Original paper

Optimizing the Extraction of Acid-soluble Collagen Inside the Eggshell Membrane

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Received November 10, 2017 ; Accepted January 24, 2018

Extraction of collagen from the eggshell membrane was optimized by the methodology of response surface. The Central Composite Design (CCD) was applied. This was done to find the optimum combination of acetic acid concentration (0.1–0.9 mol/L), temperature (5–25°C), stirring speed (100–500 rpm) and extraction time (12–36 h) to maximize the acid soluble collagen (ASC) extraction. The highest amount of extraction was theoretically predicted to be 8.55%, under the optimal. The recovery experimental ASC, was calculated to be 8.35%, which confirms the predicted value, thereby confirming the reliability of the method. Analysis of amino acids revealed glycine (308 residues/1 000 residues) as the major amino acid and showed imino acids of 229 residues/1 000 residues. ASC had minimum solubility at pH 7. No changes in solubility were observed in the presence of NaCl concentrations that did not exceed 4% (w/v). The denaturation temperature was 46.8°C which correlated suitably with imino acid contents.

Keywords: response surface methodology, eggshell membrane, optimization, acid-soluble collagen

Introduction

The processing of egg products such as liquid white and yolk generates large quantities of wastes in the form of eggshell. From 1961 to 2016, there have been 3881982 tons of eggshell produced worldwide (FAOSTAT, 2014)¹⁾. It has proved to be a safe material in terms of autoimmunity risks, i.e. it does not entail allergic reactions when consumed (MacNeil, 2001). The membrane of the eggshell is located on the outer surface of the egg's white liquid and is beneath the solid eggshell. Collagen comprises 10% of the egg's total protein make-up (MacNeil, 2001). A few collagens are already distinguished in the egg's membrane of the eggshell, i.e. type X, V and I (Arias *et al.*, 1992).

Collagen has a nature of being a protein with fibrous properties, which contributes on the fact that it has a special function involving the skin, bones, tendons and relevant tissues (Jongjareonrak *et al.*, 2005). The use of collagen is a diversified application in food, cosmetics, pharmaceutical, and cell culture

industries (Han *et al.*, 2010). The functional properties of collagen are highly influenced by their molecular structure, amino acid composition and internal linkages, which are substantially affected by processing conditions (Jeevithana *et al.*, 2014). Collagen is obtained from animals living on land, and this research aims at the poultry section. However, diseases such as foot-and-mouth disease (FMD), the bovine sponge encephalopathy (BSE) and the avian influenza are known to have restricted the trade of collagen. Therefore, alternative sources of collagen are needed to ensure safety (Liu *et al.*, 2012). Accordingly, the eggshell membrane can be considered as a prime option for obtaining collagen, and it can replace collagen sources of mammals (Ruff *et al.*, 2012).

Collagen is sensitive to temperature when it is being prepared. Oftentimes its extraction takes place at room temperature. Understanding the qualities of the conditions in which extraction is performed can make a higher yield of products containing collagen.

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Table 1. Independent variables and their levels used in the response surface design

Independent variables	Symbol	Coded factor level				
		-2	-1	0	1	2
Concentration of acetic acid (mol/L)	X ₁	0.1	0.3	0.5	0.7	0.9
Temperature (°C)	X ₂	5	10	15	20	25
Stirring speed (rpm)	X ₃	100	200	300	400	500
Time (h)	X ₄	12	18	24	30	36

This work aimed at the optimization of collagen extraction from the eggshell membrane. For this purpose, the response surface methodology (RSM) was used. The RSM is able to assess the potentials embedded in the multiple factor variables of an experimental design. Furthermore, interactions between the assessable factors have scope for simultaneous evaluations (Myers *et al.*, 2008). In this study, four main factors were employed as variables. These included the temperature, the concentration of acetic acid, and the stirring speed in addition to extraction time. These variables were applied at five levels to determine the most suitable conditions in which the extraction of collagen takes place when using the eggshell membrane as a raw material.

