Peptide extraction from silver carp (*Hypophthalmichthys molitrix*) scales via enzymatic hydrolysis and membrane filtration

Xiao-yan Zu[1,2], Ya-qi Huang[1], Ya-jing Zhao[1,3],Guang-quan Xiong[1,2], Tao Liao[1,2], Hai-lan Li[1,2]*

[1]Institute of Agro-Products Processing and Nuclear Agricultural Technology, Hubei Academy of Agricultural Sciences, Wuhan, China; [2]Key Laboratory of Agricultural Products Cold Chain Logistics, Ministry of Agriculture and Rural Affairs, Wuhan, China; [3]College of Petrochemical Technology, Lanzhou University of Technology, Lanzhou, China

***Corresponding Author:** Hai-Ian Li, Institute of Agro-Products Processing and Nuclear Agricultural Technology, Hubei Academy of Agricultural Sciences, No. 5 Nanhu Avenue, Hongshan District, Wuhan 430064, Hubei Province, China. Email: hl.li@hotmail.com

Received: 22 June 2022; Accepted: 29 December 2022; Published: 3 May 2023

PAPER

Abstract

In this work, peptides were extracted from Silver carp (SC) scales via protease hydrolysis and separated using two membranes (M1 and M2). The results revealed that the water: SC scale ratios of 50.6 mL/g, alkaline protease 1 (AP1) dose of 2313.6 U/mL, and pH of 8.14 were the optimal hydrolysis conditions, and the peptide yield reached 88.77 ± 0.32%. The optimal conditions of peptide separation were clarified: the operating pressure of the M1 (M2) was 0.25 (0.4) MPa, the liquid temperature was 30°C, and the operation time was 65 min. In this case, the permeability of the M1 (M2) reached 91.73 ± 96% (79.83 ± 7.23%), and the average molecular weight of the peptides was 758 Da (576 Da). Compared with M1 peptides, M2 peptides contained more acidic and aromatic amino acids exhibiting free-radical scavenging and tyrosinase inhibition properties. It might provide a way to utilize SC scales as a promising material to produce bioactive peptides.

Keywords: membrane filtration; peptide extraction; response surface methodology; silver carp scales

Introduction

Silver carp (SC), *Hypophthalmichthys molitrix*, a freshwater fish, has the highest aquaculture production in China. In 2020, the SC aquaculture output reached 3.81 million tons and was 2.30 times that of tilapia (*Oreochromis mossambicus*, 1.65 million tons) (China Fishery Statistical Yearbook, 2020). In the last decade, only a small part of SC scales has been used to produce local characteristic food products, while most of them are processed into inexpensive feed and even discarded as garbage, which possess serious threats to biological resources (Kraiem *et al.*, 2015). SC scales are rich in biomolecules, and other advantages include low price, small size, easy processing through enzymolysis, and low production cost (Xu *et al.*, 2021). Therefore, there is a tendency to replace tilapia scales with those of SC scales to produce fish scale peptides by inland processing companies.

Previous studies on fish scale peptides have mainly focused on the structure of fish scales and the physicochemical properties of their peptides. For instance, Okuda *et al.* (2009) reported that the hydroxyapatite content of fish scales reached 19.31%, which could directly affect the extraction rate of the peptides. Chen *et al.* (2016) clarified that the preparation of collagen from fish scales through acid extraction resulted in a substantial loss of collagen. The use of enzymatic extraction of peptides does not introduce foreign substances, ensuring the purity of the product (Liu *et al.*, 2012). Recent studies have mainly focused on biological functions and action pathways, such as fish scales peptides and glycated

ISSN 1120-1770 online, DOI 10.15586/ijfs.v35i2.2248

peptides conjugated with xylose via Maillard reaction (FSP-MRPs), which could protect against alcohol-induced liver damage. FSP-MRPs significantly reduce the elevated activities of serum aspartate aminotransferase and alanine aminotransferase, decrease the elevated levels of hepatic malondialdehyde and triglycerides, and inhibit the decrease of hepatic superoxide dismutase, catalase, and glutathione peroxidase caused by alcohol-induced liver damage (Chen *et al*., 2020). Peptides derived from crimson snapper scales possess excellent free-radical scavenging activities *in vitro*, and red tilapia scales can be used as a starting material for producing antioxidant peptides (Cai *et al*., 2021; Sierra *et al*., 2021). To the best of our knowledge, these studies rarely involved peptide extraction and separation processes, which are very important in controlling product qualities and production costs and are urgently needed in industrial production.

