Optimization of conditions for extraction of collagen from the skin of *Pangasius hypophthalmus* by response surface methodology

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Abstract: Collagen has been used widely in the production of pharmaceutical and cosmetic products. Fishbased 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 determinded 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 13.9 ^{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 130, 140 and 250 kDa, respectively.

***Keywords**: fish collagen, pangasius hypophthalmus, optimization, response surface*

Tóm tắt: Collagen được sử dụng rộng rãi trong sản xuất các sản phẩm dược phẩm, mỹ phẩm. Gần đây, collagen tách chiết từ cá được ưa chuộng hơn collagen tách chiết từ các nguồn nguyên liệu khác. Bài báo này đề cập đến việc tách chiết collagen từ da cá tra *Pangasius hypophthalmus* và xác định một số tính chất của collagen tách được. Dung môi dùng để tách chiết collagen là acid acetic và enzyme pepsin. Các yếu tố ảnh hưởng đến hiệu suất tách chiết collagen là: hàm lượng enzyme pepsin, nồng độ acid acetic, tỷ lệ lỏng/rắn và nhiệt độ. Tối ưu hóa các yếu tố ảnh hưởng đến hiệu suất tách chiết collagen bằng phương pháp đáp ứng bề mặt, xác định được điều kiện tối ưu như sau: hàm lượng enzyme pepsin: 0,25%; nồng độ acid acetic: 0,75M; tỷ lệ lỏng/rắn: 80; nhiệt độ: 13,9^{o}C. Hiệu suất tách chiết đạt 73%, với độ tin cậy 95% (p <0,05). Phân tích cấu trúc bằng máy chụp SEM cho thấy collagen tách được là một loại protein sợi cơ. Collagen tách từ da cá tra gồm ba chuỗi α_1, α_2 và β có phân tử lượng lần lượt là: 130 kDa, 140 kDa và 250kDa.

***Từ khóa:** collagen cá, pangasius hypophthalmus, tối ưu hóa, đáp ứng bề mặt*

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, lableed 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 300nm 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 (Whitford, 2005).

Collagen has been used in the biomedical, pharmaceutical (Lee et al.,2001), food and cosmetic industries (Kim and Mendis, 2006; Senaratne et al., 2006).

Response surface methodology is a statistical method that use quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equation (Triveni, Shamala and Rastogi, 2001). Besides, this experimental methodology can generate a mathematical model and optimize the process level (Bas and Boyaci, 2007; Yang, Zhao, Shi, Yang, & Jiang, 2008). 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.

Experimental

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 Le Thi Thu Huong *et al.* (2010). After removing remained flesh, the skins were washed in cold water, packed in PE bag and kept storage under -20^{o}C.

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 0.05N 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 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 0.9M. The resulting sediment was collected by centrifuging at 12,000 rpm for 30 min. The collagen precipitate was dissolved in solution of 0.5M acetic acid with the solid/ liquid ratio of 1/10 (w/v), then dialysed against 0.1M 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.

Box-Behnken design

Response surface methodology was employed for experimental design, data analysis and model building with software Design Expert 8.0.6. A Box-Behnken design with four variables was used to determine the response pattern and then to establish a model (Box & Behnken, 1960; Quang, Yang, Du, & Yi, 2008). Four independent variables used in this work were pepsin enzyme content (X_1), acetic acid concentration (X_2), liquid/solid ratio (X_3) and temperature (X_4), 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.

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

Exp	X_1 (%)	X_2 (M)	X_3	X_4 (deg 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	38.9
10	0.75	0.25	20	17	46.8
11	0.25	0.75	20	17	45.8
12	0.75	0.75	20	17	46.9
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	0.10	15.3
25	0.50	0.50	50	19.90	50.4

Gel SDS-polyacrylamide (SDS-PAGE) electrophoresis

SDS-PAGE gel electrophoresis was performed (Laemmli, 1970) using the 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 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.

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.