Materials and Methods

Materials and chemical reagents Raw membrane-bound eggshell was collected from a local confectionary shop and was immediately stored in iced water. The external membranes were carefully stripped manually and washed with deionized water. It was finally dried at ambient temperature for two d. The dried eggshell membrane was further ground to prepare powder particles. All chemicals were of analytical grade. Acetic acid, sodium chloride, Tris (hydroxymethyl) amino methane and sodium hydroxide were purchased from Merck (Darmstadt, Germany).

Extraction of ASC from eggshell membrane The ASC was extracted from the eggshell membrane according to the method of Kittiphattanabawon *et al.*, 2005 with slight modifications. Ten grams of dried eggshell membrane were precisely weighed. To remove non-collagenous proteins, the powder of the eggshell membrane was mixed with 10 volumes (v/w) of 0.1 M sodium hydroxide at 4°C, and was stirred for 24 h. The alkaline solution was changed every 2 h. After removing the supernatant, the alkali-treated samples were washed thoroughly with excessive distilled water until the pH became neutral or slightly alkaline. The treated eggshell membrane was subjected to collagen extraction by different concentration of aqueous acetic acid at different temperatures. Then, the solution of the soluble collagen was obtained from the supernatant when it was centrifuged at 18 000 gravity for 20 min. The collagen was allowed to precipitate by adding sodium chloride to a final concentration of 2.0 M in the presence of 0.05 M tris (hydroxymethyl) amino methane (pH 7.0). After being

centrifuged at 18 000 gravity for 40 min, the precipitate was dissolved in 0.5 mol/L acetic acid, and it was then dialyzed against 10 volumes of 0.1 M acetic acid in a dialysis membrane with a molecular weight cut-off of 30 kDa which occurred for 24 h at 4°C. The solution changed once in every 8 h. The dialyzed precipitate was freeze-dried to obtain the collagen. The yield of the ASC was calculated in grams of dry weight and was expressed as percentages (g/g) according to (Eq. 1):

$$\text{ASC extraction yield (\%)} = \frac{\text{Weight of extract ASC}}{\text{Weight of dry powder}} \times 100 \dots \text{Eq. 1}$$

Experimental Design and Analysis of Data The response surface methodology was used for experimental design, data analysis, and model building by the software Design Expert version 8.0.3 (Stat-Ease Inc., Minneapolis, MN), Central composite design (CCD) with four variables was used for the optimization of collagen extraction from the eggshell membrane. The CCD determines the response pattern and then establishes a model. Four independent variables used in this work were acetic-acid concentration (X₁), temperature (X₂), stirring speed (X₃), and treatment time (X₄), with five levels for each variable, while the dependent variable was the yield of the ASC collagen. The ranges and center point values of the four independent variables were based on the results of preliminary experiments. The symbols and levels are shown in Table 1.

As seen in Table 2, the CCD in the experimental design consists of 30 experimental points conducted in a random order (16 factorial points, 8 axial points and 6 center points). Six replicates were used at the central point of the designed model to estimate the pure error sum of squares. For statistical calculations, the experimental variables X_i are coded as x_i based on the following (Eq. 2):

$$Y = \beta_0 + \sum_{j=1}^4 \beta_j x_j + \sum_{j=1}^4 \beta_{jj} x_j^2 + \sum_{i < j} \beta_{ij} x_i x_j \dots \text{Eq. 2}$$

Where Y is the dependent variable (collagen contents, %); β_0 is a constant; β_j , β_{jj} and β_{ij} are the coefficients of intercept, linear, quadratic and interactive terms respectively; while X_i and X_j are the coded values of the four independent variables. Tri-dimensional and contour plots were produced from regression models by using experimental data. These response surfaces and contour plots were used in order to determine the optimum conditions applied therein (Lu *et al.*, 2008).

