Optimization of demineralization on *Cyprinus carpio haematopterus* scale by response surface methodology

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Revised: 22 August 2013 / Accepted: 29 August 2013 / Published online: 13 September 2013
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Abstract Fish scale, separated during fish mechanical processing, can serve as an additional source of proteins, especially of collagen proteins. To obtain high purity native collagen, it is required to carry out deproteinization and demineralization of fish scales. Therefore, the aim of this study was to determine the optimal conditions for demineralization of *Cyprinus carpio haematopterus* scale without loss of collagen content in HCl solution. Here, the demineralization of *Cyprinus carpio haematopterus* scale was optimized by response surface methodology. The optimum conditions were as follows: extraction time of 95 min, concentration of HCl of 1.0 M, and ratio of material to solution of 1:11. Under these conditions, the experimental yield of demineralization of scales was 92.7 ± 1.32 %, which was well consistent with the value predicted by the model.

Keywords *Cyprinus carpio haematopterus* · Scale · Demineralization · Collagen · Response surface methodology

Introduction

As one of the major components of extracellular matrix, collagen is vital for mechanical protection of tissues and organs as well as physiological regulation of cellular environment (Pati et al. 2010). It is widely used in biomedical materials, food and cosmetic industry. The scales of teleost fish are composed of hydroxyapatite and extracellular matrix, which mainly includes type I collagen fibers. Fish scale consists of two distinct regions:

an external (osseous) layer and an internal fibrillary plate (Ikoma et al. 2003). In the upper external layer, collagen fibers are randomly arranged and embedded in a proteoglycan matrix. Within the lower fibrillary layer, in contrast, the collagen fibers are co-aligned and organized in lamellae which are superimposed to produce an orthogonal and/or a double-twisted plywood pattern (Ikoma et al. 2003). Mineralization of fish scales occurs continuously throughout the life of the organism. The external layer is initially mineralized with vesicles, and then the internal layer is developed (Ikoma et al. 2003). Therefore, it is possible to increase the efficiency of collagen extraction from fish scale through previous demineralization. In addition, the appropriate acid concentration is more suitable for extraction of acid-solubilised collagen. Demineralization maintains the concentration of extraction solvent stability through extraction of acid-solubilised collagen. So, it is a critical process of demineralization for fish scale collagen extraction. Zhang's report (2009) suggested that the helix structures of grass carp scale collagen became loose in the presence of Ca^{2+} on account of Ca^{2+} combined – C=O of collagen through coordination bond resulting in the broken of the hydrogen bond inner and between the collagen molecules. Therefore, the collagen cannot form network structure. It is important of the hydrogen bond between the collagen molecules for gelation of gelatin. So Ca^{2+} has immediate impact on the gelling properties of gelatin extracted from fish scale. In addition, the mineral content is an important indicators of gelatin (the thermal denaturation products of collagen). According to the National Standard of the People's Republic of China, the ash content of gelatin may not exceed 2 %. So, the previous demineralization of scale could not only result in the benefit to the subsequent gelatin extraction but also improve the quality of gelatin. *Cyprinus carpio haematopterus* is a common species of freshwater fish in China. Therefore, the *Cyprinus carpio haematopterus* scales separated during mechanical processing can be used as an additional source of proteins, especially of

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collagens or gelatins. According to the previous researches, the collagens and gelatins were extracted from many animals, including warm-blooded animals and aquatic animals (Lin et al. 2011; Matmaroh et al. 2011; Kittiphattanabawon et al. 2010; Huang et al. 2011; Żelechowska et al. 2010; Skierka et al. 2007). And the aquatic collagens and gelatins have drawn extensive attention for its physiochemical properties, physiologically active functions and edible security. Recently, more of the research about marine animal collagen has been reported. At the same time, some researchers started their research about freshwater fish collagen. However, little information regarding the characteristics of *Cyprinus carpio haematopterus* scale collagen, as well as the extraction process of collagen and gelatin from *Cyprinus carpio haematopterus* scales. In order to develop new collagen and gelatin from *Cyprinus carpio haematopterus* scales, it is necessary to study the pretreatment technics of *Cyprinus carpio haematopterus* scales.

