Extraction of gelatin from salmon (*Salmo salar*) fish skin using trypsin-aided process: optimization by Plackett–Burman and response surface methodological approaches

HuiYin Fan^{1,3} · Marie-Josée Dumont² · Benjamin K. Simpson¹

Revised: 28 July 2017 / Accepted: 8 September 2017 / Published online: 21 September 2017
© Association of Food Scientists & Technologists (India) 2017

Abstract Gelatin from salmon (*Salmo salar*) skin with high molecular weight protein chains (α -chains) was extracted using trypsin-aided process. Response surface methodology was used to optimise the extraction parameters. Yield, hydroxyproline content and protein electrophoretic profile via sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of gelatin were used as responses in the optimization study. The optimum conditions were determined as: trypsin concentration at 1.49 U/g; extraction temperature at 45 °C; and extraction time at 6 h 16 min. This response surface optimized model was significant and produced an experimental value ($202.04 \pm 8.64\%$) in good agreement with the predicted value (204.19%). Twofold higher yields of gelatin with high molecular weight protein chains were achieved in the optimized process with trypsin treatment when compared to the process without trypsin.

Keywords Fish gelatin · Trypsin · Plackett–Burman design · Response surface methodology · Yield · Film forming ability

Introduction

Gelatin is a denatured polypeptide extracted by thermal hydrolysis from pretreated collagen sources, mainly animal skins and bones (Kim and Mendis 2006). Collagen has a molecular weight of approximately 300 kDa, which comprises of three discrete polypeptide α -chains (molecular weights ranging from 80 to 125 kDa) twisted around one another to form a triple-helical structure (Boran and Regenstein 2010). During the gelatin extraction, pre-treatments enhance the collagen extractability, followed by thermal hydrolysis during extraction which disrupts the collagen triple-helical structure and induces helix-to-coil transition, producing gelatin comprised of loosely coiled protein chains (Gómez-Guillén et al. 2002).

Fish skin has been extensively studied as a source of gelatin (Boran and Regenstein 2010). The quality of fish gelatin is mostly affected by its amino acid composition and molecular weight distribution. Its unique amino acid composition, particularly the content of imino acids (hydroxyproline and hydroxylysine), varies depending on the species and living habitat of the fish. High levels of imino acids are correlated with better physicochemical properties of gelatin (Boran and Regenstein 2010; Jongjareonrak et al. 2005). Meanwhile, the quality of gelatin is determined by the length of its protein chains. Longer protein chains of higher molecular weight produce gelatin with better functional properties (Benjakul et al. 2012). The molecular weight distribution of gelatin is greatly influenced by the extraction conditions (Gómez-Guillén et al. 2009). Milder extraction conditions (i.e. lower temperature and shorter reaction time) reduce the degradation level of the protein, thus producing gelatin with high molecular weight protein chains. However, milder extraction conditions result in lower gelatin yields (Boran and Regenstein 2010; Galea

✉ Marie-Josée Dumont
marie-josee.dumont@mcgill.ca

¹ Department of Food Science and Agricultural Chemistry, McGill University (Macdonald Campus), 21,111 Lakeshore Rd., Ste. Anne de Bellevue, QC H9X 3V9, Canada

² Department of Bioresource Engineering, McGill University (Macdonald Campus), 21,111 Lakeshore Rd., Ste. Anne de Bellevue, QC H9X 3V9, Canada

³ Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia

et al. 2000; Harris et al. 2003). In contrast, severe extraction conditions (i.e. higher temperatures and longer reaction times) produce gelatin with improved yields. However, molecular weight distribution is broader due to the greater cleavage of the protein chains into shorter chain fragments (Boran and Regenstein 2010; Kittiphattanabawon et al. 2010).

Lately, pre-treatment conditions with better efficiency have been studied to improve gelatin yield while allowing milder extraction conditions (Benjakul et al. 2012). In this regard, relatively new protease-aided pre-treatment using pepsin has successfully enhanced the conversion of collagen to gelatin and yielded approximately twofold higher amounts of fish skin gelatin as compared to those without pepsin-aided treatment (Nalinanon et al. 2008). The study also reports the use of another protease, namely trypsin, which was found to be more effective than pepsin for extracting gelatin from wastes from the leather industry (Cabeza et al. 1997). To the best of the authors' knowledge, there is no study reporting on the use of trypsin for the extraction of gelatin from fish skin.

