As Basa fish (*Pangasianodon hypopthalmus*) is one of the most consumed fish in Thailand, by-products of this fish can be one of the potential alternative raw-material for collagen extraction [10]. Many researchers had studied acid solubilized collagen (ASC) by using conventional extraction technique which employed acetic acid as a solvent. However, this technique required a number of processing steps, time-consuming, and used large quantities of chemicals [11]. Development of alternative collagen extraction methods which are considered as a green process in a way that minimizes using toxic chemicals and solvents, reducing extraction steps whilst producing competitive yields is still required [6]. So, the objective of this study was to investigate the possibility of using water acidified with carbon dioxide as an environmentally benign solvent for the extraction of collagen from skin and bone samples of Basa fish.

II. MATERIAL AND METHOD

A. Materials

Raw materials used in this study were skin and bone of Basa fish (*Pangasianodon hypopthalmus*) which were purchased from the market near our campus located in Bangkok, Thailand. Samples were cleaned with running water, followed by cutting into small pieces. The samples were sealed in polyethylene bags and stored in a -20°C freezer for further extraction process.

B. Method

Pretreatment Methods

In order to remove fat and non-collagenous substances, the pretreatment process was carried out by immersing the samples in 0.5% LASNa solution for 6 hours then followed by 1% H_2O_2 and 0.05N NaOH for 2 hours, both carried out at 4°C [11].

Collagen Extraction Using Conventional Method

After passing the pretreatment process, those two samples (skin and bone) with each weight of 20 grams were extracted using 0.75M acetic acid at 4°C. Solid-liquid ratio was controlled at 1:40 (w/v). Extraction time was varied from 3 to 24 hours (i.e. 3, 6, 12, and 24 hours). The dissolved collagen was filtered and saturated through the salting-out step with 0.9M NaCl. The purification step was carried out by dialysis using 0.1M acetic acid for 24 hours under stirring conditions where the solution was changed every 8 hours followed by a freeze-drying step for 24 hours [11].

Collagen Extraction Using Green Technique

Water acidified by carbon dioxide was used as a solvent. The extraction was conducted in a pressure vessel regulated at 37°C and a pressure of 10 bars. The solid-liquid ratio was controlled at 1: 40 (w/v), i.e. 20 grams of sample in 500 mL of distilled water. Extraction time was also varied from 3 to 24 hours (i.e. 3, 6, 12, and 24 hours). The collagen extracted in the sample was centrifuged and then dried through the freeze-drying process for 24 hours [5].

Calculation of Yield (%)

The yield (%) was determined based on the ratio between the weight of collagen extracted with the mass of the sample before the pretreatment process, and calculated based on dry weight basis [12].

Characterization – Fourier-transform Infrared Spectroscopy (FTIR)

FTIR characterization was conducted to investigate the functional group existed in collagen sample by subjecting freeze-dried collagen to ATR-FTIR (Nicolet 6700) and analyzed with the range of spectra from 4000 – 550 cm^{-1} with 4 cm^{-1} step of resolution and 16 number of scans as the result was recorded and determined using Spectragryph – optical spectroscopy software [13].

III. Result and Discussion

A. Extraction Yield (%) from Skin

A comparison study was conducted by using two different extraction techniques, i.e. conventional method and physical extraction with carbon dioxide acidified water which considered as a green technique. For the conventional method, it was found that extraction time has affected the percentage yield of the extracted collagen. Increasing extraction caused an increase in the percentage yield. As shown in Fig. 1, the highest yield was obtained when using 24 hours of extraction which was 1.7%. This finding has corresponded to the extraction yield from of previous researches reported such as collagen extracted from skin of; black drum 2.3%[14], balloon fish 4%[15], sole fish 1.93%[16], and bigeye snapper 1.59%[17] based on dry weight of ACS for 24 hours extraction process.

The effect of extraction on percentage yield was also observed when using a green technique using water acidified with carbon dioxide. When increasing extraction time from 3 to 24 hours, Percentage yields increased from 1.9% to 15.85%. This finding has corresponded to the previous research reported by Barros et al. (2014) where the application of water acidified with carbon dioxide could increase the yield extraction [6]. As can be seen in Table 1, the extraction of collagen by using green technique showed yield improvement by an average 8.06 times compared to the conventional method.

The reasons that gave a higher yield when using physical extraction with carbon dioxide acidified water related to a faster chemical reaction. Additionally, the reaction under pressurized fluid extraction in the vessel caused an increase in dissolving power and diffusion coefficients, low viscosity and high solvent strength. These conditions promoted the penetration of solvent into the solid matrix and enhanced the dissolution process that allowed the cellular matrix to desorb [18].