Demineralization process of *Megalobrama amblycephala* scales, *Ctenopharyngodon idellus* scales and *Gadus morhua* backbones has been reported (Wang et al. 2010; Wu et al. 2008; Skierka et al. 2007). Nevertheless, collagen from different species and habitats might be different in terms of molecular compositions and properties. For example, fish collagen particularly from species living in cold waters has different solubility in acid medium and thermal denaturation temperature as compared with that from warm-blooded animals. Therefore, the methods used for demineralization of other materials cannot be applied for *Cyprinus carpio haematopterus* scales.

To dissolve mineral salts from fish scales, EDTA (Ethylene Diamine Tetraacetic Acid) can be used. But the effectiveness of this process was not determined (Wang et al. 2008; Matmaroh et al. 2011; Duan et al. 2009). The process of fish scale demineralization is normally carried out by immersing the sample into a variety of strong or weak acids. HCl has been applied for the demineralization of fish backbones and bones (Figueiredo et al. 2011; Castro-Ceseña et al. 2011; Skierka et al. 2007). In the case of HCl, the major inorganic constituents of bone were converted into monocalcium phosphate and calcium chloride. It was reported that the best effect of demineralization of cod back bones, almost 100 %, was achieved with 1 M HCl solution during 72 h extraction. And the loss of collagen amounted to only about 0.2–3.2 % (Skierka et al. 2007). In contrast to HCl, EDTA does not cause any loss of collagen, but the efficiency of demineralization is lower (Skierka et al. 2007). Fish collagen can be dissolved at the high concentration of HCl solution. In general, extraction efficiency of a compound is influenced by multiple parameters, such as temperature, time and solvent polarity. The optimum demineralization conditions and the influences of parameters, such as HCl concentration, extraction time and ratio of material to solution, on the yield of demineralization from *Cyprinus carpio haematopterus* scale have not been reported. Response surface methodology (RSM) has been successfully used to model and optimize biochemical and biotechnological processes

related to food systems including the extraction of polysaccharides from *Lycium ruthenicum* (Liu et al. 2013), the extraction of protein from red pepper seed (Firatligil-Durmus and Evranuz 2010), the extraction of stachyose from *Stachys floridana* Schuttl. ex Benth (Zhong et al. 2013) and the extraction of antioxidants from wheat bran (Singh et al. 2012). According to the literature, the vast majority of existing studies focused on the structure and properties of collagen from various species. The authors think that the studies of collagen and gelatin extraction technology such as demineralization process and removal of non-collagenous protein are needed to adapt the process to a large scale. Therefore, the aim of this work is to optimize the process for demineralization of *Cyprinus carpio haematopterus* scale in HCl solution using response surface methodology. And a central composite design (CCD) is adopted to study the influence of treatment conditions on demineralization.

Materials and methods

Raw material

Live *Cyprinus carpio haematopterus* with average weight range of 3.0–3.2 kg were purchased from a local market in Luoyang, China. Fish scales were mechanically separated from fresh *Cyprinus carpio haematopterus*. Samples were cut into 2–3×2–3 mm² with scissors, and were stored at –20 °C in polyethylene bags prior to demineralization.

Demineralization process

The scales were demineralized with HCl solution. The demineralized scales were then filtered through cotton-cloth. The dry weight and ash content in the residue and the hydroxyproline content in supernatant were determined. The yield of the demineralization was calculated using the following formula:

$$W = (A - B) / A \times 100 \quad (1)$$

Where: W — the yield of demineralization of scales (%); A — concentration of ash in the raw material (%); B — concentration of ash in the demineralized sample (%).

Collagen loss (%) was determined using an indirect method: the ratio of hydroxyproline extracted with HCl solution to their initial concentration in the raw material.