Table 2. Central composite design and responses of the dependent variables for Collagen

No.	Acetic acid concentration (mol/L)(X1)	Temperature (°C)(X2)	Stirring speed (rpm)(X3)	Time (h)(X4)	Yield (%)
1	0.5	15	300	24	8.21
2	0.5	25	300	24	6.99
3	0.1	15	300	24	5.76
4	0.5	15	300	24	8.35
5	0.5	15	300	24	8.18
6	0.7	20	400	18	6.50
7	0.3	10	200	18	6.13
8	0.7	20	200	30	5.90
9	0.3	10	400	18	7.32
10	0.3	20	200	30	7.04
11	0.7	10	400	30	7.58
12	0.5	15	300	24	8.38
13	0.3	20	200	18	6.90
14	0.5	15	300	36	8.12
15	0.5	15	300	24	8.32
16	0.7	20	400	30	6.78
17	0.5	15	300	12	7.47
18	0.5	15	300	24	8.38
19	0.3	20	400	30	7.98
20	0.9	15	300	24	4.20
21	0.7	20	200	18	5.25
22	0.3	10	400	30	7.58
23	0.5	15	500	24	8.55
24	0.7	10	400	18	7.13
25	0.5	15	100	24	6.48
26	0.3	20	400	18	7.81
27	0.7	10	200	30	6.67
28	0.5	5	300	24	7.41
29	0.3	10	200	30	6.78
30	0.7	10	200	18	5.82

Prediction error The error of prediction extraction was obtained by (Eq. 3):

$$\text{Relative error\%} = \frac{\text{Experimental value} - \text{Predicted value}}{\text{Experimental value}} \times 100 \dots \text{Eq. 3}$$

Amino acid analysis Ten milligrams of lyophilized ASC was dissolved in 3 mL of 6 N hydrochloric acid and the mixture was hydrolyzed in a vacuum-sealed glass at 110°C for 24 h. The hydrolysate was analyzed on an auto-analyzer of amino acids (Hitachi 835-50, Shimadzu Seisakusho Co. Ltd., Kyoto, Japan). The types of amino acids were determined by being derived with ninhydrin, and were then measured via absorbance at 570 nm, except for proline and hydroxyproline, which were measured by being treated with the absorbance at 440 nm. The amino acid content was expressed by the number of residues per 1 000 residues.

Collagen solubility test Optimum solubility at different pH and salt concentrations was determined according to the

method of Jongjareonrak *et al* (2005) with a slight modification (Jongjareonrak *et al.*, 2005). Collagen sample was dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/mL and the mixture was stirred at 4°C for 12 h.

Effect of pH on collagen solubility Eight mL of the collagen solution were poured into several centrifuge tubes, and their pH values were modified to range from 1 to 10 by adding proportionate levels of 6 M NaOH or 6 M hydrochloric acid. Distilled water was added to the sample solution to reach a total volume of 10 mL. The solution was stirred lightly for 30 min at 4°C and then was subject to the rotation of a centrifuge at 10 000 × g for 30 min. From each tube, an aliquot (1 mL) of the supernatant was gathered, and the Lowry method was used in order to measure the protein content (Lowry *et al.*, 1951). The relative solubility of collagen was calculated and was set in contrast with the pH that yielded the most effective solubility.

Effect of NaCl on collagen solubility Five mL of collagen

Table 3. Estimated coefficients of the fitted quadratic polynomial equation for different responses

Parameter	Regression coefficient	Standard error	F value	P value	Indication
Linear					
X ₁	-0.37625	0.2	448.19	< 0.0001	significant
X ₂	-0.0704167	0.2	15.70	0.0013	significant
X ₃	0.51375	0.2	835.63	< 0.0001	significant
X ₄	0.197917	0.2	124.02	< 0.0001	significant
Quadratic					
X ₁ ²	-0.837396	0.19	2537.25	< 0.0001	significant
X ₂ ²	-0.282396	0.19	288.55	< 0.0001	significant
X ₃ ²	-0.203646	0.19	150.06	< 0.0001	significant
X ₄ ²	-0.133645	0.19	64.63	< 0.0001	significant
Interaction					
X ₁ X ₂	-0.293125	0.25	181.35	< 0.0001	significant
X ₁ X ₃	0.031875	0.25	2.14	0.1637	Not significant
X ₁ X ₄	0.063125	0.25	8.41	0.0110	significant
X ₂ X ₃	-0.014375	0.25	0.44	0.5190	Not significant
X ₂ X ₄	-0.060625	0.25	7.76	0.0139	significant
X ₃ X ₄	-0.070625	0.25	10.53	0.0054	significant

were transferred into 0.05 M acetic acid and then mixed with 5 mL NaCl in 0.05 M acetic acid at different concentrations (0–12% w/v). Accordingly, a final concentration of 1–6% (w/v) was achieved. The solution was stirred gently for 30 min at 4°C and centrifuged at $10\,000 \times g$ for 30 min. The relative solubility was calculated and was set in contrast with the relative solubility of the salt concentration, thereby showing the greatest level of solubility.