TABLE 1
Percentage Yield (%) of Collagen Extracted From Skin of Basa Fish *(Pangasianodon hypopthalmus)*

Extraction Time (hour)	Extraction of collagen						Yield Improvement (Times)
	Conventional method			Green technique			
	gram of collagen extracted (dry weight)	gram of collagen / kg of raw material (dry weight)	% Yield	gram of collagen extracted (dry weight)	gram of collagen / kg of raw material (dry weight)	% Yield	
3	0.04	2	0.21	0.38	19	1.90	9.04
6	0.08	4	0.43	0.63	31.5	3.15	7.32
12	0.17	8.5	0.85	1.12	56	5.60	6.57
24	0.34	17	1.70	3.17	158.5	15.85	9.32
Mean							8.06

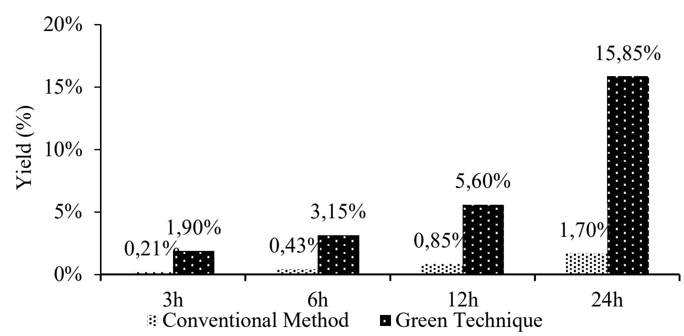

Fig. 1. Yield (%) of collagen extracted from Skin of Basa fish (*Pangasianodon hypopthalmus*).

B. Extraction Yield (%) from Bone

A comparison of the extraction yield obtained from the two methods was also carried out on bone samples. The same data pattern was observed as the data obtained from skin samples. The results showed that the extraction yield increased when the extraction time increased. As presented in Fig. 2, the highest yield was obtained when using 24 hour-extraction time, which was 1.70% and 15.85% from the conventional method and green technique, respectively. The average yield improvement was approximately 7 times as shown in Table 2. Furthermore, the percentage yield of bone collagen extraction was generally less than those values obtained from skin samples. This information corresponded to the data of previous research in a way that yield of ASC extracted from the skin of big eye tuna (*Thunnus obesus*) was higher than that obtained from its bone (13.5% and 2.6%) [19].

TABLE 2
PERCENTAGE YIELD (%) OF COLLAGEN EXTRACTED FROM BONE OF BASA FISH (*PANGASIANODON HYPOPTHALMUS*)

Extraction Time (hour)	Extraction of collagen						Yield Improvement (Times)
	Conventional method			Green technique			
	gram of collagen extracted (dry weight)	gram of collagen / kg of raw material (dry weight)	% Yield	gram of collagen extracted (dry weight)	gram of collagen / kg of raw material (dry weight)	% Yield	
3	0.02	1	0.11	0.11	5.5	0.55	5.02
6	0.05	2	0.26	0.46	23	2.30	8.84
12	0.08	4	0.42	0.78	39	3.90	9.28
24	0.17	8.5	0.84	1.37	68.5	6.85	8.15
Mean							7.76

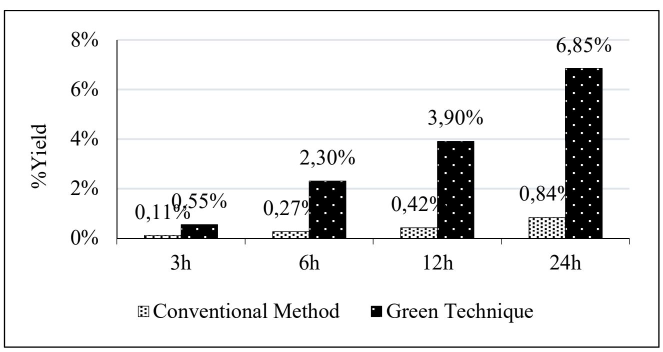

Fig. 2. Yield (%) of collagen extracted from bone of Basa fish (*Pangasianodon hypopthalmus*).