To maximize the yield and the quality of extracted gelatin, several optimization studies have been conducted involving processing variables such as the concentration of pre-treating agent, and the temperature and time of pre-treatment and extraction. These optimization studies were performed on New Zealand hoki skins (Mohtar et al. 2010, 2013), surimi processing waste (Norziah et al. 2014), and African catfish skin (Alfaro et al. 2014). Recently, statistical experimental approaches including Plackett–Burman (PB) design and response surface methodology (RSM) have been used to optimize multiple parameters that affect the extraction efficiency (Alfaro et al. 2014; Reddy et al. 2008; Sai-Ut et al. 2014; Zhou et al. 2011). PB design enables an effective determination of significant factors from a large number of process variables for subsequent optimization studies (Plackett and Burman 1944). RSM helps in evaluating the process factors to build models of variables with optimized conditions, and further exposes responses with the highest desirability (Sai-Ut et al. 2014).

Atlantic salmon (*Salmo salar*) that is mostly farm-raised, is a salmon of high commercial importance. Because of the increased demand of skinless salmon food products, large amount of skins (5 wt% of the whole fish) are produced as by-products that can become a potential source of gelatin (Gómez-Guillén et al. 2002, 2009). However, there is no efficient protocol reporting the extraction of gelatin from salmon fish skin using trypsin. Therefore, the objective of this study was to optimize the extraction conditions of gelatin from salmon skin using trypsin-aided process in order to maximize the yield of gelatin with high molecular weight protein chains.

Materials and methods

Chemicals

Atlantic salmon (*S. salar*) skins were obtained from the Jean-Talon fish market, Montreal, Canada. Trypsin from porcine pancreas (EC 3.4.21.4; powdered; 90.97 U/mg) was obtained from ICN Biomedicals Inc. (Ohio, USA); 2-propanol, citric acid anhydrous, glycine, methanol, sodium dodecyl sulfate (SDS) and Tris base were purchased from Fisher Scientific (Fair Lawn, NJ, USA); acetic acid and hydrochloric acid were purchased from Fisher Scientific (Nepean, Ontario, Canada); sodium hydroxide was purchased from Merck (KGaA, Darmstadt, Germany); 2-mercaptoethanol (2-ME), activated charcoal, chloramine-T hydrate, Coomassie Brilliant Blue R-250, Ehrlich's solution, *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and *trans*-4-hydroxy-L-proline (Hyp) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Laemmli sample buffer was purchased from Bio-Rad Laboratories (Hercules, CA, USA); high-molecular-weight protein markers of 53–220 kDa were purchased from GE Healthcare (Buckinghamshire, UK). All chemicals and reagents used were of analytical grade.

Fish skins preparation

Fish skins were manually removed at the fish market and immediately packed in polyethylene bags, kept in ice with a skin/ice ratio of 1:2 (w/w) in a polystyrene box, and transported to the Department of Food Science, McGill University, within 1 h. Upon arrival, residual meat was removed manually from the skin and washed with tap water. The skins were cut into small pieces ($1.5 \times 1.5 \text{ cm}^2$) with scissors and placed in polyethylene bags. The skins were stored at -20°C for further use.

Extraction of gelatin from fish skins

Removal of non-collagenous proteins

The frozen fish skins were thawed overnight in the refrigerator before use. The samples were degreased by tumbling in warm (35°C) water (Muyonga et al. 2004). Non-collagenous proteins from the skin were removed by stirring in 0.45 M NaCl at 4°C for 3 min, and washed with distilled water (Rahman et al. 2008). The skin/solution mass ratio was 1:6 (w/v).

Extraction method for optimization of gelatin extraction

As pre-treatment, collagenous-rich fish skins were soaked in 50 mM tris-HCl buffer (pH 8.0) in the presence of trypsin at different concentrations, and stirred continuously at room temperature (22–25 °C) for different periods of time, then filtered with a Whatman no. 4 filter paper and washed with distilled water. The extraction step was conducted by gently stirring the mixture of pretreated skins and distilled water using a shaking water bath (model 25, Precision Scientific, USA) at different temperatures for different periods of time. The protein solutions were then centrifuged (7000×g, 15 °C, 30 min) and the supernatant was lyophilized using a freeze dryer (Modulyod-115, ThermoSavant, Holbrook, NY, USA) at −50 °C and 120 mBar for 48 h. The lyophilized proteins were stored at −20 °C. The skin/solution ratio was 1:6 (w/v) throughout the process.