Differential scanning calorimetry (DSC) measurements

The denaturation temperature (T_d) of collagen from the eggshell membrane was measured by the DSC device (Perkin-Elmer DSC pyres-1, USA). About 5 mg of the freeze-dried sample were solubilized in 50 mM acetic acid and were then sealed in aluminum pans. The sample was then measured by the DSC. An aluminum pan equipped with 0.5 mol/L acetate buffer solution was used as reference. The temperature increased from 20 to 110°C, at a heating rate of 5°C/min. An average of values from the triplicate was reported. The endothermic peak value of the curve corresponded with the T_d of collagen.

Results and Discussion

Data analysis and evaluation of the fitted model All of the 30 experiments were assessed, and the variable being dependent (Y) was considered (Table 2). The gathered data were subjected to analytical processes and, as a result, model equation no.2 was obtained on the regression. The quadratic coefficients and the linear showed effects of significance ($P > 0.05$). Meanwhile, parameters of X_1X_2 , X_1X_4 , X_2X_4 and X_3X_4 of the interaction coefficients showed significance and difference ($P > 0.05$). Two of the non-significant parameters

were removed at the 95% statistical level, and this allowed the establishment of the response surface model equation according to the following.

The model was highly significant ($F = 21.76$, $p < 0.0001$), and only a 0.01% chance exists for a big model F value to happen because of noise.

$$Y = +8.30 - 0.38X_1 - 0.070X_2 + 0.51X_3 + 0.20X_4 - 0.29X_1X_2 + 0.063X_1X_4 - 0.061X_2X_4 - 0.071X_3X_4 - 0.84X_1^2 - 0.28X_2^2 - 0.20X_3^2 - 0.13X_4^2 \quad \dots \text{Eq. 4}$$

The synergistic effect is indicated by the positive sign of the digits, whereas the negative sign denotes an antagonistic effect (Rodrigues *et al.*, 2006).

ANOVA determined the significance of the coefficients in the model. The values for regression coefficients present in the equation have been documented (Table 4). To check a coefficient's significance, the P values were acquired as a tool. This subsequently determined the interactions among the present variables. Zhang *et al.* stated that any particular model can host any parameter capable of exhibiting a regression coefficient which is considerably large and one P value which is substantially small. This implies that the response variables can be affected more notably (Zhang *et al.*, 2010).

From Table 3, it is evident that all coefficients with linear qualities and the quadratic coefficient terms are significantly available, while their P values are very small ($P < 0.05$). The interaction between the coefficients, except X_1X_3 and X_2X_3 , also had significant effects on the proposed model. The model was highly significant (Table 4). The coefficient of determination (R^2) shows the ratio of the explained variation to the total variation, and measures the fitness degree (Nath *et al.*, 2007). The model is able to fit suitably with actual data if R^2

Table 4. Analysis of variance (ANOVA) for the response surface

Source	Degrees of freedom	Coefficient	Sum of squares	Mean sum of squares	F value	P-value
Model	14		32.48	32.48	306.08	<0.0001
Residual	15		0.11	0.11		
Lack of fit	10		0.81	0.81	1.24	0.4308
Pure error	5		0.033	0.033		
Cor total	29		32.60	32.60		
Adj-R ²		0.918				
CV		1.22				
PRESS		0.51				
Standard deviation		0.087				
Adequate precision		70.088				

gets close to the value 'one' (Sin *et al.*, 2006). Through analysis of variance, the value of R^2 in this model can be calculated to reach a value of 0.9965. This proved that the regression model could explain appropriately the system's authentic response patterns. The determination coefficient (adjusted) ($\text{Adj}R^2 = 0.9933$) approved the model's strong significance, thereby offering an agreeable correlation between the estimated values and the empirical values of the collagen's actual yield. A precise measure of the S: N (signal to noise ratio), if exceeding four, would designate a distinguishing process by the model which is appropriate (Myers *et al.*, 2008). The error analysis showed that the fit test is lacking (0.4308), and posed to be not significant at the 95% statistical level. This is in confirmation regarding the model's validity. Meanwhile, the lower values of the variation's coefficient ($\text{CV} = 1.22$) shows great accuracy, assuming that the empirical values are reliable (Song *et al.*, 2011).