Results

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

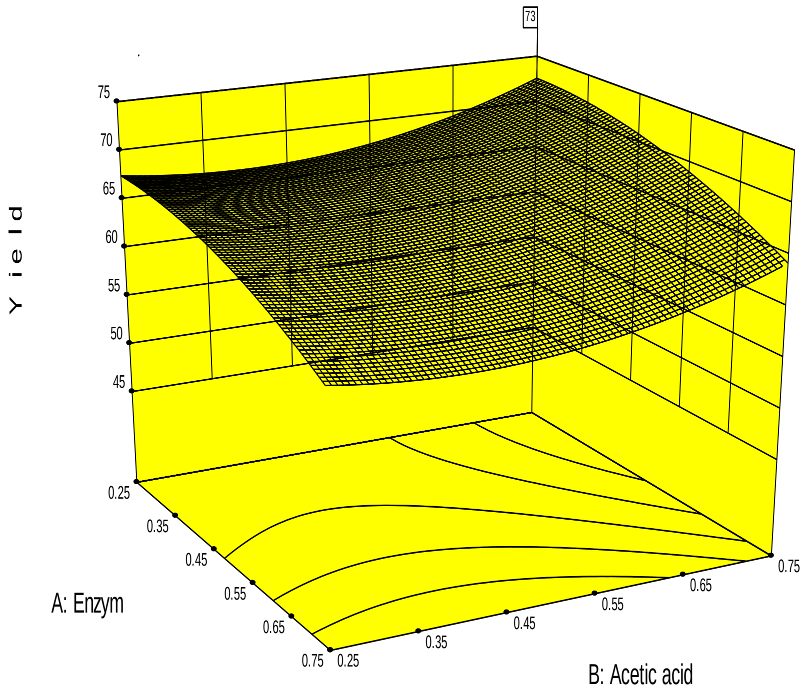

Fig.1 Response surface plot showing the effect of pepsin enzym content and acetic-acid concentration on the extraction yield of collagen. The ratio L/S was constant at 80; the temperature was constant at 13.9^{o}C.

The effects of pepsin enzym 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 enzym 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.

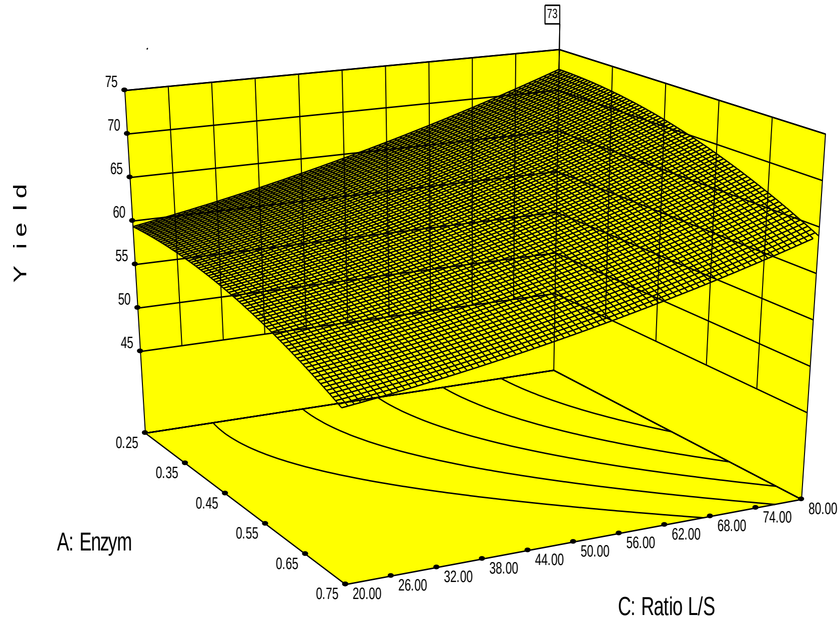

Fig.2 Response surface plot showing the effect of pepsin enzym content and ratio L/S on the extraction yield of collagen. The acetic acid concentration was constant at 0.75M ; the temperature was constant at 13.9^{o}C.