Hydroxyproline content

The hydroxyproline content was determined after hydrolysis of the material in 6 M HCl for 6 h at 105 °C, using the colorimetric method recommended by ISO3496 (Anonymous 1994). In the

calculation of the collagen in scales from hydroxyproline content, the used established conversion factor was 14.7 (Żelechowska et al. 2010).

Experimental design for single factor and response surface methodology

The aim of single factor tests was to optimize the main effective factors and the better ranges of parameters for RSM (Table 1). Here, the factors included time, the concentration of HCl and the ratio of material to solution. The initial step of the preliminary experiment was to select an appropriate demineralization time. Scale samples (5 g) were immersed in 40 mL of HCl (1.0 M) at 4 °C. The second step of the preliminary experiment was to select the appropriate concentration of HCl using the best demineralization time chosen in the previous step. The concentration of HCl varied from 0.6 to 1.4 M with the ratio of material to solution at 1:8. Final step of the preliminary experiment was to select the appropriate ratio of material to solution. With the demineralization time of 90 min obtained from the first step, scales were demineralized under various ratios of material to solution range from 1:4 to 1:12 at the given HCl concentration of 1.0 M obtained from the second step. Based on the results, a central composite design (CCD) was used to investigate the effects of the three independent variables on the yield of demineralization (Y). Time (X_1), concentration of HCl (X_2) and ratio of material to solution (X_3) were chosen as independent variables. The range and centre point values of three independent variables presented in Table 2 were based on the results of single-factor experiment (Table 1). For statistical calculation, the variables were coded as follows:

$$X = (X_i - X_0) / \Delta X_i \quad (2)$$

Where, X is the independent variable coded value; X_i is the independent variable real value; X_0 is the independent variable real value on the centre point and ΔX_i is the step change value.

The whole design consisted of 20 experimental points carried out in random order. Eight factorial points, six centre

points and six axial points at the centre of the design was used for the estimation of a pure error sum of squares. The yield of demineralization (Y, %) was selected as the dependent variable. The generalized second-order polynomial model used in the response surface analysis was as follows (Kim et al. 2013):

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (3)$$

Where, Y is the dependent variable; β_0 is a constant; β_i , β_{ii} and β_{ij} are regression coefficients; X_i and X_j are levels of the independent variables.

Statistical analysis

The optimal demineralization conditions were estimated through regression analysis and three-dimensional (3D) response surface plots of the independent variables and each dependent variable. Design-Expert 8.0 software was used to analyze the experimental data and the results with p -values less than 0.05 were considered to be statistically significant.

Results and discussion

The result and analysis of single factors

The effects of each factor on the yield of demineralization were shown in Table 1. The amount of dissolved minerals was increased with the increase of treatment time and reached the peak value about 91.6 ± 1.12 % when the treatment time was 90 min. The demineralization yield after the immersion for 120 min and 150 min was not significantly different to that for 90 min ($p > 0.05$). Skierka et al. (2007) have reported that the largest percentage of the total content of minerals (about 100 %) was removed in Baltic cod (*Gadus morhua*) backbone treated with 1.0 M HCl solution for 3 days with the solution replacement once a day. And the amount of dissolved minerals after 24 and 48 h treatment was similar (Skierka et al. 2007). Therefore, demineralization is less efficient for the scales immersed with 1.0 M HCl solution over 150 min. The yield of demineralization continued to increase with the rising HCl concentration and reached the peak value (90.7 ± 1.15 %) at concentration 1.0 M. However, the yield of demineralization was no longer increased when the concentration exceeded 1.0 M. So the concentration of HCl of 1.0 M is applicable for demineralization of *Cyprinus carpio haematopterus* scale. At the ratio of material to solution of 1:10, the yield of demineralization was the highest.