Experimental design

Plackett–Burman design

In order to select significant variables for gelatin extraction, the concentration of trypsin used in pre-treatment, the pre-treatment time, extraction temperature and extraction time were tested through PB design experiments. A 12-run PB design was applied to evaluate eleven factors inclusive of four selected variables (A–D) and seven dummy variables (E–K). Each variable was represented at two levels, coded as −1 for the low level and +1 for the high level.

Each level's experimental value was established based on preliminary experimental results. Briefly, gelatin with high molecular weight protein chains were successfully obtained from fish skins pre-treated at a trypsin concentration of 1.0 U/g for 4 h and extracted at 50 °C for 3 h; however, low yields were obtained (data not shown). In this study, each variable and their corresponding levels to be studied were generated. Trypsin concentration ranging from 0.5 (−1) to 1.5 U/g (+1) was generated by referring 1.0 U/g as center level. Trypsin pre-treatment time ranging from 1.5 (−1) to 5 h (+1) was used to minimize the effect of longer pre-treatment time that can lead to a decreased yield of fish skin gelatin extracted (Yang et al. 2007). Extraction temperature ranging from 40 (−1) to 70 °C (+1) was generated to induce the transition of collagen to gelatin which occurs at 40 °C and above (Eastoe and Leach 1977), while excessive cleavage of the protein chains happens at 80 °C and beyond (Djagny et al. 2001). Extraction times ranging from 5 (−1) to 15 h (+1) was performed to increase gelatin yield by increasing extraction times (Muyonga et al. 2004).

Two responses were recorded, namely the yield of the extracted gelatin (calculated based on Hyp content) (response 1, Y_1) expressed in Eq. (1), and the intensity of the protein chains (α -chains) quantified densitometrically via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (response 2, Y_2). The response values (Y_1 and Y_2) were the average of the experiments performed in triplicates. Analysis of the regression coefficients and significant variables with confidence levels above 95% were conducted to the yield (Y_1) and intensity of α -chains (Y_2) in extracted gelatin

Yield of gelatin (based on Hyp content)

$$= \frac{\text{Hyp content of gelatin} \left(\frac{\text{mg}}{\text{g}} \right) \times \text{weight of gelatin obtained (g)}}{\text{Hyp content of fish skin} \left(\frac{\text{mg}}{\text{g}} \right) \times \text{weight of fish skin used (g)}} \times 100\% \quad (1)$$

Response surface methodology

A Box–Behnken design (BBD) of RSM was adopted to determine the effects of the independent variables and to generate the optimum gelatin extraction conditions for maximum response. A total of 18 experiments were conducted with six replicates of the center point. Three significant variables identified from the Plackett–Burman (PB) design were used: trypsin concentration (X_1), extraction temperature (X_2), and time (X_3). Each variable was assessed at three coded levels (−1, 0, +1), by employing the optimal levels estimated from the PB design as level zero. In this regard, the pre-treatment time was fixed at 4 h 58 min, which was the estimated optimal level. The yield of gelatin extracted based on the intensity of the α -chains (Y) was taken as the response calculated by using Eq. (2). Gelatin extracted according to the method described by Kołodziejaska et al. (2008) without enzyme-aided process was used as control for the calculation of the response. Briefly, fish skins were pretreated by stirring in 0.45 M NaCl at 4 °C for 3 min, and subjected to gelatin extraction in distilled water at 45 °C for 60 min. The response value (Y) was the average of the experiments performed in triplicates

Yield of gelatin (based on intensity of α -chains)

$$= \frac{I_1 \times W_1}{I_2 \times W_2} \times 100\% \quad (2)$$

where I_1 is the intensity of α -chains in gelatin with trypsin-assisted process (%); W_1 , the weight of gelatin with trypsin-assisted process (g); I_2 , the intensity of α -chains in gelatin without trypsin-assisted process (%); W_2 , the weight of gelatin without trypsin-assisted process (g).

Values obtained from experimental runs of RSM on gelatin extraction were subjected to analysis of variance (ANOVA), using the design expert (Version 7.0.0) software. A quadratic model was used to fit the response (dependent variable) to the independent variables expressed as coded factors (X_1 , X_2 and X_3). The coefficient of determination (R^2) was used to evaluate the fit of the response surface quadratic model. The three dimensional response surface plots were built as a function of the independent variables (X_1 , X_2 and X_3) to assess the interactive relationship among significant variables and to generate optimal conditions for the gelatin extraction.