Extraction optimization of peptides usually refers to the degree of hydrolysis (DH). The higher the DH, the more complete the enzymatic hydrolysis, but this does not mean that the peptide has high biological activity (Gbogouri *et al*., 2004). In industry, the yield of peptides with specific molecular weights (i.e., target peptides) is the key indicator for measuring the production efficiency and the quality of products. In this study, considering peptide yield as an indicator, peptides of SC scales (SCSPs) were extracted by protease hydrolysis subjected to response surface methodology (RSM). The SCSPs were then separated and purified through membrane filtration, followed by analysis of the amino acid (AA) composition by ion-exchange chromatography with ninhydrin postcolumn derivatization. This work provides reference data for further utilization of SC scales and the efficient production of peptides.

Materials and Methods

Materials

SC scales were purchased from Liangzihu Aquatic Products Processing (Wuhan, China). Alkaline protease 1 (AP1, 2,400,000 U/mL, liquid) and neutral protease (NP, 1600 AU/g, powder) were purchased from Genencor Bioengineering (Jiangsu, China). Alkaline protease 2 (AP2, 2400 U/mg, powder) and flavor protease (FP, 500 U/mg, powder) were provided by Novozymes Biotechnology (Jining, China). Alkaline protease 3 (AP3, 200 U/mg, powder) was acquired from Yuanye Biotechnology (Shanghai, China). The filtration membranes were supplied by Langji (Shanghai, China). All reagents in the experiments were of analytical grade.

Preparation of peptides

To remove impurities from the SC scales, 4% (v/v) HCl was added at a ratio of 10:1 (mL/g, v/w). An ultrasonic cleaner (KQ3200db, Kunshan Ultrasonic Instrument Co., Ltd., China) with 90 W was used to assist decalcification for 2 h. The decalcified scales were washed repeatedly with flowing water and put into a blast drying oven (DHG-9203A, Shanghai Yiheng Scientific Instrument Co., Ltd., China) at 50°C for 18 h. After the procedure, transparent, decalcified SC scales were obtained. Proteases were then used to hydrolyze the SC scales. Membrane filtration was carried out by using laboratory membrane separation equipment (LNG-NF-101, Shanghai Langi Membrane Separation Equipment Engineering Co., Ltd., China). Finally, white peptide powder was obtained by freeze-drying.

Selection of proteases

Five grams of decalcified scales were put into distilled water. At the appropriate temperature and pH conditions, five proteases (AP1, AP2, AP3, NP, and FP) were used to hydrolyze the SC scales, with all proteases at the same enzyme activity of 4800 U/mL. The enzymatic hydrolysis conditions were as follows: liquid to solid ratio of 10:1 (v/w), enzyme content of 2 μL, and a hydrolysis time of 4.5 h. To obtain the supernatants, the inactivated liquid was centrifuged at 2775 g for 20 min. The peptide yield of the supernatant was used as the indicator of the enzymatic hydrolysis effect for selecting protease.

Enzymatic hydrolysis optimization based on response surface methodology

After screening protease, the yield of peptide was used as the index of enzymatic hydrolysis effect to conduct single-factor experiment. The effects of water to SC scale ratios (10–60 mL/g), protease dose (0.6–1.6 μL/mL), and pH value (7.5–10.0) on peptide yield were carefully studied. The factors of fish scale mass, enzymatic hydrolysis time, and temperature remained at 1 g, 4 h, and 50°C, respectively.

Combined with the results of the single-factor experiments and considering the peptide yield as the response value, the Box–Behnken design of RSM was employed to conduct a three-factor and three-level experiment for further optimization of the hydrolysis conditions. Three-dimensional response surface and contour maps were generated to analyze the contributions of the factor to the response value as well as the interaction among factors (Davidovich-Pinhas *et al*., 2015).

Table 1. Process factors and levels.

Variables	Code	Unit	Coded variable levels		
			−1	0	1
Water:SC scale	A	mL/g	45	50	55
AP1 dose	B	μL/mL	0.9	1.0	1.1
pH	C		7.5	8.0	8.5

As shown in Table 1, the three variables and their respective ranges were selected on the basis of single-factor tests.