The procedure's optimization The 3D surface response and the 2D plots offered graphical depictions of the regression equation (Fig. 1). Therefore, visualizing the association among responses and empirical levels can be achieved for each variable (Liu *et al.*, 2014; Yu and Chao, 2013). Patterns of the corresponding contour plots show whether the shared interactions among the independent variables have a substantial occurrence (Zhang *et al.*, 2010). Elliptical contours can be achieved under conditions of a fine interface among the independent variables (Muralidhar *et al.*, 2001, Chang *et al.*, 2007), and the surface indicates the highest estimated value which is restricted to the minimum ellipse of the contour's illustration (Zhang *et al.*, 2010). The combined results by concentrations of acetic-acid and temperatures affect the acid soluble yield of collagen obtained by the eggshell membrane (Fig. 1a). Such plots show the outcome by functioning on double factors, thereby maintaining the other variable stable by the mid-point of it. The yield of collagen increased when there was an acetic acid concentration between 0.5 and 0.7 mol/L and a temperature between 15 and 20°C. The extraction yield decreased parallel to the increase in the concentration of acetic

acid (0.7–0.9 mol/L) along with temperature (20–25 h). Therefore, it can be said that the concentration of acetic acid (X_1) and temperature (X_2) can significantly affect the yield and extraction (Table 3).

The yield was shown to be different, because different concentrations of acetic acid were used, and this could have been the result of differences in how much the collagen can be soluble in acidic media used for extraction. The acid concentration being used in the process had roles in determining the extraction bulk's pH value. Therefore, as the interaction among the electrostatic platform occurs, modifications in the acid's concentration could affect the proteins' structure, and the proteins' charge density can determine the value of pH (Verheul *et al.*, 1998). In this study, greater yields were achieved close to the 0.5 M concentration. Lower yields were seen when concentrations ranged between of 0.9–1.1 M. This could be because of the fact that collagen denatures at fairly lower pH values (Carvalho *et al.*, 2003).

Collagen is not a stable protein when confronting temperature, and is prone to denaturation at room temperature. Its chemical structure is partly the reason behind its susceptibility to temperature (Gudmundsson and Hafsteinsson, 1997). Lower levels of hydroxyproline in collagen entail a weaker stability in terms of temperature (Muyonga *et al.*, 2004). Therefore, controlling temperatures during the extraction of tissues relevant to collagen is necessary for the maintenance of collagen's natural structure in its original form. As temperatures increase, collagen is more easily decomposed to form gelatin. This improves the collagen's extraction yield from the eggshell membrane.

The effects of stirring speed are evident on the yield of ASC obtained from the eggshell membrane (Fig. 1b, d and f). The yield of ASC increased by increasing the stirring speed, especially when the speed was within the range of 300–400 rpm. A higher solubility of the eggshell membrane in acetic acid was achieved, resulting in a greater driving force for collagen particles to diffuse from the eggshell membrane into the medium. A slight improvement was shown in this regard,

Fig. 1. Effects of different variables (X_1 : Acetic acid concentration, X_2 : Temperature, X_3 : Stirring speed, X_4 : Treatment time) on collagen extraction yield

however, when the stirring speed became faster than 300 rpm. A greater solubility of the eggshell membrane in acetic acid was observed, thereby exhibiting a higher potential for collagen's particles to be extracted from the eggshell membrane and to enter the medium. A further increase in the speed, making it faster than 400 rpm, however, failed to yield substantial improvements regarding extraction results (Kiew and Don, 2012).

