OPTIMIZATION OF CONDITIONS FOR EXTRACTION OF COLLAGEN FROM THE SKINS OF BASA FISH *(PANGASIUS HYPOPHTHALMUS)* BY THE RESPONSE SURFACE METHOD

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ABSTRACT

Collagen has been used widely in the production of pharmaceutical and cosmetic products. Fish based collagen has still more advantages than other sources of collagen and becomes an object of interested researches recently. Extraction and some properties of collagens from the skin of *Pangasius hypophthalmus* fish were investigated. Pepsin enzyme and acetic-acid solution was used as an extracting solvent. The optimal conditions for collagen extraction were determined by response surface methodology. The effects of four independent variables (pepsin enzyme content, acetic acid concentration, liquid/solid ratio, and temperature) on the extraction yield of collagen from *Pangasius hypophthalmus* skin were evaluated. The optimal conditions to obtain the highest yield were determined as follows: pepsin enzyme content of 0.25 %, acetic acid concentration of 0.75 M, liquid/solid (w/w) ratio of 80, temperature of 14 ^{o}C. The predicted yield was 73 % which was in agreement with the actual value ($P < 0.05$). The molecular weights of α_1, α_2 and β chains in collagen were estimated to be 115 and 125 and 240 kDa, respectively.

Keywords: fish collagen, extraction, *Pangasius hypophthalmus.*

1. INTRODUCTION

Collagen is the most abundant protein in vertebrates making up approximately 30 % of total protein. Collagen is a major component of connective tissue, muscle, teeth, bone and skin. There are 19 types of collagen, labelled I-XIX. Collagen is composed of three similarly sized triple helix polypeptid chains. Each chain contains about 1000 amino acid residues in size and has an average length of 300 nm and diameter of 1.4 nm. Collagen has a repetitive primary sequence of which every third residue is glycine. The sequence of the polypeptide chain can be described as Gly-X-Y, in which X and Y are often found to be proline and hydroxyproline forming a left-hand super helix with the other two chains [1]. Collagen has been used in the biomedical, pharmaceutical [2], food and cosmetic industries [3, 4].

Response surface methodology is a statistical method that use quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equation [5]. Besides, this experimental methodology can generate a mathematical model and optimize the process level [6, 7]. So far, available publications on collagen extraction with response surface methodology are very limited. The objective of this work was to investigate the effects of above four variables on the yield of collagen solution extracted from the skin of *Pangasius hypophthalmus* fish by response surface methodology. Optimization of the extraction was also performed.

2. EXPERIMENTAL

2.1. Material

The skins of *Pangasius hypophthalmus* fish were supplied by the Viet An Company in An Giang province in Vietnam. Pre-treatment method was adopted from [8]. After removing remained flesh, the skins were washed in cold water, packed in PE bag and kept in a storage tank under -20 ^oC.

2.2. Collagen extraction

The skins of *Pangasius hypophthalmus* fish 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 0.05 N 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 skin were extracted with acetic acid and pepsin solution. Pepsin enzyme content, acetic acid concentration, the ratio of liquid/solid and temperature were chosen as variables with different levels. The extract was filtered out with filter cloth and this was followed by vacuum-filtering with a Whatman No.1 filter paper. The collagen was precipitated by adding NaCl to the final concentration of 0.9 M. The resulting sediment was collected by centrifuging at 12,000 rpm for 30 min. The collagen precipitate was dissolved in solution of 0.5 M acetic acid with the solid/ liquid ratio of 1/10 (w/v), then dialysed against 0.1 M acetic acid distilled water sequentially. The collagen was obtained by freeze-drying and this was followed by removing lipid from crude collagen with supercritical carbon dioxide.

2.3. Box-Behnken design

Response surface methodology was employed for experimental design, data analysis and model building with software Design Expert 8.0.6 Trial. A Box-Behnken design with four variables was used to determine the response pattern and then to establish a model [9, 10]. Four independent variables used in this work were pepsin enzyme content (X1), acetic acid concentration (X2), liquid/solid ratio (X3) and temperature (X3), with three levels for each variable, while the dependent variable was the extraction yield of collagen. The symbols and levels are shown in table 1. Six replicates at the central point of the designed model were used to estimate the pure error sum of squares. Experiments were randomised to maximise the effects of unexplained variability in the observed response, due to extraneous factors.