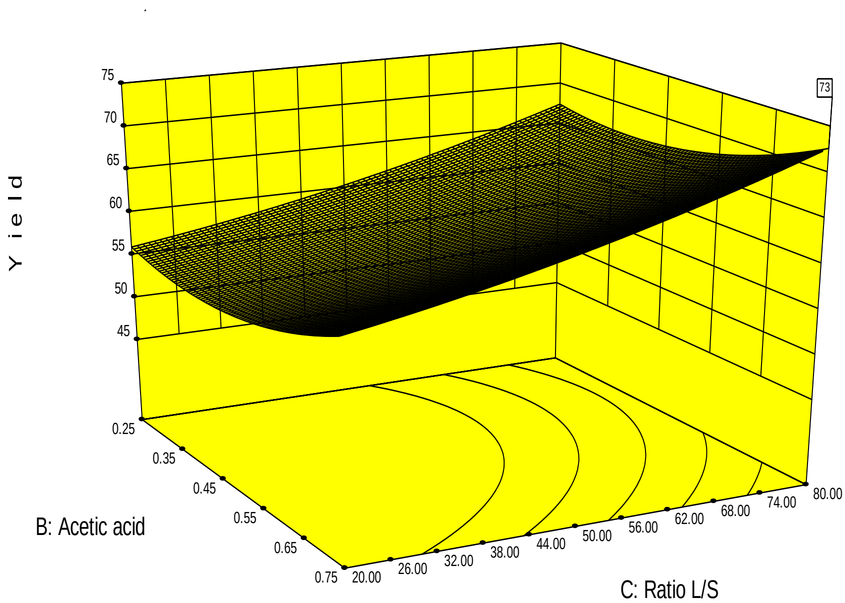

Fig.3 Response surface plot showing the effect of acetic-acid concentration and ratio L/S on the extraction yield of collagen. The pepsin enzym content was constant at 0.25%; the temperature was constant at 13.9^{o}C.

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 X_1, X_2, X_3 and X_4 are the code variables for pepsin enzym 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 enzym content of 0.25%, a acetic-acid concentration of 0.75M, a liquid/solid ratio of 80, a temperature of 13.9^{o}C. 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 the extraction yield of Carp fish skin *Cyprinus carpio* ASC (41.3%) (Rui Duan et al.,2009) and higher than the extraction yield of big-eyed snapper PSC (65.03%) (Sitthipong Nalinanon et al., 2007) for 24h.

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			

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 (Yan et al, 2007) and chanel cat fish (Liu et al, 2007).

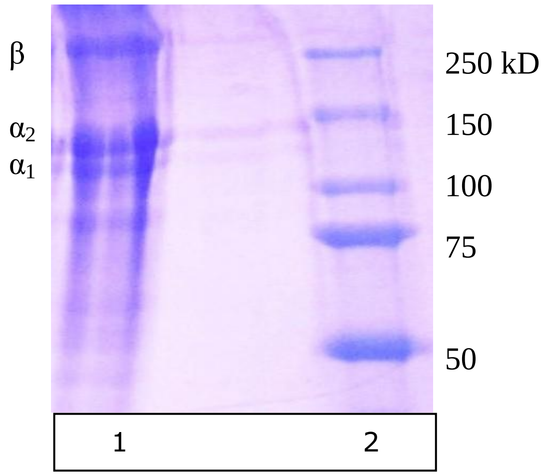

Fig.4 SDS-PAGE pattern of pepsin-solubilized collagen from *pangasius hypophthalmus* skin. Lane: 1, pepsin-solubilized collagen; 2, protein markers.

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 (Zhongkai Zhang et al., 2005).