Hydroxyproline content

The hydroxyproline (Hyp) content of gelatin was determined according to the method of Bergman and Loxley (1963) with minor modifications. The sample (1.0 g) was hydrolyzed with 6 M HCl (8.0 ml) at 105 °C in an oven for 24 h. The hydrolysate was clarified with 200 mg of activated charcoal and filtered. The filtrate was neutralized with 10.0, 1.0 and 0.1 M NaOH to pH 6.0–6.5. An aliquot of 0.1 ml neutralized sample was transferred into an amber tube and isopropanol (0.2 ml) was added and mixed well. To the mixture, 0.1 ml of an oxidant solution [a mixture of 7% (w/v) chloroamine T and acetate/citrate buffer, pH 6, at a ratio of 1:4 (v/v)] was added and mixed thoroughly. Subsequently, 1.3 ml of Ehrlich's reagent solution [a mixture of 2g 4-dimethylamino-benzaldehyde in 98 ml of 8% (v/v) hydrochloric acid] and isopropanol at a ratio of 3:13 (v/v) was added. The mixture was mixed and heated at 60 °C for 25 min in a shaking water bath and cooled in running water for 2–3 min. The solution was diluted to 5 ml with isopropanol. Absorbance was measured within 30 min at $A_{558\text{ nm}}$ using an UV/Vis spectrophotometer (model DU 800, Beckman Coulter, USA). Hydroxyproline standard curve was prepared using absorbance readings obtained from standard solutions with concentrations ranging from 10 to 60 ppm. Distilled water was used as the blank. The Hyp content was calculated and expressed as mg/g sample.

Protein electrophoretic profile analysis

SDS–PAGE was used for gelatin electrophoretic profile analysis according to the method of Laemmli (1970) with minor modifications. The gelatin samples (0.01 g) were dissolved completely in 1.0 ml distilled water. Solubilized samples were mixed at a 1:1 (v/v) ratio with Laemmli sample buffer (62.5 mM Tris–HCl (pH 6.8), 25% glycerol, 2% SDS and 0.01% Bromophenol Blue) in the presence of 5% 2-ME, and heated at 100 °C for 10 min. Fifteen micrograms of protein of each sample were loaded onto

each well (15 µg/well) of polyacrylamide gels (1.5 mm thickness) comprising of 5% stacking gel and 10% resolving gel, and subjected to electrophoresis using a Mini-PROTEAN II unit (Bio-Rad, USA). Electrophoresis was conducted at constant voltage of 80 V for stacking gel and 120 V for resolving gel for approximately 90 min of total running time. The gel was stained with 1 g Coomassie Brilliant blue R-250 in a 4.5:4.5:1 solution of methanol–water–acetic acid and de-stained several times by gentle shaking with a 8:1:1 solution of water–methanol–acetic acid. High-molecular weight markers of 53–220 kDa (GE Healthcare UK) were used to estimate the molecular weight of the protein fractions. The intensity of the protein fractions was quantified densitometrically using the Quantity One version 4.6.2 software from Bio Rad (USA).

Results and discussion

Screening of significant variables using Plackett–Burman design

The PB experimental design matrix and the corresponding responses are shown in Table 1. The effects of the variables on the yield of gelatin extracted based on Hyp content (Y_1) and statistical analysis of the PB design are shown in Table 2. The concentration of trypsin (A) and extraction temperature (C) were found to significantly increase the yield of extractability of the gelatin, while the effects of trypsin incubation time (B) and extraction time (D) were insignificant. Cabeza et al. (1997) reported that increased concentrations of trypsin may affect the cleavage of the cross-links of collagen and further enhance the gelatin extractability. Meanwhile, increased extraction temperatures produced higher gelatin yield from fresh salmon skin as a result of an increase in collagen solubility (Kolodziejska et al. 2008). In comparison, the effect of the extraction temperature was found to be slightly more significant with a higher contribution level of 32.42% and a lower probability value (p value) of 0.0106 when compared to trypsin concentration with contribution of 32.34% and a p value of 0.0182 (data of p value not shown).