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

SDS-PAGE gel electrophoresis was performed in a mini-PROTEAN Tetra cell manufactured by BIORAD, using the buffer system of 0.1 % SDS, 0.025 M Tris and 0.192 M glycine [11]. 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 % (v/v) 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 kDa to 250 kDa.

Table 1. Box-Behnken design and the response for extraction yield of collagen from *Pangasius hypophthalmus* skin.

Exp	X1 (%)	X2 (M)	X3 (v/w)	X4 (^{0}C)	Yield (%)
1	0.25	0.25	20	3	30.6
2	0.75	0.25	20	3	31.8
3	0.25	0.75	20	3	35.6
4	0.75	0.75	20	3	34.7
5	0.25	0.25	80	3	37.6
6	0.75	0.25	80	3	39.5
7	0.25	0.75	80	3	36.3
8	0.75	0.75	80	3	38.9
9	0.25	0.25	20	17	46.8
10	0.75	0.25	20	17	45.8
11	0.25	0.75	20	17	46.9
12	0.75	0.75	20	17	45.8
13	0.25	0.25	80	17	60.8
14	0.75	0.25	80	17	53.6
15	0.25	0.75	80	17	69.2
16	0.75	0.75	80	17	61.1
17	0.50	0.50	50	10	59.6
17	0.50	0.50	50	10	45.7
17	0.50	0.50	50	10	60.7
17	0.50	0.50	50	10	56.4
17	0.50	0.50	50	10	60.7
17	0.50	0.50	50	10	56.6
18	0.15	0.50	50	10	69.2
19	0.85	0.50	50	10	40.5
20	0.50	0.15	50	10	60.5
21	0.50	0.85	50	10	68.4
22	0.50	0.50	7.57	10	59.4
23	0.50	0.50	92.43	10	62.4
24	0.50	0.50	50	0.1	15.3
25	0.50	0.50	50	19.9	50.4

2.5. Scanning electron microscopy

Collagen samples was diluted to equal concentrations of 3 mg/ml with milliQ water, were coated on clean glass coverslips and air dried at room temperature in clean air flow of a laminar flow hood. Dried samples were then platinum coated and examined in SEM 7410F – JMS – JEOL – Japan, at 50.000x magnification.

3. RESULTS

3.1. Effect of pepsin enzyme content, acetic-acid concentration, liquid/solid ratio and temperature on the extraction yield of collagen

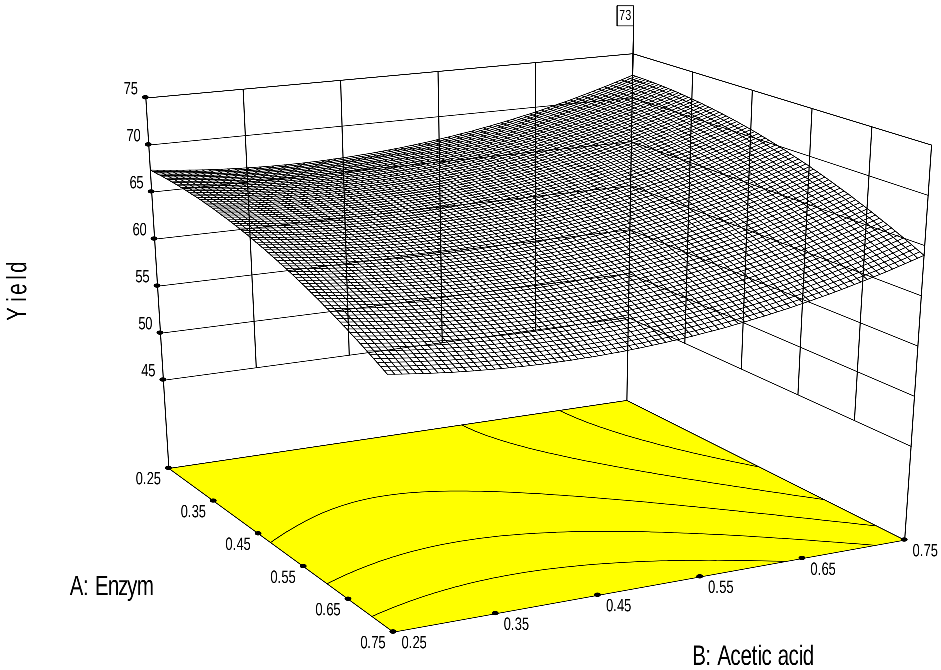

Figure 1. Response surface plot showing the effect of pepsin enzym content and acetic-acid concentration on the extraction yield of collagen. The ratio L/S was constant at 80; the temperature was constant at 13.87 °C.