C. Processing Time

In order to assess the reaction efficiency, processing time of the two methods was recorded. Time-saving process is one of the indicators of achieving greener process [20]. As shown in Table. 3, using water acidified by carbon dioxide as a solvent instead of acetic acid could save processing time by 48 hours due to less processing step. As reported by Silva et al. (2016), this method is a one-step extraction process since it does not require salting out and dialyzing steps. From our observation, collagen was precipitated immediately after the extraction with water acidified by carbon dioxide had stopped [5].

TABLE 3
COMPARISON OF PROCESSING TIME BETWEEN CONVENTIONAL EXTRACTION AND EXTRACTION USING GREEN TECHNIQUE

Methods	Processing Time (hours)					Total
	Pre-treatment	Extraction	Salting out	Dialyzing	Freeze drying	
Conventional extraction	8	24	24	24	24	104
Green Technique	8	24	-	-	24	56

D. *Characterization by Fourier Transformed Infrared Spectroscopy (FTIR)*

From Fig. 3, FTIR spectra of the extracted collagen samples showed the presence of amide A, amide B, amide I, amide II, amide III as the main absorbance bands. As reported by the previous researcher. Amide A has a range of peaks between 3400-3440 cm^{-1} indicated N-H stretching vibration [21]. Since the sample used for extraction showed a lower number than those obtained from the Type 1 collagen standard. It was associated with a hydrogen bond formed with carbonyl groups present in the peptide chain**.** The Amide B band appeared approximately at 2900 cm^{-1} which associated with asymmetrical stretching of CH_2 [22]**,** where all collagen extracted showed those bands.

TABLE 4
FTIR SPECTRA PEAK LOCATIONS FOR COLLAGEN FROM BASA FISH (*PANGASIANODON HYPOPTHALMUS*)

Region	Type I Collagen Standard (cm^{-1})	Skin with Conventional Method (cm^{-1})	Bone with Conventional Method (cm^{-1})	Skin with Green Technique (cm^{-1})	Bone with Green Technique (cm^{-1})
Amide A	3307.17	3292.28	3279.63	3298.75	3278.50
Amide B	2952.41	2924.58	2920.20	2924.67	2917.43
Amide I	1628.25	1632.08	1632.10	1632.59	1628.45
Amide II	1549.58	1534.44	1540.46	1537.98	1537.95
Amide III	1235.96	1237.58	1238.99	1236.41	1236.79

Typical of collagen bands are shown by Amide I, II, and III [23] where the vibration ranges of those three bands are 1600-1700 cm^{-1}, 1500-1600 cm^{-1}, and 1200-1300 cm^{-1}, respectively. Amide I was associated with stretching vibration of carbonyl group C=O and hydrogen bonds between the carboxyl group and N-H which attributed to collagen triple helical structure [24].

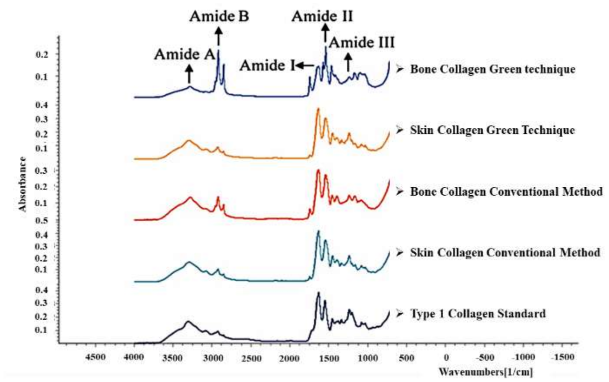

Fig. 3. FTIR spectra of collagen from Basa fish compared with type 1 collagen standard.

Furthermore, Amide II band attributed to both N-H bending and C-N stretching vibration [25]**.** Amide III corresponded to the deformation of N-H bend coupled with C-N stretching which included in the complex intermolecular interactions in collagen. Approximately, 1.0 ratio between bands shown at 1450 cm^{-1} has indicated the intact of triple helical structure of collagen was intact [26]. The result showed that collagen extracted both by conventional and green technique had the similarity on the native structure of collagen**.**

IV. CONCLUSION

In conclusion, both green technique and the conventional method can be used to extract acid soluble collagen from the skin and bone of Basa fish (*Pangasianodon hypopthalmus*). Additionally, the green technique could improve the percentage yield of approximately 8 and 7 times for skin and bone samples, also saved processing time up to 48 hours. In the case of characterization, FTIR confirmed the presence of Amide A, B, I, II, III as the native structure of collagen. According to the presented results, it can be concluded that water acidified with carbon dioxide as a green solvent may have great potential for the production of acid soluble collagen from Basa fish (*Pangasianodon hypopthalmus*).

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