Optimization of enzyme-assisted extraction and characterization of collagen from Chinese sturgeon (*Acipenser sturio Linnaeus*) skin

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Submitted: 14-07-2012 Revised: 19-09-2012 Published: 07-09-2013

ABSTRACT

Background: Sturgeon (*Acipenser sturio Linnaeus*) skin contains high amount of nutrients including unsaturated fatty acids and collagen. A pepsin-assisted extraction procedure was developed and optimized for the extraction of collagen from Chinese sturgeon (*Acipenser sturio Linnaeus*) skins. **Objective:** To determine the optimum conditions with the maximum yield of the pepsin-soluble collagen (PSC) extraction. **Materials and Methods:** The conditions of the extraction were optimized using response surface methodology. The Box–Behnken design was used to evaluate the effects of the three independent variables (extraction time, enzyme concentration, and solid–liquid ratio) on the PSC yield of the sturgeon skin. **Results:** The optimal conditions were: solid–liquid ratio of 1:11.88, enzyme concentration of 2.42%, and extraction time of 6.45 h. The maximum yield of 86.69% of PSC was obtained under the optimal conditions. This value was not significantly different from the predicted value (87.4%) of the RSM ($P < 0.05$). **Conclusion:** The results of this study indicated that the production of PSC from sturgeon skin is feasible and beneficial. The patterns of sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns (SDS-PAGE) indicated that the sturgeon skin contains type I collagen, which is made of α-chain and β-chain. The infrared spectra of the collagens also indicated that pepsin hydrolysis does not affect the secondary structure of collagen, especially triple-helical structure.

Key words: Extraction, pepsin-soluble collagen, response surface methodology, sturgeon skin, SDS-PAGE

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.117859

Quick Response Code:

INTRODUCTION

Sturgeon (*Acipenser sturio Linnaeus*) is one of the most common freshwater fish consumed worldwide. This is as results of its high nutrient (unsaturated fatty acids and proteins) content with a good mouth-feel taste. Chinese sturgeon is a prehistoric fish that attracts price for its high quality flesh, and it is suitable for processing to other fish products. The nutritional value coupled with the high patronage of the sturgeon has resulted in increased production ("Sturgeon Aquaculture").[1] Statistics available indicates that the annual production of sturgeon in China alone exceeds 10,000 tonnes.[2] Sturgeon can be used for sturgeon fillet production. During processing, solid waste is mostly generated, especially at the skins and cartilages. Sturgeon's skin is a form of connective tissue that contains a lot of collagen, proteoglycans, and water,[3] and that can be used as a potential raw material for collagen extraction.

According to Kittiphattanabawon *et al.*,[4] sturgeon skin contains collagen that is used to manufacture leathers. The collagen also used in the film industries, pharmaceuticals, and for cosmetic, food, and biomedical materials. Collagen is commonly isolated from the skins and bones of sturgeon fish. It is a crucial structural protein responsible for the formation of tissues such as skin, bone, and tendon. Collagen also provides support for organs and protect tissues.[5] The molecules of collagen have common triple-helical domains with each of the three α-helices containing the repeating sequences Gly-X-Y (X and Y are proline and hydroxyproline residues, respectively).[6]

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The extraction of collagen from fish skin was carried out using pepsin and acid.[7,8] As far as we are concerned, not much research has been conducted on the optimal conditions for the optimum yield of pepsin-soluble collagen (PSC). Factors such as extraction time, enzyme concentration, and solid–liquid ratio affect the extraction ability of the collagen. In this study, response surface methodology (a statistical method that uses quantitative data from an appropriate experimental design for determining or simultaneously for solving multivariate equations) was use to generate mathematical model and optimize the process levels.[9–11] The aim of the study was to investigate the effect of extraction time, enzyme concentration, and solid–liquid ratio on the yield of PSC extracted from sturgeon skin using response surface methodology and to determine the structure of sturgeon skin characterization.

MATERIALS AND METHODS

Materials and Chemicals

Pepsin was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). High molecular weight protein marker and calf-skin collagen were produced at TaKaRa Biotechnology (Dalian) Co., Ltd. and Sigma Chemical Co., respectively. All solvents and chemicals were of analytical grade and obtained from Zhenjiang Chemical Company (Zhenjiang, Jiangsu, China). Distilled water was used throughout the study. The live sturgeons (average weights in the range of 1.5–2.5 kg) were obtained from Lianchuang Aquatic Science and Technology Demonstration Park of Zhenjiang, China. After killing the sturgeons, the skins were washed with water and frozen at −18 °C for 2 weeks (prior to collagen extraction).