A quadratic model is given in the following formula (Atukuri *et al.*, 2019):

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X_i^2 + \sum_{\substack{i=1 \\ i\langle j}}^{k} B_{ij} X_i X_j + \varepsilon \quad (1)$$

Where, Y is the response function; B_0 is the center point of the system; ε is the random error; B_i, B_{ii}, and B_{ij} represent the coefficients of the linear, quadratic, and interactive effects, respectively; and X_i, X_i^2, and X_iX_j represent the linear, quadratic, and interactive effects of the independent variables (water:SC scale, AP1 dose, and pH value), respectively.

Peptides isolated via membrane filtration

A total of 3 L SCSP samples (12.2 mg/mL) were prepared. Polyethersulfone (PES) resin with a filtration area of 0.24 m^2 was used as the NF membrane. In the experiment, a 5000 Da membrane (M1) was operated to remove proteases and macromolecular impurities from the samples. Filtrates with molecular weights less than 5000 Da were further separated through a 3000 Da membrane (M2). Then, the 3000–5000 Da filtrates were marked as M1 peptides, and filtrates < 3000 Da were marked as M2 peptides.

The effects of operating pressure (0.10–0.45 MPa), operating time (5–80 min), and operating temperature (10–40°C) were analyzed for membrane flux. In the operation time experiment, starting from the first drop of filtrate in the measuring cylinder, samples were taken every 5 min. In other experiments, sampling was concluded after 5 min of operation.

Determination of membrane flux and peptide transmittance

Membrane flux (J_m, L/[m^2•h]) refers to the volume of filtrate that passes through the membrane per unit time and unit membrane area under certain operating conditions. This is usually used to measure the permeation rate and capacity of membrane elements to the material liquid. Membrane flux is expressed by the following formula (2):

$$Jm\ (\mathrm{L/m^2 \cdot h}) = \frac{v}{t \times \mathrm{Am}} \quad (2)$$

Where, v (L) is the filtrate volume, t (h) is the operating time, and A_m (m^2) is the membrane filtration area.

Two groups of 5 L SCSP solutions (pH 7.5, concentration 12.2 mg/mL) were prepared, and filtrates were collected through the M1 and M2 under their optimal operating pressures. The membranes were washed repeatedly with deionized water, and the eluate was collected and combined with the filtrates. The total volume of filtrates was then determined. According to the law of material balance, the permeability of peptides (P, %) was calculated by the below formula (3):

$$P\ (\%) = \frac{c_1 \times v_1}{c_0 \times v_0} \times 100\% \quad (3)$$

Where, c_1 (mg/mL) is the mass concentration of the filtrate, v_1 (mL) is the filtrate volume, c_0 (mg/mL) is the initial mass concentration of the solution, and v_0 (mL) is the initial volume of the solution.

Determination of peptide yield

Peptide yield can be characterized by the biuret method via the colors of the complexing reactant between the peptide bond and copper sulfate. It was determined by the method with minor modifications (Zhang *et al.*, 2019). A standard solution of 10 mg/mL bovine serum albumin was diluted to different concentrations and mixed with 4 mL of biuret reagent. After reacting for 30 min at room temperature, the absorbance value of the solution was measured at 540 nm with distilled water as the blank control. Drawing a standard curve, the regression equation was obtained as follows: y = 0.0549 x + 0.002 (R^2 = 0.9992 in the concentration range of 0–10 mg/mL), where y (A) is the absorbance and x (mg/mL) is the concentration of bovine serum protein.

The SCSP sample was added to a 15% (w/v) trichloroacetic acid solution with a volume ratio of 1:1 for precipitating proteins. After vortexing, the mixture was reacted for 10 min and then centrifuged at 2775 g for 20 min. The supernatant was mixed with 4 mL of biuret reagent, left to react for 30 min at room temperature, and its absorbance was measured at 540 nm using a double beam UV spectrophotometer (UH5300, Hitachi Co., Ltd., Japan). The corresponding concentration was obtained by comparison with the standard curve, and the yield of peptide (Y, %) was calculated according to Equation (4).

$$Y\ (\%) = \frac{c_1 \times f \times v}{m} \times 100\% \quad (4)$$

Where, c_1 (mg/mL) is the peptide concentration in the test solution, f is the dilution ratio, v (mL) is the total volume of test liquid, and m (mg) is the total mass of the sample.