Extraction time and yield correlated positively (Figs. 1c and 2e). The yield of ASC increased as the duration of extraction was prolonged, ranging from 12–24 h. The mass transfer rates of analyte from the matrix play important roles in the extraction's efficiency (Bartle *et al.*, 1991). In this study, collagen's yield increases partly because of analyte's better recovery over extended durations.

Among the four extraction variables in this study, acetic

acid concentration was the most significant factor affecting the yield of ASC in the extraction process, followed by stirring speed, treatment time and temperature. This was proved by the regression coefficients and significance of the quadratic model (Table 3), besides the gradient of slope in the 3D response surface plot (Fig. 1). The results of this work confirmed previous reports by Kiew and Don (2012) and Wang *et al.* (2008), who concluded that collagen can be extracted more efficiently from cultured catfish and grass carp by extending the treatment time and stirring speed. However, longer extraction durations require higher expenditures of energy. For the same reason, the duration of extraction should be controlled and optimized. To reduce time and save costs, the central composite rotatable design was used in the experiment to replace the conventional design of studying factors one by one.

Predictive model's verification The equations that can

Table 5. Optimized collagen extraction conditions predicted by software and experimental responses obtained.

Optimized condition				Predicted response	Experimental response
Acetic acid concentration (mol/L)	Temperature (°C)	Stirring speed (rpm)	Time (h)		
0.44	11.87	399.97	25.89	8.55	8.35

Fig. 2. Solubility of Acid soluble collagen at different pHs (A) and NaCl concentrations (B).

estimate the optimum response values were examined, and the resultant optimum conditions were an acetic acid concentration (X_1) of 0.44 mol/L, a temperature (X_2) of 11.87°C, a stirring speed (X_3) of 399.97 rpm and a duration (X_4) of 25.89 h (Table 5). The prediction error is a relative error which is entered into the results of the experiment. Optimizations for the yield of collagen extraction was 8.55%. The RSM model was validated by the fact that a value of 8.35 was observed in the actual experiments, thereby demonstrating that the used model is appropriate for the purpose of extraction.

Amino acid composition Analyzing the amino acids of ASC revealed variations among the results (Table 6). Collagen is triple helical in nature and is characterized by a unique composition of amino acids (Gly-Pro-Hyp)_n (Singh *et al.*, 2011). Accordingly, glycine (Gly) was the most abundant compound and showed 309 per 1 000 residues in the ASC from the eggshell membrane. The stabilization of conformation requires the existence of glycine residues in a pattern that includes one glycine in every three positions of the amino acid sequence. The ratio of hydroxyproline and proline acid residues are 104 and 125 residues per 1 000 amino acid residues, respectively. The amount of imino acid (hydroxyproline and proline) content is extremely important because it affects functional properties, i.e. solubility, cross-linking ability and the thermal stability of collagen (Gomez-Guillen *et al.*, 2011). Small fractions of histidine, methionine, phenylalanine and tyrosine residues were found with 8, 9, 10 and 9 residues per 1 000 residues, respectively. These results

are in accordance with previous results reported by Zhao and Chi (2009). The composition of amino acids and the low cysteine level in collagen obtained from the eggshell membrane confirm the presence of type I collagen.

Effect of pH and NaCl concentration on solubility The effect of pH value and NaCl concentration on the solubility of ASC obtained from the eggshell membrane is shown in Fig. 2. The results showed that the highest solubility of ASC was found at the pH value of 5, and the solubility decreased when the pH value exceeded 5. There was a sudden fall in solubility at the neutral pH value. Generally, ASC is more soluble in pH ranges that are acidic (Foegeding *et al.*, 1996). This result confirmed a previous report regarding the solubility of eel collagens which reached the lowest value of solubility at a pH value of 7 (Veeruraj *et al.*, 2013). Furthermore, Kittiphattanabawon *et al.* suggested that variations among the molecular conformations of collagen cause differences in solubility when pH values vary (Kittiphattanabawon *et al.*, 2005). In this study, the solubility of ASC obtained from the eggshell membrane remained at a constant level parallel to the increase in the NaCl concentration which rose up to 3%. A dramatic decrease in the solubility of ASC was observed at 4% or above. The maximum values of solubility of ASC and PSC, obtained from the eel skin, were measured when the salt concentration ranged between 3–4% (Veeruraj *et al.*, 2013). Jongjareonrak *et al.* (2005) demonstrated that adding salt can reduce the solubility of collagens as the ionic strength increases and the hydrophobic interactions among protein chains

Fig. 3. Thermal denaturation curves of acid soluble collagen.

improve, thereby causing protein precipitation (Jongjareonrak *et al.*, 2005). Different contents of hydrophobic amino acids and the isoelectric point of collagens can cause variations among the levels of solubility (Jongjareonrak *et al.*, 2005).