Preparation of collagen from sturgeon skin

The collagens were extracted using the method of Nalinanon *et al*.[8] with slight modification. All the preparation procedures were carried out at a temperature of 4 °C.

Pretreatment of sturgeon skin

The skins of sturgeons were first thawed and washed with water. The residual meat on the skins were removed manually and washed with water. The skins were then chopped into lengths of 0.5–1.0 cm and further washed with water. To remove noncollagenous proteins, the prepared sturgeon skins were mixed with 3.5% NaCl at a solid ratio of 1:10 (w/v) followed by continuous stirring at 200 rpm for 24 h. The NaCl solution was changed every 4 h. The skins were then washed with water and defatted with 0.5% Na_2CO_3 at a solid–liquid ratio of 1:20 (w/v), followed by continuous stirring at 200 rpm for 48 h. The 0.5% Na_2CO_3 solution was changed every 8 h. The defatted skins were washed with water till the neutral point, and then freeze-dried (prior to collagen extraction).

Pepsin-soluble collagen extraction

The defatted skins were subjected to collagen extraction using pepsin solution. Extraction time, enzyme concentration, and solid–liquid ratio were the chosen variables with five levels. The resulting viscous solution was centrifuged at 5000 rpm for 20 min in order to remove the insoluble substances. The collagen was then precipitated by adding $(NH_4)_2SO_4$ to the final concentration of 2.6 M in the presence of 0.05 M tris(hydroxymethyl)aminomethane (pH 7.0). The resulting sediments were collected by centrifugation at 5000 rpm for 20 min and dissolved in water, then sequentially dialyzed against water. The dialyzates were freeze-dried and denoted as PSC.

Box–Behnken design

Among the PSC extraction, the one with the highest content was chosen for further optimization with RSM (software Design Expert 6.0, Stat-Ease Inc., USA). A Box–Behnken design with three variables was used to determine the response pattern and to establish the statistical model.[12] The effects of extraction time (X_1), solid–liquid ratio (X_2), and enzyme concentration (X_3) on the enzymatic extraction of the PSC from sturgeon skin were then determined with RSM at a three-variable and five-level. The observed values for the PSC extracted at different combinations of the independent variables are presented in Table 1. The

Table 1: Box–Behnken design and the response for the content of pepsin-soluble collagen from sturgeon skin

No.	Coded levels			Response
	Extraction time, X_1	Solid–liquid ratio, X_2	Enzyme concentration, X_3	Yield of pepsin-soluble collagen (%)
1	0	0	0	77.33
2	1	1	0	84.04
3	1	0	−1	62.23
4	1	0	1	70.20
5	0	0	0	77.21
6	0	0	0	81.84
7	−1	−1	0	25.75
8	−1	1	0	64.21
9	0	1	1	74.31
10	0	0	0	84.91
11	−1	0	−1	47.82
12	0	1	−1	67.73
13	0	−1	1	46.64
14	1	−1	0	52.47
15	0	0	0	84.69
16	−1	0	1	64.62
17	0	−1	−1	23.57

X_1, extraction time; X_2, solid–liquid ratio; X_3, enzyme concentration

coded levels of the independent variables used in BBD are also listed in Table 2.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; DYCZ-24DN mini double vertical electrophoresis apparatus and DYY-6C-type bi-stable electrophoresis power supply, Beijing 61 Instrument Factory, China) was performed to determine the purity of PSC described by Suphatharaprateep.[13] The samples were dissolved in a 50 g/L SDS solution. The mixtures were then heated to 100 °C for 15 min, followed by centrifugation at 8500 rpm for 5 min to remove undissolved debris. The solubilized samples were mixed with the buffer sample (0.5 mol/L Tris–HCl, pH 6.8 containing 40 g/L SDS, 4% glycerol in the presence or absence of 20% β ME) in the ratio of 1:1 (volume ratio). The mixtures were loaded onto a polyacrylamide gel, which was made of 75 g/L separating gel and 40 g/L stacking gel and then subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, the gels were stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 30 min. The gels were then de-stained with a mixture of 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight protein markers were used to estimate the molecular weight of the proteins. Type I collagens from calf-skins were used as standard collagens.

Table 2: Coded levels of independent variables used in the RSM design

Coded variable levels	Extraction time (X_1) (h)	Solid–liquid ratio (X_2)	Enzyme concentration (X_3) (%, w/w)
1	9	1:14	3
0	6	1:10	2
–1	3	1:6	1

Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) (Nexus 470 Fourier transform infrared spectrometer, Thermo Nicolet Co., USA) spectroscopy of collagens were analyzed using the method of Cao *et al*. and Xu *et al*.[14,15] The FTIR spectra were obtained at a resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1} at room temperature. The FTIR spectra were obtained with discs containing 2 mg samples in approximately 100 mg potassium bromide (KBr disk) with a Fourier transform IR instrument.