The effects of the variables on the intensity of the α -chains are shown in Table 3. The intensity of the α -chains (Y_2) from extracted gelatin showed that the extraction temperature (C) and time (D) imposed significant influence with negative effects (lower intensity of α -chains measured), while trypsin concentration (A) and trypsin incubation time (B) were insignificant. This is in agreement with the production of shorter chain fragments from greater gelatin degradation attributed to the exposure of higher extraction temperature and longer extraction time (Galea et al. 2000; Kittiphattanabawon et al. 2010). In comparison,

Table 1 Plackett–Burman experiment design with actual experimental values and coded values (in bracket) and response values for gelatin extraction

Exp run	Trypsin (U/g), A	Incubation time (h), B	Extrac. temp (°C), C	Extrac. time (h), D	E	F	G	H	I	J	K	Yield (by Hyp) (%), Y_1	Intensity of α -chains (%), Y_2
1	1.5 (1)	1.5 (−1)	40 (−1)	5 (−1)	1	−1	1	1	−1	1	1	31.7	55.18
2	1.5 (1)	5 (1)	40 (−1)	15 (1)	1	1	−1	−1	−1	1	−1	43.2	49.80
3	0.5 (−1)	5 (1)	70 (1)	5 (−1)	1	1	1	−1	−1	−1	1	36.7	32.76
4	1.5 (1)	1.5 (−1)	70 (1)	15 (1)	1	−1	−1	−1	1	−1	1	42.0	22.56
5	1.5 (1)	1.5 (−1)	70 (1)	15 (1)	−1	1	1	1	−1	−1	−1	45.2	19.47
6	0.5 (−1)	5 (1)	70 (1)	15 (1)	−1	−1	−1	1	−1	1	1	36.8	20.65
7	0.5 (−1)	1.5 (−1)	40 (−1)	15 (1)	−1	1	1	−1	1	1	1	27.3	45.13
8	0.5 (−1)	1.5 (−1)	70 (1)	5 (−1)	1	1	−1	1	1	1	−1	32.9	41.57
9	1.5 (1)	5 (1)	70 (1)	5 (−1)	−1	−1	1	−1	1	1	−1	56.8	53.25
10	0.5 (−1)	5 (1)	40 (−1)	15 (1)	1	−1	1	1	1	−1	−1	30.1	45.42
11	0.5 (−1)	1.5 (−1)	40 (−1)	5 (−1)	−1	−1	−1	−1	−1	−1	−1	11.1	54.08
12	1.5 (1)	5 (1)	40 (−1)	5 (−1)	−1	1	−1	1	1	−1	1	26.2	54.56

Table 2 Effects of the variables on yield of gelatin extracted (based on Hyp content) (Y_1) and statistical analysis of data from the Plackett–Burman design

	Effect	Coefficient	Sum of squares	% Contribution
Intercept		35.00		
Trypsin concentration (U/g), A	11.70	5.85	410.67	32.34 ^a
Pre-treatment time (h), B	6.60	3.30	130.68	10.29
Extraction temp (°C), C	16.57	8.28	411.68	32.42 ^a
Extraction time (h), D	7.97	3.98	95.20	7.50
E (dummy)	5.30	2.65	42.13	3.32
F (dummy)	−2.60	−1.30	10.14	0.80
G (dummy)	9.03	4.52	122.40	9.64
H (dummy)	0.73	0.37	0.81	0.064
J (dummy)	−1.33	−0.67	2.67	0.21
K (dummy)	3.13	1.57	14.73	1.16
AB	9.30	4.65	28.83	2.27

 $R^2 = 0.8811$; Adj- $R^2 = 0.7384$
^a5% significance level

the effect of the extraction temperature was found to be more significant with higher contribution of 37.59% and a lower p value of 0.0091, as compared to the effect of the extraction time with 17.65% of contribution and a p value of 0.0258.

Based on both response analyses from PB experimental designs, three significant variables were identified, namely trypsin concentration during pre-treatment (A), extraction temperature (C) and extraction time (D). Those variables were further optimized in the subsequent studies. The optimal levels of these variables estimated from PB with the highest desirability were used as zero levels in the subsequent optimization studies. To estimate the optimal levels, variables were set as ‘in range’ for goal, while both

responses were set as ‘maximize’ for goal, with the intensity of the α -chains set as the highest importance (++++), and the yield of the extracted gelatin (based on Hyp content) set as moderate importance (+++). The optimal levels estimated were trypsin concentration at 1.5 U/g, extraction temperature at 40 °C, and extraction time of 5 h.