During the demineralization in HCl, partial collagen contained in scales was dissolved. The solubility of collagen

Table 1 The results of single factors on the yield of demineralization

Time (min)	Y (%)	Concentration of HCl (M)	Y (%)	The ratio of material to solution	Y (%)
30	83.2±1.03	0.6	80.5±1.21	1:4	76.3±1.07
60	87.8±0.93	0.8	87.5±1.54	1:6	83.2±0.93
90	91.6±1.12	1.0	90.7±1.15	1:8	88.9±1.07
120	90.2±1.05	1.2	90.3±1.55	1:10	91.2±1.25
150	90.5±1.23	1.4	90.4±1.41	1:12	90.6±1.10

Mean values ± SD from five separate samples

Table 2 Independent variables and their levels used in the response surface design

Independent variables	Factor level				
	−1.682	−1	0	1	1.682
Time (min)	39.5	60	90	120	140.5
Concentration of HCl (M)	0.66	0.8	1.0	1.2	1.34
The ratio of material to solution	1:6.6	1:8	1:10	1:12	1:13.4

depended on the concentration of acid, time of the process and ratio of material to solution. Loss of collagen from scales was slightly increased with increase in treatment time and ratio of material to solution. And the amount of dissolved collagen arrived at minimum value about 0.08 % at HCl concentration of 1.0 M. In low concentration acidic medium, collagen swells and this facilitates its solubility. But at low pH, for example, backbones were decalcified in 1.0 M HCl solution, the uptake of collagen is significantly lower (Skierka et al. 2007). The loss of collagen amounted to only 0.08–1.9 %, which was less than the results of Żelechowska et al. (2010). This phenomenon is due to the less extraction time. Thus, treatment time of 90 min, HCl concentration of 1.0 M and the ratio of material to solution of 1:10 were considered to be central points of RSM (Table 2) to optimize the process of demineralization without considering the loss of collagen.

Model fitting

Experimental design and corresponding experimental data obtained were shown in Table 3. The response surface regression procedure of Design Expert (version 8.0) software was used to analyze the experimental data. Through multiple regression analysis on the experimental data, the response variable and the test variables were represented by the following second-order polynomial equation (in terms of coded levels):

$$Y = 94.8 + 0.91X_1 + 1.21X_2 + 1.73X_3 - 1.1X_1X_2 + 0.2X_1X_3 + 0.35X_2X_3 - 2.49X_1^2 - 2.76X_2^2 - 2.92X_3^2 \quad (4)$$

Where, Y is the yield of demineralization; X_1 , X_2 and X_3 are the coded variables for treatment time, HCl concentration and ratio of material to solution, respectively.

In general, exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fitness. The statistical significance of the regression model was checked by *F*-test and *p*-value, and the analysis of variance (ANOVA) for the response surface quadratic model was shown in Table 4. A small value of R^2 indicated a poor relevance of the dependent variables in the model. High value of determination coefficient ($R^2=0.9596$), obtained by ANOVA of the quadratic regression model (Table 4), indicated that the

model was adequate for prediction within the range of experimental variables.

The *p*-value were used as a tool to check the significance of each coefficient, and the smaller the *p*-value was, the more significant the corresponding coefficient was (Yin et al. 2011). For the present experiments, the model terms were significant when the value of *p* was less than 0.05. It could be seen from Table 5 that the linear coefficients (X_1 , X_2 and X_3), the quadratic term coefficients (X_1^2 , X_2^2 and X_3^2) and cross product coefficient ($X_1 \times X_2$) were significant with very small *p*-value ($p < 0.05$). The coefficients of other terms were not significant ($p > 0.05$).

In the present experiment, the model *F*-value of 26.41 (Table 4) implied that the model was significant. Meanwhile, the lack of fit value of the model was 3.43 (Table 4), which was not significant. Therefore, the model fitness was good.