RESULTS AND DISCUSSION

Optimization of conditions for extraction of PSC

The analysis of variance (ANOVA) of RSM on the enzymatic extraction of PSC from the sturgeon skins is shown in Table 3. The exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fit, which makes checking of the model adequacy essential.[16] The *P* value of the model was less than 0.0001. Lack of fitness value of the model was 0.2389, which was not statistically significant. These two values confirmed the goodness of the model fitness.

Coefficient of determination (*R*-Squared) is defined as the ratio of the variation of the variables under consideration to the total variation, which measures the degree of fitness.[17] The value of *R*-Squared less than 1 indicates a poor relevance of the dependent variables in the model. The model can fit well with the actual data when *R*-Squared approaches unity (0).[18] The coefficient of determination (*R*-Squared) of the model was 0.9747, which indicated that the model adequately represented the real relationship between the chosen parameters. The results of the error analysis indicated that the lack of fitness was insignificant ($P > 0.05$). The coefficient of variation (CV) was less than 5% which indicated that the model was reproducible.[19] The predicted residual sum of squares for the model that describes the measure of how a particular model fits each

Table 3: Analysis of variance for the response surface quadratic model of the content of pepsin-soluble collagen from sturgeon skin

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value
Model	5744.37	9	638.26	30.02	<0.0001
Residual	148.81	7	21.26		
Lack of fit	91.56	3	30.52	2.13	0.2389
Pure error	57.25	4	14.31		
Total	5893.18	16			
R-Squared	0.9747		Adj-*R*-Squared	0.9423	
CV%	7.19		PRESS	1554.43	

point in the design was 1554.43. The model *F*-value (30.02) implied that the model was significant. The predicted second-order polynomial model was:

$$Y = 81.20 + 8.32X_1 + 17.73X_2 + 6.80X_3 - 1.72X_1X_2 - 2.21X_1X_3 - 4.12X_2X_3 - 8.21X_1^2 - 16.37X_2^2 - 11.77X_3^2$$

where Y is the yield of PSC, X_1, X_2, and X_3 are the coded variables for extraction time, solid–liquid ratio, and enzyme concentration, respectively.

The effect of extraction time and solid–liquid ratio on the yield of PSC at the fixed reaction enzyme concentration of 2.0% is shown in Figure 1. From Figure 1, it is observed that at a low solid–liquid ratio, the PSC yield increased with an increase in solid–liquid ratio. This is probably due to the increase in the reaction area of the enzyme. The PSC yield did not increase with a further increase in the solid–liquid ratio, which could be due to the complete reaction of sturgeon skin with the liquid. In addition, the extraction time indicated positive effects on the yield of PSC. The yield of PSC increased with the extension of time, especially between 3 and 7 h. When the extraction time was longer than 7 h, an increase in the yield was relatively slight. The optimal value of the extraction time and the solid–liquid ratio was 6.45 and 1:11.88, respectively.

The effect of enzyme concentration and extraction time on the PSC yield was determined at the a fixed solid-liquid ratio of 1:10, which was shown in Figure 2. Enzyme concentration indicated the positive effect on the yield of PSC. The yield of PSC was increased with an increase in enzyme concentration to a certain value (approximately 2.4 M), thereafter decreased. Pepsin is the most popular reagent used for collagen preparation due to the high extractability. The solubility of collagen in pepsin solution can affect the extraction of the collagen from sturgeon skin. The concentration of the pepsin solution can change the number of contact sites. In this study, a relatively higher yield was obtained at the concentration of 2.42%. This could be attributed to more positively charged amine groups of the collagen at this concentration. The effect of solid–liquid ratio and enzyme concentration on the PSC yield at the fixed extraction time of 6 h is shown in Figure 3. Evidently, there was significant interaction difference between the solid–liquid ratio and enzyme concentration. The optimal conditions obtained with RSM at a three-variable and five-level central composite design include a solid–liquid ratio of 1:11.88, an enzyme concentration of 2.42%, and an extraction time of 6.45 h. Under the optimal conditions, the content of the PSCs was 86.69%.