Optimization of significant variables using response surface methodology

The BBD of RSM design matrix and the corresponding results of RSM experiments, together with the experimental values and coded levels of independent variables

Table 3 Effects of the variables on intensity of α -chains (Y_2) in extracted gelatin and statistical analysis of data from the Plackett–Burman design

	Effect	Coefficient	Sum of squares	% Contribution
Intercept		41.20		
Trypsin concentration (U/g), A	2.54	1.27	19.28	2.64
Pre-treatment time (h), B	3.07	1.54	28.37	3.88
Extraction temp (°C), C	−13.53	−6.76	274.46	37.59 ^a
Extraction time (h), D	−9.27	−4.64	128.90	17.65 ^a
E–F	5.48	2.74	45.10	6.18
F–G	−6.77	−3.38	68.68	9.41
G–H	6.79	3.39	69.16	9.47
H–J	2.00	1.00	6.02	0.82
J–K	−0.37	−0.18	0.20	0.028
K–L	0.66	0.33	0.66	0.09
AB	16.37	8.19	89.38	12.24

 $R^2 = 0.9491$; Adj- $R^2 = 0.8601$
^a5% significance level

are shown in Table 4. The intensity of the α -chains of all extracted gelatins were determined using SDS–PAGE analysis and are used as part of the result calculation. It was found that high molecular weight protein chains (α -chains) with molecular weight ranging from 95 to 117 kDa were observed at different intensities (figure not shown). However, no α -chain or other protein fractions were obtained for all gelatin extracted at 30 and 40 °C for 1 h, which showed insufficient extraction heat and time applied for the

conversion of water-insoluble collagen into water-soluble gelatin (Benjakul et al. 2012; Gómez-Guillén et al. 2002).

The ANOVA analysis for the response surface quadratic model for the gelatin yield based on α -chains intensity indicated that the model was significant ($p < 0.05$) at the 95% probability level, with p value of 0.0004. The coefficient of determination (R^2) for the model was 0.9449 (a value >0.75 indicates fitness of the model), indicating that the model is capable of explaining 94.49% of the variation

Table 4 Box–Behnken experiment design with actual experimental values and coded values (in bracket) and response values of the yield of gelatin extracted calculated based on α -chains band intensity

Exp run	Trypsin concentration (U/g), X_1		Extraction temperature (°C), X_2		Extraction time (h), X_3		Yield (by α -chains intensity) (%), Y
1	1.5	(0)	40	(0)	5	(0)	162.26
2	2.5	(1)	30	(−1)	5	(0)	0.79
3	1.5	(0)	40	(0)	5	(0)	215.69
4	1.5	(0)	30	(−1)	9	(1)	3.74
5	2.5	(1)	40	(0)	9	(1)	114.82
6	1.5	(0)	50	(1)	1	(−1)	62.60
7	1.5	(0)	40	(0)	5	(0)	185.52
8	1.5	(0)	40	(0)	5	(0)	210.00
9	2.5	(1)	50	(1)	5	(0)	110.92
10	2.5	(1)	40	(0)	1	(−1)	6.82
11	0.5	(−1)	30	(−1)	5	(0)	0.67
12	0.5	(−1)	40	(0)	9	(1)	90.72
13	1.5	(0)	40	(0)	5	(0)	142.71
14	0.5	(−1)	40	(0)	1	(−1)	4.40
15	1.5	(0)	30	(−1)	1	(−1)	0.44
16	1.5	(0)	40	(0)	5	(0)	192.18
17	1.5	(0)	50	(1)	9	(1)	143.61
18	0.5	(−1)	50	(1)	5	(0)	135.13

Fig. 1 Three-dimensional response surface plots for optimization of gelatin extracted with major protein band intensity, as a function of **a** trypsin concentration and extraction temperature; **b** trypsin concentration and extraction time; **c** extraction temperature and extraction time

in response. The lack of fit for the model was insignificant ($p < 0.05$) with p value of 0.5131, suggesting that the experimental data obtained was a good fit with the model.

The ANOVA analysis of the optimization study indicated that the model terms, the linear coefficients of X_2 , X_3 , and the quadratic coefficients of X_1^2 , X_2^2 and X_3^2 were significant ($p < 0.01$). The linear coefficients of X_1 (trypsin concentrations) were not significant, indicating that these trypsin concentrations could not influence the yields of gelatin with α -chains. This suggested that trypsin, similar to some proteases, enabled collagen structure disruption by cleaving the cross-links at the terminal telopeptide regions, but did not cause cleavage of the collagen triple-helical domain allowing production of gelatin with high molecular weight protein chains (Cabeza et al. 1997; Galea et al. 2000). This was observed with the relatively high yields of gelatin with α -chains obtained when high trypsin concentration (2.5 U/g) was used (refers to Table 4 exp runs 5 and 9). Meanwhile, the extraction temperature and time affected the response significantly, where more severe extraction conditions (higher temperature and time) decreased the yields of gelatin with α -chains. This indicated the random cleavage of gelatin structures, leading to the production of shorter chain fragments and the lower yields (Benjakul et al. 2012; Kittiphattanabawon et al. 2010). From this study, the gelatin yield (Y) can be expressed in terms of the following regression equations as follow:

$$Y = 184.73 + 0.30X_1 + 55.83X_2 + 34.83X_3 - 6.08X_1X_2 + 5.42X_1X_3 + 19.43X_2X_3 - 60.63X_1^2 - 62.22X_2^2 - 69.91X_3^2$$