Determination of peptide molecular weight

The molecular weight of the peptides was determined by gel permeation chromatography (GPC, Waters 2690 D, Waters Co., USA). The chromatographic conditions (Jia *et al.*, 2010) with some modifications were as follows: using an aqueous-phase separation column, the flow rate was 0.6 mL/min, the mobile phase was 0.1 M $NaNO_3$ aqueous solution, and the buffer solution was sodium acetate solution at pH 2.7. Here, a Waters 2410 refractive index detector was used, and both the column and detector temperatures were kept at 313 K. The molecular weight of the peptide was determined by using polyethylene oxide as the standard.

Amino acid analysis

The AA compositions were analyzed by an AA analyzer (L-8900, Hitachi Co., Japan) according to the method reported by Matsuo *et al.* (2019). The enzymatic hydrolysate and peptides were dissolved in 0.02 M HCl solution, and the suspension was filtered through a 0.45 μm filter. Then, the samples were sent to the Food Quality Supervision, Inspection, and Testing Center of the Ministry of Agriculture (Wuhan, China) for AA analysis. The samples were analyzed by ninhydrin postcolumn derivatization through an AA analyzer equipped with a C18 column (4.6 × 150 mm, 5 μm, Waters Co., USA). The AA contents were calculated using standard AAs.

Statistical analysis

Data were analyzed via SPSS 20.0 Statistical Analysis System (SPSS Inc., USA). One-way analysis of variance (ANOVA) with Duncan's multiple range test was used to examine significant (at P values of 0.05) differences between comparable treatments. One-way ANOVA is used to study the difference of the average value of the dependent variable among different groups under different levels of a single factor. Data were analyzed by RSM using Design-Expert statistical software (DX 10.0.4, Stat-Ease Inc, USA) to optimize the enzymatic hydrolysis conditions. All experiments were performed with three independent replicates, and the means ± standard deviations (SD) of the replicates were reported.

Results and Discussion

Different proteases affect the yield of Silver carp scale peptides

Results are expressed as mean ± SD from triplicate determinations. Different lowercase letters show a significant ($P < 0.05$) difference among the time groups, and different capital letters show a significant ($P < 0.05$) difference among the protease groups.

As shown in Table 2, AP resulted in higher yields of peptides during the entire enzymolysis process than NP and FP. In addition to hydrolyzing peptide bonds, AP has Ser active sites that can also hydrolyze ester bonds and amide bonds, as well as promote transesterification and transpeptidation (Puri *et al.*, 2002).

In the case of APs, the SCSP yield in the AP1 and AP3 groups was significantly higher ($P < 0.05$) than that in the AP2 group during the entire enzymatic hydrolysis process, indicating that the hydrolyzing ability of AP relates to its state and that higher enzyme activity can be observed in the liquid state than in the solid state. Compared with AP3, AP1 led to a superior yield of SCSPs most of the time, where the yield of SCSPs reached a maximum of 31.27 ± 1.22%. The SCSP yields

Table 2. Effect of different proteases on the yield of peptides.

	Peptide yield (%)				
Time (min)	AP1	AP2	AP3	NP	FP
30	26.99 ± 0.14^{Da}	26.06 ± 0.89^{Da}	23.36 ± 1.10^{Ca}	17.14 ± 1.87^{Ba}	11.93 ± 1.83^{Aa}
60	31.27 ± 1.22^{Db}	28.84 ± 1.61^{Cb}	27.23 ± 0.90^{Cb}	24.62 ± 0.50^{Bb}	17.85 ± 1.39^{Ab}
90	31.90 ± 0.22^{Dbc}	30.77 ± 1.39^{Dbc}	28.02 ± 0.98^{Cbc}	26.25 ± 1.18^{Bbc}	19.98 ± 0.03^{Ac}
120	32.15 ± 0.39^{Cbcd}	31.22 ± 1.04^{Cc}	28.49 ± 0.82^{Bbc}	27.89 ± 0.18^{Bcd}	21.16 ± 0.25^{Acd}
150	32.67 ± 0.58^{Dcd}	30.94 ± 1.02^{Cc}	28.58 ± 1.13^{Bbc}	28.24 ± 0.65^{Bde}	22.01 ± 0.56^{Ade}
180	32.54 ± 0.52^{Cbcd}	31.52 ± 0.91^{Cc}	29.29 ± 0.75^{Bc}	29.48 ± 0.57^{Bde}	22.00 ± 0.65^{Ade}
210	32.45 ± 0.74^{Cbcd}	31.39 ± 0.77^{Cc}	28.85 ± 1.28^{Bbc}	29.45 ± 1.05^{Bde}	22.40 ± 1.10^{Ade}
240	33.29 ± 0.66^{Cd}	31.98 ± 0.79^{Cc}	28.48 ± 0.50^{Bbc}	29.44 ± 1.59^{Bde}	22.52 ± 0.51^{Ade}
270	33.46 ± 0.96^{Dd}	31.78 ± 1.40^{Dc}	28.53 ± 0.90^{Cbc}	30.15 ± 0.79^{Be}	23.50 ± 0.59^{Ae}