The effects of pepsin enzyme content and acetic-acid concentration on the extraction yield of collagen from *Pangasius hypophthalmus* skin are shown in Fig. 1. The extraction yield of collagen was decreased with the increase of pepsin enzyme content. The extraction yield was slightly decreased when the acetic-acid concentration increase to a certain value (approximately 0.30 to 0.51 M) thereafter increased. The effect of liquid/solid ratio on the extraction yield of collagen is shown in Figs. 2 and 3. The extraction yield of collagen was increased when the liquid/solid ratio increase.

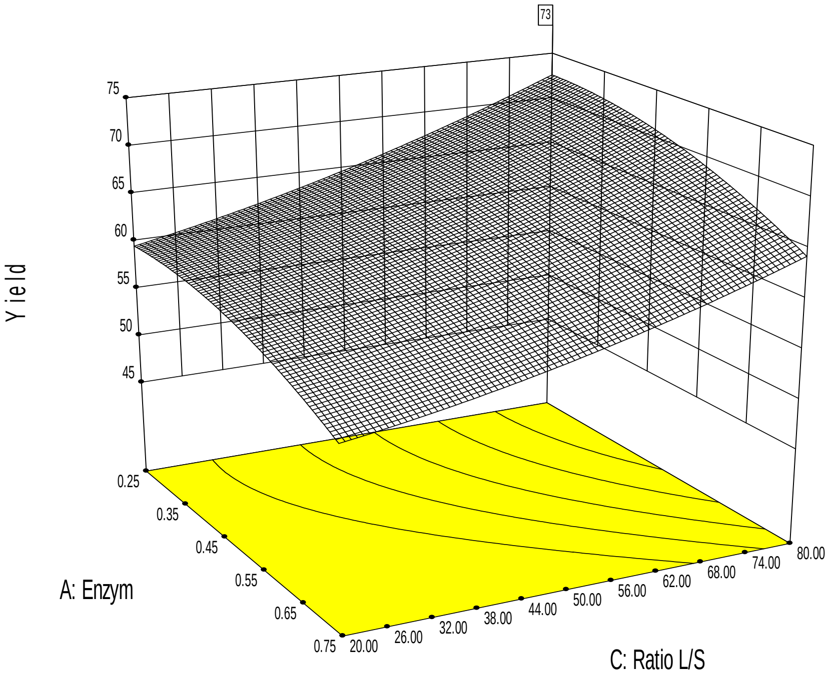

Figure 2. Response surface plot showing the effect of pepsin enzyme content and ratio L/S on the extraction yield of collagen. The acetic acid concentration was constant at 0.75 M; the temperature was constant at 13.87 ^{o}C.

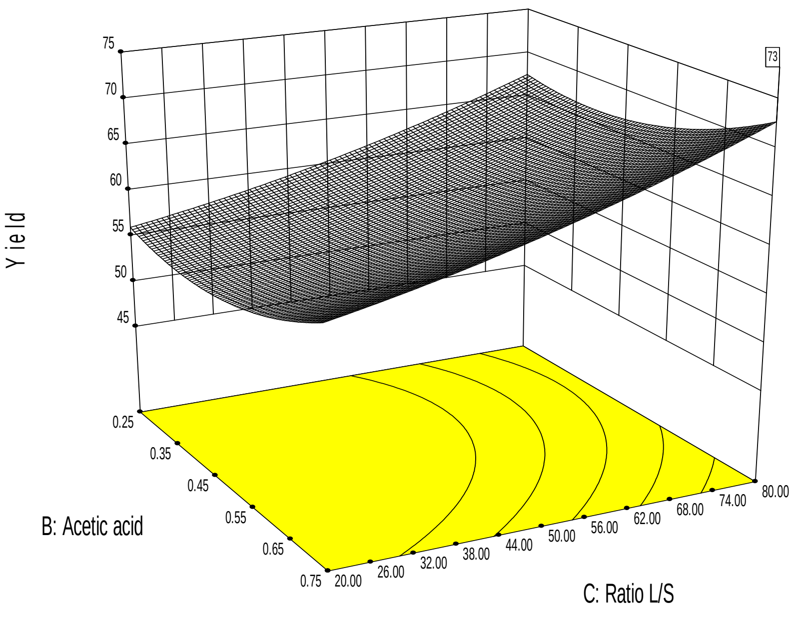

Figure 3. Response surface plot showing the effect of acetic-acid concentration and ratio L/S on the extraction yield of collagen. The pepsin enzyme content was constant at 0.25 % ; the temperature was constant at 13.87 ^{o}C.