The optimal levels of each variable for a maximum yield of gelatin extracted with high α -chains band intensity were determined by creating three-dimensional response surface plots (Fig. 1a–c). These plots were made with the response (Y) on the Z-axis against any two independent variables, while keeping another variable at the centre point value (coded value = 0). The yield of gelatin with α -chains increased with the increase in trypsin concentration (X_1) and extraction temperature (X_2) (Fig. 1a), trypsin concentration (X_1) and extraction time (X_3) (Fig. 1b), and extraction temperature (X_2) and extraction time (X_3) (Fig. 1c). However, an increment of these variables beyond the optimum value imposed depletion of the response, indicating excessive destabilization and cleavage of the extracted gelatin, resulting in the decrease in gelatin's α -chains intensity. Excessive extraction conditions,

particularly high extraction temperatures and prolonged extraction times, imposed extensive degradation by cleaving gelatin's high molecular weight protein chains (α -

Fig. 2 SDS–PAGE patterns of salmon fish skin gelatins extracted in triplicate under optimal conditions: (lane 1–3) gelatins obtained in triplicates; HMW denoted for high molecular weight protein markers

chains), which resulted in the concomitant formation of shorter chain fragments. An increase in shorter chain fragments and a decrease in intensity of the gelatin high molecular weight protein chains were observed in gelatin extracted from skin of brown-banded bamboo shark, blacktip shark (Kittiphattanabawon et al. 2010) and African catfish (Alfaro et al. 2014) when using higher extraction temperatures.

The optimum levels of each variable obtained from RSM analysis were: trypsin concentration (X_1) = 1.49 U/g, extraction temperature (X_2) = 45 °C, and extraction time (X_3) = 6 h 16 min, with predicted gelatin yield value of 204.19%. This value was further verified by conducting experiments in triplicates under the optimal conditions. A gelatin yield of $202.04 \pm 8.64\%$ was obtained and was not significantly different ($p > 0.05$) when compared to the predicted value, which demonstrated the validity of the model. The electrophoretic profile analysis (Fig. 2) shows that under these optimal conditions, gelatin contained distinct bands of α -chains. This indicated that gelatin with high molecular weight protein chains can be obtained under these optimized conditions.

Conclusion

The optimized conditions for trypsin-aided extraction of gelatin from salmon fish skin has been achieved using the PB and BBD of RSM. The optimum levels of three significant variables (trypsin concentration, extraction temperature, and extraction time) successfully maximized the yield of gelatin with high molecular weight protein chains. Approximately twofold higher yields of gelatin with high

molecular weight protein chains were attained with the optimized process using trypsin treatment when compared to the process without trypsin.

Acknowledgements The authors would like to thank to Dr. Shudong He, School of Food Science and Engineering, Harbin Institute of Technology, China for his valuable technical suggestions provided in the SDS–PAGE analysis and optimization studies. A grateful acknowledgement goes to the Ministry of Education Malaysia (MOE) and Universiti Malaysia Sabah (UMS) for awarding PhD studentship to HuiYin Fan for her study.