show little improvement by prolonging the enzymolysis time beyond 60 min. Thus, AP1 and a 60 min hydrolysis time were selected for subsequent experiments.

Single-factor experiments

From Figure 1A, the yield of SCSP increased with increasing water:SC scale ratio. At a ratio of 50 mL/g, the peptide yield was 86.30 ± 4.55%, which was significantly ($P < 0.05$) higher than that of the lower-ratio groups. No changes were observed among the 50 and 60 mL/g groups. Per Figures 1B and 1C, the SCSP yield increased initially and then decreased with a further increase in AP1 dose and pH value. When the AP1 dose was 1 μL/mL, the SCSP yield reached 87.97 ± 2.03% and was significantly ($P < 0.05$) higher than that of the other groups. When the pH value was 8.0, the peptide yield reached a maximum of 88.23 ± 1.25%, which was significantly higher than that of the pH 8.5–10.0 groups ($P < 0.05$). Thus, a ratio of 50 mL/g, an AP1 dose of 1 μL/mL, and a pH value of 8.0 were implemented in the subsequent experiments.

Notably, the effect of pH on the enzymatic hydrolysis reaction is mainly manifested in changing the spatial conformation and active site of the enzyme molecule (Anwar and Saleemuddin, 1998). Therefore, insufficient or excess pH will cause enzyme denaturation and change the specific binding of the enzyme molecule to the substrate, thus reducing the enzymatic hydrolysis effect and peptide yield.

Optimization of enzymatic hydrolysis conditions by response surface methodology

Response surface design and regression model variance analysis

As shown in Table 3, the highest yield of SCSPs reached 90.7 ± 4.50% under the following conditions: pH = 8.0, ratio of liquid to material = 50 mL/g, and AP1 amount = 1.0 μL/mL. The yield results had a high error and were further verified through ANOVA.

The results of ANOVA are shown in Table 4. The regression equation was obtained as follows: $Y = 88.16 + 0.39\ A - 0.26\ B + 2.40\ C - 3.25\ AB - 3.32\ AC - 4.27\ BC - 2.61\ A^2 - 2.56\ B^2 - 6.33\ C^2$. For the model, $P = 0.0023 < 0.01$, indicated that the model was highly significant. The lack-of-fit phase $P = 0.1606 > 0.05$ indicated that the data were reliable and that the fitting degree was good. High correlation coefficients of R^2 and R^2_{adj} revealed that there were good correlations between each single factor (A, B, C) and the response value of Y (SCSP yield) and could explain 93.41% of the test results. The *F* and P values (Prob > *F*) represent the influence of every factor on the response value (Ikoma *et al.*, 2003). As shown in the results, the contributions of the three single factors to the peptide yield were in the order of pH, water:SC scale ratio, and AP1 dose. The primary term C, the interaction terms AB and AC, and the quadratic terms A^2 and B^2 all significantly affected the peptide yield at the $P < 0.05$ level, and both the interaction terms BC and the