SDS-PAGE patterns of PSC

The SDS-PAGE patterns of the collagens from the skin of the sturgeons were determined under nonreducing and

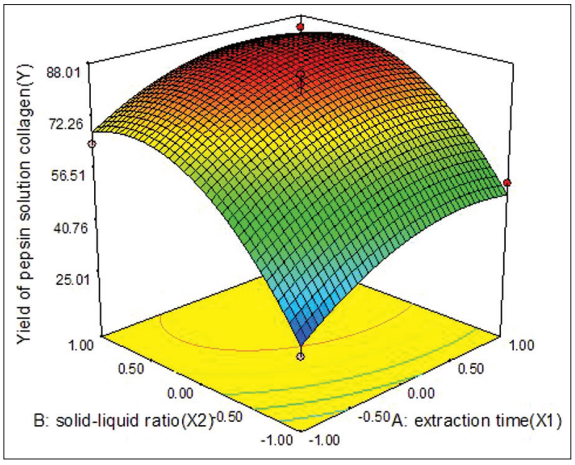

Figure 1: Response surface plot showing the effects of extraction time and solid–liquid ratio on the yield of pepsin-soluble collagen: the enzyme concentration was constant at 2.0%

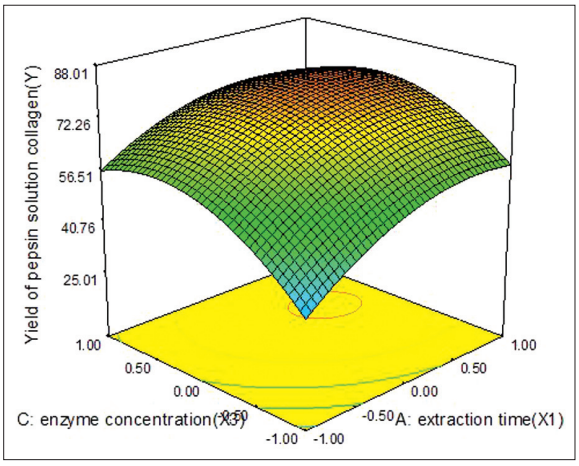

Figure 2: Response surface plot showing the effects of extraction time and enzyme concentration on the yield of pepsin-soluble collagen: the solid–liquid ratio was constant at 1:10

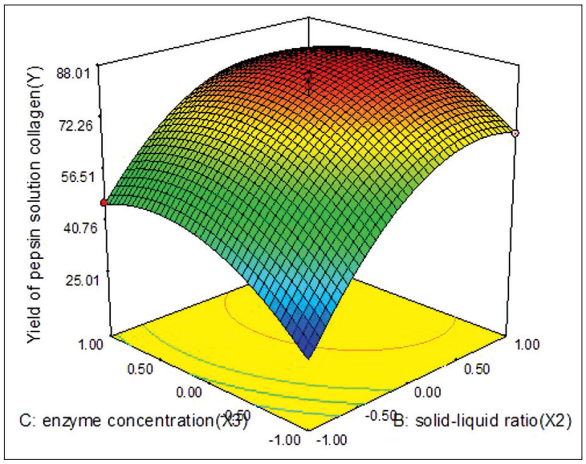

Figure 3: Response surface plot showing the effects of solid–liquid ratio and enzyme concentration on the yield of pepsin-soluble collagen: the extraction time was constant at 6 h

reducing conditions, which was shown in Figure 4. It is observed that the major components of collagens comprised α-chain and β-chain. The α_1-chain, α_2-chain, and β-chain patterns were similar to those of standard collagen type I from calf skin (lane 1). The α-chain and β-chain had subunits molecular weights of 116 and 200 kDa, respectively. It was, therefore, evident that the sturgeon skin collagen is type I collagen and the electrophoretic patterns of collagens under nonreducing and reducing conditions were quite similar. The major component of type I collagen are two α_1- and one α2-chain ($[\alpha_1]_2\alpha_2$). The sturgeon skin collagen molecule depicted $[\alpha_1]_2\alpha_2$. It is obvious that type I collagen is the main collagen in collagen extracted with pepsin from the sturgeon skin. This observation is in agreement with the findings reported by Muyonga and Kittiphattanabawon.[20,21]

FTIR spectra of PSC from sturgeon skin

Fourier transform infrared spectroscopy was used to study changes in the secondary structure of collagen.[22] The FTIR spectra in the range of 4000–400 cm^{-1} of collagens from the sturgeon skin are presented in Figure 5. The main absorption bands were amide A (3333 cm^{-1}), amide B (2922 cm^{-1}), amide I (1653 cm^{-1}), amide II (1539 cm^{-1}), and the amide III (1238 cm^{-1}).