Table 3 Experimental design and results of Response Surface Analysis for the demineralization

Run	Coded variable levels			Y(%)
	X_1	X_2	X_3	
1	−1	−1	−1	81.3
2	1	−1	−1	86.5
3	−1	1	−1	87.6
4	1	1	−1	84.3
5	−1	−1	1	85.3
6	1	−1	1	87.2
7	−1	1	1	88.9
8	1	1	1	90.5
9	−1.682	0	0	85.9
10	1.682	0	0	90.1
11	0	−1.682	0	85.6
12	0	1.682	0	88.9
13	0	0	−1.682	83.4
14	0	0	1.682	90.2
15	0	0	0	94.3
16	0	0	0	95.1
17	0	0	0	94.6
18	0	0	0	96.2
19	0	0	0	93.8
20	0	0	0	94.7

Table 4 Analysis of variance for fitted quadratic polynomial model of demineralization

source	DF	Sum of square	Mean square	F-value	Probability (<i>p</i>)
Model	9	352.21	39.13	26.41	<0.0001*
Residual	10	14.82	1.48		
Lack of fit	5	11.47	2.29	3.43	0.10
Pure error	5	3.35	0.67		
Cor total	19	367.03			
	$R^2=0.9596$				

All the results proved that the model was fully applicable. The full model was made with three dimensional and contour plots to predict the relationships between the independent variables and the dependent variables.

Analysis of response surfaces

Response surface graphs were plotted between two independent variables while the remaining independent variables were kept at the zero code level. The relationship among the variables was illustrated by these surface plots. The contour map could indicate the interaction strength. The ellipse represented significant interaction and the rotundity represented unapparent interaction (Zhong et al. 2013). The graphical representations of the regression equation (Eq. (4)), the response surfaces and the contour plots were obtained using Design-Expert. The maximum value predicted by the surface was confined in the smallest ellipse in the contour diagram.

The effects of three factors as well as their interactive effects on the yield of demineralization were shown in Figs. 1, 2 and 3. Figure 1 denoted the three dimensional surface plots of the effect of extraction time (X_1) and concentration of HCl (X_2) on response: when the extraction time was increased from 60 to 120 min and the concentration of HCl was increased from 0.8 to 1.2 M, Y gradually mounted up to the highest value (103 min and 1.08 M, respectively), and then declined gradually. The interaction of extraction time (X_1) and the concentration of HCl (X_2) was significant for the contour map was close to ellipse (Fig. 1) and the *p*-value was only 0.0286 (Table 5).

When the extraction time was increased, Y gradually mounted up to the highest value (103 min), and then declined gradually. One possible reason is that the minerals are not dissolved fully within short time. So, the minerals are not released totally, which causes a lower yield of demineralization. Higher concentration of HCl results in the shrinking of collagenous fibres in the solution with low pH. The solution with a very low pH value reduces the absorption of water by collagen. Thus, the structures of collagenous fibres are enhanced (Skierka and Sadowska 2007). A large increase in collagen solubility was observed in the pH range from 4 to 7 (Żelechowska et al. 2010), indicating that low pH was disadvantageous for collagen

swelling. The yield of demineralization was decreased when the concentration of HCl was over 1.08 M because there is less space for HCl among the macromolecules.

The trends in the three dimensional surface plots by the interaction between concentration of HCl (X_2) and the ratio of material to solution (X_3) (Fig. 2) and the interaction between extraction time (X_1) and the ratio of material to solution (X_3) (Fig. 3) were the same, indicating that there was no significant interaction. Figure 2 showed the 3-D response surface plot and the contour plot at varying extraction time and ratio of material to solution at fixed concentration of HCl. The yield of demineralization was increased slowly during the extraction time from 60 min to 95 min and then dropped slowly from 95 min to 120 min. And the yield of demineralization was increased rapidly with the rising ratio of material to solution from 1:8 to 1:10.5. And the yield of demineralization reached the plateau region and declined slightly when the ratio was beyond 1:10.5. Figure 3 showed the 3-D response surface plot and the contour plot with varying concentration of HCl and the ratio of material to solution at fixed extraction time. It indicated that the maximum yield of demineralization could be achieved when concentration of HCl and ratio of material to solution were 1.03 M and 1:10.5, respectively.