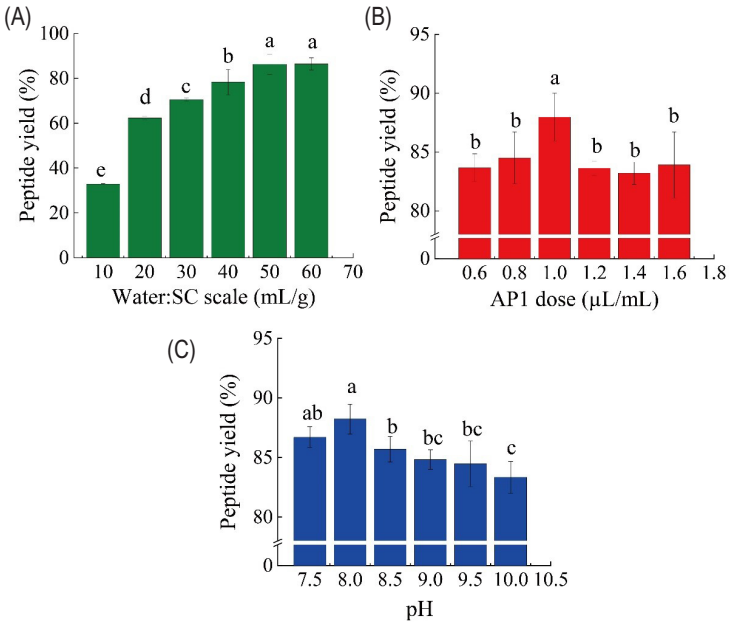

Figure 1. Effects of different water: SC scale values (A); AP1 doses (B); and pH values (C) on peptide yield. The different lowercase letters show significant ($P < 0.05$) differences among the columns.

Table 3. Independent variables and response value.

Run no.	A Water:SC scale (mL/g)	B AP1 dose (μL/mL)	C pH	Y Peptide yield (%)
1	50	1.1	7.5	79.1 ± 2.33
2	45	0.9	8.0	79.3 ± 5.04
3	45	1.0	7.5	72.7 ± 3.21
4	55	1.1	8.0	80.2 ± 3.50
5	55	1.0	8.5	79.1 ± 2.41
6	45	1.1	8.0	87.8 ± 5.58
7	50	1.0	8.0	87.6 ± 5.18
8	50	1.1	8.5	76.4 ± 2.51
9	50	1.0	8.0	87.0 ± 7.05
10	55	1.0	7.5	82.0 ± 4.75
11	50	1.0	8.0	88.6 ± 2.01
12	55	0.9	8.0	84.7 ± 2.55
13	50	1.0	8.0	90.7 ± 4.50
14	50	0.9	7.5	73.6 ± 5.83
15	50	0.9	8.5	88.0 ± 3.23
16	45	1.0	8.5	83.1 ± 4.50
17	50	1.0	8.0	86.9 ± 5.05

quadratic term C^2 significantly affected the peptide yield at the $P < 0.01$ level.

Analysis of the response surface plot

According to the interaction results from Table 3 and Figure 2, with increasing AP1 dose, water:SC scale ratio, and pH value, the SCSP yield initially increased and then decreased. Among them, the pH and AP1 dose surface was steep, and the interaction between them showed a strong effect on the yield of peptides ($P = 0.0051 < 0.01$). In Figure 2, the contour map under the interaction of each factor shows an oval shape and suggests the significant interaction of each factor, which is consistent with the results of ANOVA of the regression model. The contour density along the pH value was higher than that along the AP1 dose or water:SC scale ratio, indicating that pH value had the most significant effect on the peptide yield. This result is consistent with the data in Table 4 ($P = 0.0155 < 0.05$). In addition, there was a synergistic effect among the three factors of AP1 dose, pH, and water:SC scale ratio. The synergy of these factors significantly ($P < 0.05$ or $P < 0.01$) affected the yield of peptides. For example, the single factor AP1 dose showed little effect on the yield of SCSPs, whereas the interaction of AP1 dose with pH significantly affected SCSP yield ($P = 0.0051 < 0.01$).

Method validation

The optimal process parameters were obtained by Design-Expert 10.0.4 Software. When the water:SC scale ratio was 50.613 mL/g, the AP1 dose was 0.964 μL/mL, and the pH value was 8.140. The highest peptide yield was 88.57%. Thus, after optimization, the parameters water:SC scale ratio of 50.6 mL/g, an AP1 dose of 0.96 μL/Ml, and a pH of 8.14 were chosen. The experiment was conducted in triplicate, and the peptide yield was 88.77 ± 0.32%. There was no significant difference between the obtained SCSP yield and the predicted value of the regression equation, indicating that the model was valid.