DSC measurements To investigate the thermal stability of collagen solutions, DSC was used in order to determine the T_d value of collagen in the eggshell membrane. As shown in Fig. 3, the DSC spectra of ASC showed a single endothermic peak. The denaturation temperature was observed from the maximum value of the endothermic peak. The T_d of the ASC averaged 46.8°C. The T_d reflects the transformation of collagen from a triple helical structure to a random coil (Pietrucha, 2005). A broad endothermic peak for collagen in Fig. 3 proved that this peak results from the complex thermos transition that includes the disruption of protein/water interactions, the rupture of hydrogen bonds and the evaporation or vaporization of the bound water (Rochdi *et al.*, 1999). The temperature of denaturation may be influenced by the degree of hydroxylation of the Pro and the Gly–Pro–Hyp sequence in collagen (Kittiphattanabawon *et al.*, 2005). In the present study, the T_d of collagen obtained from the eggshell membrane was quite higher than the T_d of collagens obtained in previous studies on fish and calf samples which ranged from 19.4 to 40.8°C (Kittiphattanabawon *et al.*, 2005; Cho *et al.*, 2004; Veeruraj *et al.*, 2013; Wang *et al.*, 2014). In addition, a report suggested that the intramolecular hydrogen bonds of the triple helix structure of collagen are prone to disruption due to the repulsion of collagen molecules in an acetic acid solution. The pyrrolidine rings of imino acids restrict the conformation of polypeptide chains and strengthen the triple helix. The imino acid content is primarily responsible for thermal stability and the formation of the triple stranded helix (Ikoma *et al.*, 2003). There is a positive correlation between the hydroxyproline content and the temperature of denaturation. A positive correlation also exists between the content of imino acids and the temperature of denaturation.

Conclusion

This study investigated the optimum conditions for the extraction of the eggshell membrane collagen by using the

Table 6. Amino acid composition of collagens from eggshell membrane

Amino acids	Residues/1 000 amino acids
Aspartic acid (Asp)	49
Serine (Ser)	34
Alanin (Ala)	93
Glutamic acid (Glu)	63
Cysteine (Cys)	4
Methionine (Met)	9
Leucine (Leu)	28
Threonine (Thr)	18
Glycine (Gly)	309
Histidine (His)	8
Arginine (Arg)	69
Proline (Pro)	125
Hydroxyproline (Hyp)	104
Tyrosine (Tyr)	9
Valine (Val)	27
Isoleucine (Ile)	16
Leucine (Leu)	25
Phenylalanine (Phe)	10
Total	1 000

RSM and CCD. All of the variables (acetic-acid concentration, temperature, stirring speed and time) demonstrated substantial effects on the extraction of ASC. The model presented an R^2 of 0.9965 and a P value of less than 0.0001, which implies that the predicted values are close to the actual values of the yield of ASC. These results approved the model in general. The composition of amino acids revealed the typical composition of collagen and confirmed the presence of type I collagen. ASC had higher solubility at acidic values of pH and in the presence of salt concentrations above 3%. The temperature of denaturation pertaining to the studied ASC correlated with the imino content of the ASC. Even though our results could be useful for the production of ASC from the eggshell membrane, further studies are needed to be directed on the structural chemistry and functional biology of collagen, in order to discover more aspects of this realm.

Acknowledgments We are grateful to the Department of Food Science, Engineering and Technology (Tehran University of Agricultural Engineering and Technology and Shiraz University of Agricultural Engineering and Technology) for support of this data. This paper has been resulted from the student PhD thesis of Tehran University.

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