Amide A peak of PSC was found at 3333 cm^{-1}. However, there was a slight shift of the amide A peak of PSC to lower wave number when compared with other proteins. Normally, a free N–H stretching vibration commonly occurs in the range of 3400–3440 cm^{-1}, and the position is shifted to the lower frequencies when the hydrogen bonds formed stabilized the helix structure.[23] The amide B peak of the collagens (2922 cm^{-1}) was attributed to the asymmetrical stretch of CH_2 (2920–2922 cm^{-1}) stretching vibration.[24]

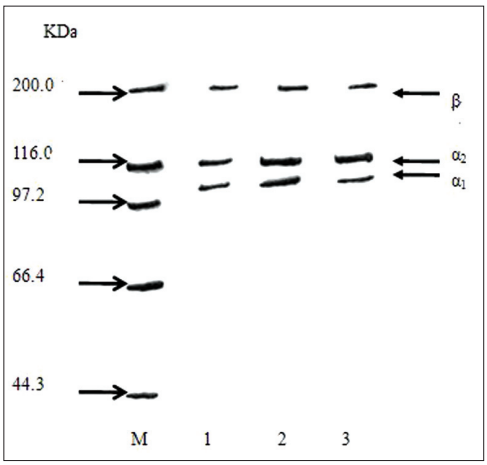

Figure 4: SDS-PAGE pattern of collagen from sturgeon skin under reducing and nonreducing conditions. Lane M: high MW protein markers; lane 1: calf-skin collagen; lane 2: collagen from sturgeon skin under reducing conditions; lane 3: collagen from sturgeon skin under nonreducing condition

Amide I peak of PSC was also found at 1653 cm^{-1}, which was associated with C=O stretching vibration or hydrogen bond coupled with COO^-.[25] The amide I band is said to be the most useful amide for the infrared spectroscopic analysis of the secondary structure of proteins.[26] The amide II peak of the collagens was observed at 1539 cm^{-1}, resulting from N–H bending vibration coupled with the stretching vibration of CN (1536–1544 cm^{-1}).[27] The amide III peak of the collagens was observed at 1238 cm^{-1}. The amide I peak was associated with the triple-helical structure 1 (1234–1235 cm^{-1}). On the basis of the slight differences with other proteins, the triple-helical structure might be slightly affected by pepsin digestion during collagen extraction.[28]

CONCLUSION

From the study, various factors including, extraction time, enzyme concentration, and solid–liquid ratio had significant effects on the yield of PSC. The optimal conditions include a solid–liquid ratio of 1:11.88, an enzyme concentration of 2.42%, and the extraction time of 6.45 h. Under the optimal conditions, the maximum content of PSC was 86.69%, which was not significantly different from the predicted yield (87.4%) ($P < 0.05$). The results of the study therefore indicated that the production of PSC from sturgeon skin is feasible and beneficial. From the SDS-PAGE patterns, it was concluded that the sturgeon skin collagen was type I collagen, with the collagen molecule depicted as $[\alpha_1]_2\alpha_2$. The FTIR spectra, therefore, suggested that pepsin hydrolysis does not affect the triple-helical structure.

ACKNOWLEDGEMENT

This work was supported financially by Graduate Innovative Projects in Jiangu Province (CXZZ11-0589).The authors gratefully acknowledge Lianchuang Aquatic Science and

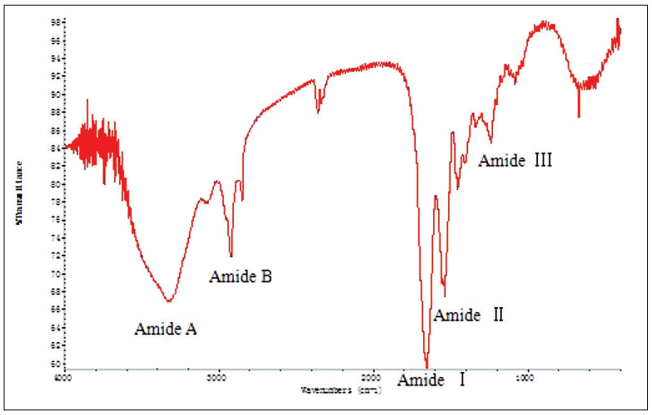

Figure 5: The FTIR spectra of pepsin-soluble collagen

Technology Demonstration Park of Zhenjiang(China) for providing sturgeon. We also appreciate Dr. Mohanmmed Takase for language polishing.

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Cite this article as: Feng W, Zhao T, Zhou Y, Li F, Zou Y, Bai S, *et al*. Optimization of enzyme-assisted extraction and characterization of collagen from Chinese sturgeon (*Acipenser sturio Linnaeus*) skin. Phcog Mag 2013;9:32-7.

Source of support: Nil. **Conflict of interest:** None declared.