When the ratio of material to solution was at 1:10.5, Y was the highest in the study. The effect of the ratio of material to solution on the yield was very significant ($p < 0.01$). When the ratio was lower (1:8), the rate of removal of minerals became

Table 5 Regression coefficient of polynomial function of response surface of the yield of demineralization

Term	Coefficient estimate	Standard error	<i>p</i>
X_1	0.74	0.32	0.0198
X_2	1.39	0.32	0.0043
X_3	1.91	0.32	0.0004
X_1X_2	−0.65	0.42	0.0286
X_1X_3	0.65	0.42	0.6521
X_2X_3	−0.10	0.42	0.4350
X_1^2	−2.59	0.32	<0.0001
X_2^2	−2.85	0.32	<0.0001
X_3^2	−3.01	0.32	<0.0001

Fig. 1 Response surface (3-D) and contour plots showing the effect of extraction time and concentration of HCl on the response Y

less with the consumption of HCl and Y was lower. The higher ratio of material to solution (1:10.5) was applicable to the diffusion of the minerals.

Among the studied three parameters, according to the regression coefficient significance of the quadratic polynomial model (Table 5) and gradient of slope in the 3-D response surface plot (Figs. 1, 2 and 3), the most significant factor that affects the yield of demineralization was the ratio of material to solution, followed by the concentration of HCl and extraction time.

Optimization and verification

The suitability of the model equation for predicting the optimum response values was verified with the selected optimal

Fig. 2 Response surface (3-D) and contour plots showing the effect of extraction time and ratio of material to solution on the response Y

Fig. 3 Response surface (3-D) and contour plots showing the effect of concentration of HCl and ratio of material to solution on the response Y

conditions. The maximum predicted yield and experimental yield of demineralization were given in Table 6. In terms of the actual production, it was difficult to control these optimum conditions. For convenient operation, experiment rechecking was performed according to these modified optimal conditions: time of 95 min, HCl concentration of 1.0 M and ratio of material to solution of 1:11. The experiments with five replications of *Cyprinus carpio haematopterus* scale were carried out according to the above conditions and the obtained mean value of demineralization yield was 92.7 ± 1.32 % (Table 6). This value was not significantly different from the predicted value of 93.5 % within 95 % confidence interval. Previous studies showed that the demineralization yield of *Megalobrama amblycephala* scale was 79.18 % with the optimum condition: treatment time of 86 min, concentration of HCl 0.43 M and the ratio of liquid to material 62:1 (Wang et al. 2010). Wu et al. (2008) have reported that the demineralization yield of *Ctenopharyngodon idella* scale can reach 87.62 % with HCl solution. Though the 92.7 % yield of demineralization was slightly lower than 100 % yield reported by Skierka et al. (2007), processing time was largely reduced. According to the report by Skierka et al. (2007), the solubility of minerals after

Table 6 Predicted and experimental values of the responses at optimum conditions

	Time (min)	Concentration of HCl (M)	The ratio of material to solution	Y (%)
Optimum conditions	94.2	1.038	1:10.6	93.5 (predicted)
Modified conditions	95	1.0	1:11	92.7 ± 1.32 (actual) ^a

^a Mean values $\pm$ SD from five separate samples

24 and 48 h of treatment respectively amounted to about only 83 % and 87 %. Verification experiments demonstrated that experimental values were reasonably close to the predicted values and confirmed the validity of the RSM model. Therefore, the model was adequate for the demineralization process.

Conclusion

RSM was used to estimate and optimize the experimental variables: extraction time (min), HCl concentration (M) and the ratio of material to solution. The high correlation of the model indicated that the second-order polynomial model could be used to optimize the demineralization of *Cyprinus carpio haematopterus* scale. The optimal demineralization conditions were as follows: treatment time of 95 min, HCl concentration of 1.0 M and the ratio of material to solution of 1:11. Under these conditions, the experimental yield of demineralization was 92.7 ± 1.32 %, which was not significantly different with the predicted yield value (Table 6). All of these results indicated that RSM was successfully applied to determine the optimum factors for demineralization of *Cyprinus carpio haematopterus* scale with HCl solution.

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