3.2. Model fitting and optimization

The mathematical model representing the extraction yield of collagen as a function of the independent variables within the region under investigation was expressed by the following equation:

$$Y = 51.17-2.66X_1 +1.66X_2 +4.16X_3 +9.73X_4 -0.15X_1X_2 -0.56X_1X_3-1.39X_1X_4$$
$$+0.38X_2X_3 +0.0.63X_2X_4 +2.49X_3X_4 -1.93X^2{}_1 +2.87X^2{}_2 +1.09X^2{}_3 -12.93X^2{}_4 \quad (1)$$

where Y is the extraction yield of collagen, whereas X1, X2, X3 and X4 are the code variables for pepsin enzyme content, acetic-acid concentration, liquid/solid ratio and temperature, respectively.

The analysis of variance for the response surface quadratic model of the extraction yield collagen from *Pangasius hypophthalmus* skin was shown in Table 2. The P-value of the model was less than 0.0003; meanwhile, the lack of fit value of the model was 0.4819 which was not significant. These two values confirmed that the model fitness was good.

By analysis of variance, the R^2 value of this model was determined to be 0.89, which proved that the regression model defined the true behavior of the system.

By prediction with computing program, the optimal conditions to obtain the highest yield of collagen were determined as follows: a pepsin enzyme content of 0.25 %, a acetic-acid concentration of 0.75 M, a liquid/solid ratio of 80, a temperature of 13.87 ^oC. After extraction under these optimal conditions, the extraction yield of collagen was 72.8 0.3 % and this value was not significantly different from the predicted value 73 % within 95% confidence interval.

This result indicated that there was abundant collagen in *Pangasius hypophthalmus* skin. The extraction yield of collagen from *Pangasius hypophthalmus* skin is higher than that of Carp fish skin *Cyprinus carpio* ASC of 41.3 % [12] and big-eyed snapper PSC of 65.03 % [13] for 24 h.

Table 2. Analysis of variance for the response surface quadratic model of the extraction yield collagen from *Pangasius hypophthalmus* skin.

Source	Sum of squares	df	Mean square	F	p
Model	4213.74	14	300.98	7.26	0.0003
Residual	580.73	14	41.48	-	-
Lack of fit	431.66	10	43.17	1.16	0.4819
Pure error	149.07	4	37.27	-	-
Total	5166.47	29	-	-	-

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

The electrophoresis patterns of the samples are shown in Fig. 4. Collagen displayed one β band (250 kDa), and two α bands (α_1-130 kDa and α_2 -140 kDa). These patterns were similar to those of pepsin soluble collagen from the skin of Grass carp [14] and channel cat fish [15].

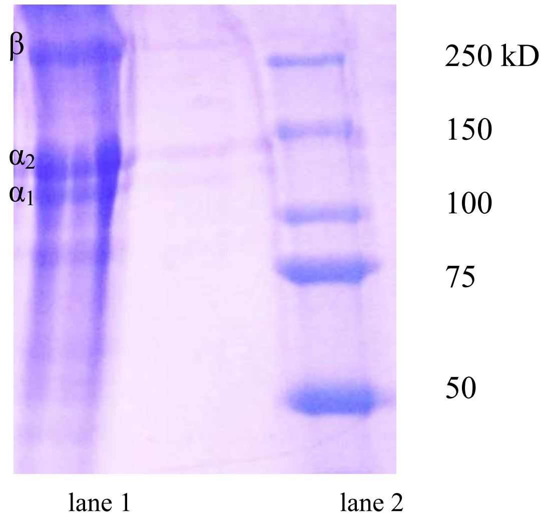

Figure 4. SDS-PAGE pattern of pepsin-solubilized collagen from *Pangasius hypophthalmus* skin. Lane: 1, pepsin-solubilized collagen; lane 2, protein markers.