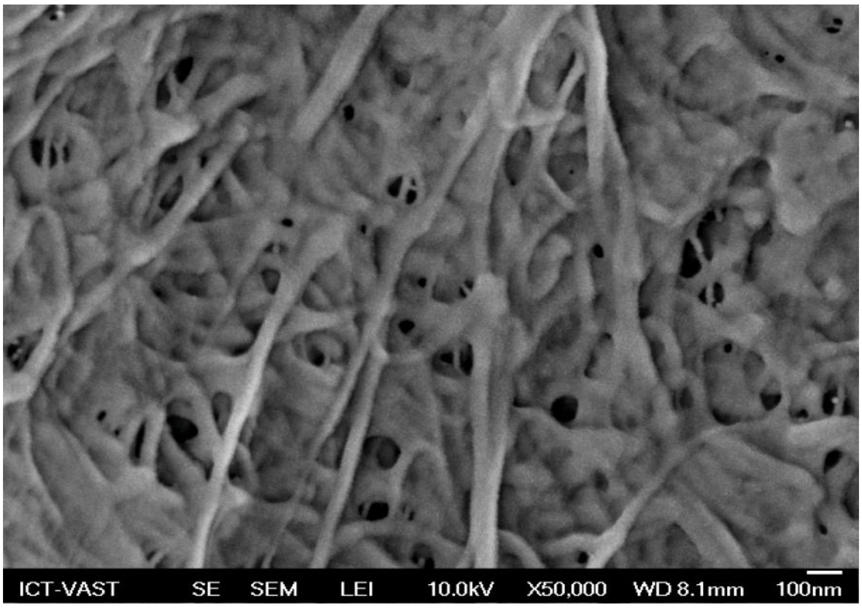

Fig.5 Surface morphologies of the collagen from *pangasius hypophthalmus* skin observed by SEM.

Conclusions

The following conclusions are deduced from this study:

The effects of four variables (pepsin enzym content, acetic-acid concentration, liquid/solid ratio and temperature) on the extraction yield of collagen from the skin of *Pangasius hypophthalmus* fish by response surface methodology. The optimal conditions to obtain the highest yield were determinded as follows: a pepsin enzym content of 0.25%, a acetic-acid concentration of 0.75 M, a liquid/solid ratio of 80, a temperature of 13.9 ^{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 was fibril pattern. The large amount of type I collagen having subunit composition α_1, $\alpha_{2,}$ and β.

Acknowledgements

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References

[1] Bas, D., and Boyaci, I. H., 2007. Modeling and optimization I: Usability of responce surface methodology. *Jounal of Food Engineering*. Vol.78, pp 836-845.

[2] Box, G. E. P., and Behnken, D. W., 1960. Some new three level design for study of quantitative variables. *Technometric*. Vol.2, pp 455-475.

[3] Kim, S.K. and E. Mendis., 2006. Bioactive compounds from marine processing byproducts- a review. *Food Research Intl*. Vol.39, pp 383-393.

[4] Lee, C.H et al., 2001. Biomedical applications of collagen. *Inter.J. of Pharma*. Vol.221, pp 1-22.

[5] Le Thi Thu Huong, Nguyen Ngoc Truong, Nguyen Hoang Dung, Phan Dinh Tuan., 2010. Treatment of Tra fish *(pangasius hypophthalmus)* skin for collagen extraction. *Journal of Science and Technology.* Vol. 48, 6A, pp 319-328.

[6] Liu, H et al., 2007. Studies on collagen from the skin of channel catfish (*Ictaurus punctaus*). *Food Chemistry*. Vol. 101, pp 621-625.

[7] Rui Duan, Junjie Zhang, Xiuqiao Du, Xingcun Yao and Kunihiko Konno., 2009. Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chemistry*. Vol.112, pp 702-706.

[8] Senaratne, L. S et al., 2006. Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Bioresource Tech*. Vol.97, pp 191-197.

[9] Sitthipong Nalinanon, Soottawat Benjakul, Wonnop Visessanguan and Hideki Kishimura., 2007. Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry*. Vol.104, pp 593-601.

[10] Triveni, R et al., 2001. Optimised production and utilization of exopolysaccharid from Agrobacterium radiobacter. *Processing Biochemistry*. Vol.36, pp 787-795.

[11] Wang, L. Z et al., 2008. Optimisation of supercritical fluid extraction of flavonoids from *Pueraria lobata*. *Food chemistry*. Vol.108, pp 737-741.

[12] Whitford, D., 2005. The structure and function of fibrous proteins. *Protein: structure and function*, John Wiley & Sons Ltd., pp 92-97.

[13] Yan, Z., et al., 2007. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp *Ctenopharyngodon idella*. *Food chemistry*. Vol. 103, pp 906-912.

[14] Yang, B et al., 2008. Effect of ultrsonic treatment on the recovery and DPPH radical scavenging activity of polysaccharides from longan fruit pericarp. *Food chemistry*. Vol. 106, pp 685-690.
[15] Zongkai Zang, Guoying Li and Bi Shi., 2005. Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal of the Society of Leather Technologists and Chemists*. Vol.90, pp23.