References

- Alfaro AT, Biluca FC, Marquetti C, Tonial IB, Souza NE (2014) African catfish (*Clarias gariepinus*) skin gelatin: extraction optimization and physical–chemical properties. *Food Res Int* 65:416–422
- Benjakul S, Kittiphattanabawon P, Regenstein JM (2012) Fish Gelatin. In: Simpson BK, Nollet LML, Toldra F, Benjakul S, Paliyath G, Hui YH (eds) *Food biochemistry and food processing*, 2nd edn. Wiley, Iowa, pp 388–405
- Bergman I, Loxley R (1963) Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal Chem* 35:1961–1965
- Boran G, Regenstein JM (2010) Fish gelatin. *Adv Food Nutr Res* 60:119–143
- Cabeza L, Taylor MM, Brown EM, Marmer WM (1997) Influence of pepsin and trypsin on chemical and physical properties of isolated gelatin from chrome shavings. *J Am Leather Chem As* 92:200–208
- Djagny KB, Wang Z, Xu S (2001) Gelatin: a valuable protein for food and pharmaceutical industries: review. *Crit Rev Food Sci Nutr* 41(6):481–492
- Eastoe JE, Leach AA (1977) Chemical constitution of gelatin. In: Ward AG, Courts A (eds) *The science and technology of gelatin*. Academic, New York, pp 73–105
- Galea CA, Dalrymple BP, Kuypers R, Blakeley R (2000) Modification of the substrate specificity of porcine pepsin for the enzymatic production of bovine hide gelatin. *Protein Sci* 9(10):1947–1959
- Gómez-Guillén MC, Turnay J, Fernández-Díaz MD, Ulmo N, Lizarbe MA, Montero P (2002) Structural and physical properties of gelatin extracted from different species. A comparative study. *Food Hydrocoll* 16(1):25–34
- Gómez-Guillén MC, Pérez-Mateos M, Gómez-Estaca J, López-Caballero E, Giménez B, Montero P (2009) Fish gelatin: a renewable material for developing active biodegradable films. *Trends Food Sci Technol* 20(1):3–16
- Harris P, Normand V, Norton IT (2003) Gelatin. Unilever Research Laboratory, Sharnbrook, Bedford, Bedford
- Jongjareonrak A, Benjakul S, Visessanguan W, Nagai T, Tanaka M (2005) Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brown striped snapper (*Lutjanus vitta*). *Food Chem* 93(3):475–484
- Kim S-K, Mendis E (2006) Bioactive compounds from marine processing byproducts—a review. *Food Res Int* 39(4):383–393
- Kittiphattanabawon P, Benjakul S, Visessanguan W, Shahidi F (2010) Comparative study on characteristics of gelatin from the skins of brownbanded bamboo shark and blacktip shark as affected by extraction conditions. *Food Hydrocoll* 24(2–3):164–171
- Kołodziejka I, Skierka E, Sadowska M, Kołodziejki W, Nieci-kowska C (2008) Effect of extracting time and temperature on

- yield of gelatin from different fish offal. Food Chem 107(2):700–706
- Laemmli UK (1970) Cleavage of structure protein during the assembly of head bacteriophage T4. Nature 227:680–685
- Mohtar NF, Perera C, Quek S-Y (2010) Optimisation of gelatine extraction from hoki (*Macruronus novaezelandiae*) skins and measurement of gel strength and SDS–PAGE. Food Chem 122(1):307–313
- Mohtar NF, Perera C, Quek S-Y, Hemar Y (2013) Optimisation of gelatine gel preparation from New Zealand hoki (*Macruronus novaezelandiae*) skins and the effect of transglutaminase enzyme on gel properties. Food Hydrocoll 13:204–209
- Muyonga JH, Cole CGB, Duodu KG (2004) Extraction and physico-chemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatin. Food Hydrocoll 18(4):581–592
- Nalinanon S, Benjakul S, Visessanguan W, Kishimura H (2008) Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. Food Hydrocoll 22(4):615–622
- Norziah MH, Kee HY, Norita M (2014) Response surface optimization of bromelain-assisted gelatin extraction from surimi processing wastes. Food Biosci 5:9–18
- Plackett RL, Burman JP (1944) The design of optimum multifactorial experiments. Biometrika 33(4):305–325
- Rahman MS, Al-Saidi GS, Guizani N (2008) Thermal Characterization of gelatin extracted from yellowfin tuna skin and commercial mammalian gelatin. Food Chem 108(2):472–481
- Reddy LVA, Wee Y-J, Yun J-S, Ryu H-W (2008) Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett–Burman and response surface methodological approaches. Bioresour Technol 99:2242–2249
- Sai-Ut S, Benjakul S, Sumpavapol P, Kishimura H (2014) Optimization of gelatinolytic enzyme production by *B. amyloliquefaciens* sp. H11 through Plackett–Burman design and response surface methodology. IAR 6(1):1–10
- Yang H, Wang Y, Jiang M, Oh J, Herring J, Zhou P (2007) 2-Step optimization of the extraction and subsequent physical properties of channel catfish (*Ictalurus punctatus*) skin gelatin. J Food Sci 72(4):C188–C195
- Zhou J, Yu X, Ding C, Wang Z, Zhou Q, Pao H, Cai W (2011) Optimization of phenol degradation by *Candida tropicalis* Z-04 using Plackett–Burman design and response surface methodology. J Environ Sci 23:22–30