3.4. Scanning electron microscopy

The scanning electron micrograph at 50,000x magnification are shown in Fig. 5. the surface morphologies of the collagen there were fibril networks with a rough membranous structure for the collagen membrane, the same as the surface morphology of the collagen membrane of bovine [16].

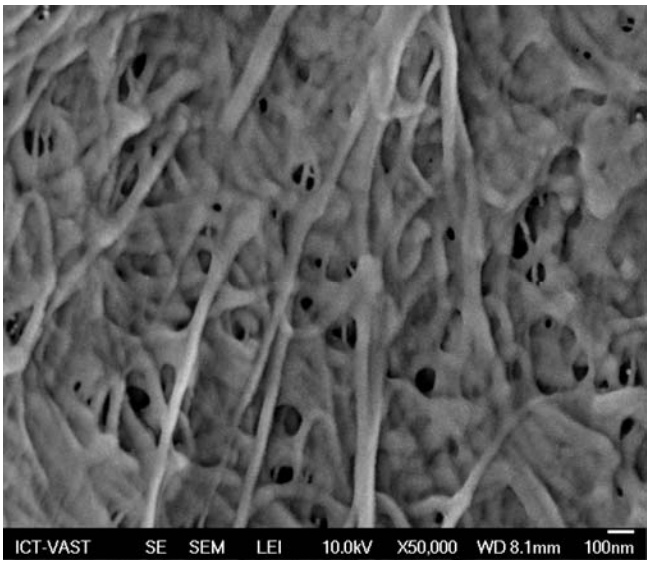

Figure 5. SEM surface morphologies of the collagen from *Pangasius hypophthalmus* skin.

4. CONCLUSIONS

The following conclusions are deduced from this study:

The effects of four variables (pepsin enzyme content, acetic acid concentration, liquid/solid ratio and temperature) on the extraction yield of collagen from the skin of *Pangasius hypophthalmus* fish were studied by response surface methodology. The optimal conditions to obtain the highest yield were determined as follows: pepsin enzyme content of 0.25 %, acetic-acid concentration of 0.75 M, a liquid/solid ratio of 80, a temperature of 13.87 ^{o}C. The predicted yield was 73 % which was in agreement with the actual value ($P < 0.05$).

The results of research on the structural characteristics of collagen show that surface morphologies of collagen were fibril pattern. The large amount of type I collagen has subunit composition α_1, $\alpha_{2,}$ and β.

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TỐI ƯU HÓA QUÁ TRÌNH TRÍCH LI COLLAGEN TỪ DA CÁ TRA (*PANGASIUS HYPOPHTHALMUS)* BẰNG PHƯƠNG PHÁP BỀ MẶT ĐÁP ỨNG

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TÓM TẮT

Collagen là một loại protein được ứng dụng rộng rãi trong các ngành sản xuất thực phẩm, mỹ phẩm và dược phẩm. Collagen cá có nhiều ưu điểm hơn collagen từ các nguồn khác, do đó gần đây các nhà khoa học tập trung nghiên cứu công nghệ tách chiết collagen từ cá. Bài báo hướng đến mục tiêu tối ưu hóa hiệu suất trích li collagen từ da cá Tra (*Pangasius hypophthalmus*) bằng phương pháp bề mặt đáp ứng, đồng thời xác định một số tính chất của collagen trích li được. Khảo sát ảnh hưởng của các biến số (X1- hàm lượng enzyme pepsin, X2- nồng độ acid acetic, X3- tỉ lệ dung dịch/da cá, X4- nhiệt độ) đến hiệu suất trích li (Y). Hiệu suất tối ưu là 73 % ($P < 0,05$) đạt được khi trích li da cá với hàm lượng enzyme pepsin là 0,25 %, acid acetic: 0,75 M, tỉ lệ dung dịch/da cá là 80/1 (v/w) và nhiệt độ 14 ^{o}C. Kết quả chụp SEM cho thấy collagen có cấu trúc sợi; hình chụp điện di cho thấy collagen thuộc loại I gồm các chuỗi đơn vị là α_1, α_2 và β có phân tử lượng lần lượt là 115 kDa, 125 kDa và 240 kDa.

Từ khóa: collagen cá, trích li, *Pangasius hypophthalmus.*