Table 4. Response surface quadratic model ANOVA and regression coefficient estimation.

Source	Sum of squares	df	Mean square	F	P	Prob > F
Model	452	9	50.22	11.03	0.0023	Significant
A-Water:SC scale	1.2	1	1.20	0.26	0.6234	
B-AP1 dose	0.55	1	0.55	0.12	0.7381	
C-pH	46.08	1	46.08	10.12	0.0155	
AB	42.25	1	42.25	9.28	0.0187	
AC	44.22	1	44.22	9.71	0.0169	
BC	73.10	1	73.10	16.05	0.0051	
A^2	28.57	1	28.57	6.27	0.0407	
B^2	27.49	1	27.49	6.04	0.0437	
C^2	168.71	1	168.71	37.05	0.0005	
Residual	31.88	7	4.55			
Lack of fit	21.99	3	7.33	2.96	0.1606	Not significant
Pure error	9.89	4	2.47			
Cor total	483.88	16				
	$R^2 = 0.9341$		$R^2_{Adj} = 0.8494$		CV = 2.58%	

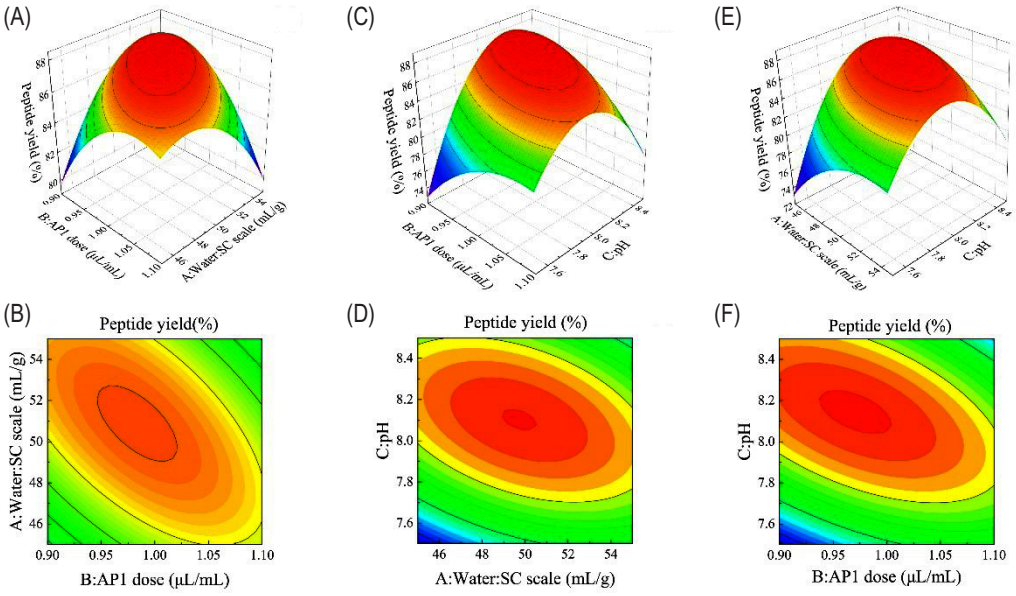

Figure 2. Three-dimensional response surface plots (A, C, and E) and contour maps (B, D, and F) for the interactions of enzymatic hydrolysis conditions.

Membrane flux and peptide permeability of each component

As shown in Figure 3A, with increasing operating pressure, the membrane flux increased gradually and then plateaued. In general, the internal and external pressure difference forms turbulence on the membrane surface, which increases the tangential shear force of the filtrates, thus leading to the increase of the flow rate and permeability per surface unit membrane (Nghiem *et al.*, 2005). However, the membrane flux remained unchanged with increasing pressure when the two membrane pressures (Figure 3) were greater than 0.25 and 0.4 MPa for M1 and M2, respectively. This is because the concentration of solute molecules in the membrane surface area is high, resulting in more macromolecule solutes accumulating on the surface of the membrane to form a gel layer (Fugère *et al.*, 2005). When the pressure is too high, the thickening of the dense gel layer produces strong resistance to the liquid material, thereby counteracting the driving force generated by the pressure and reducing the flux of the membrane.

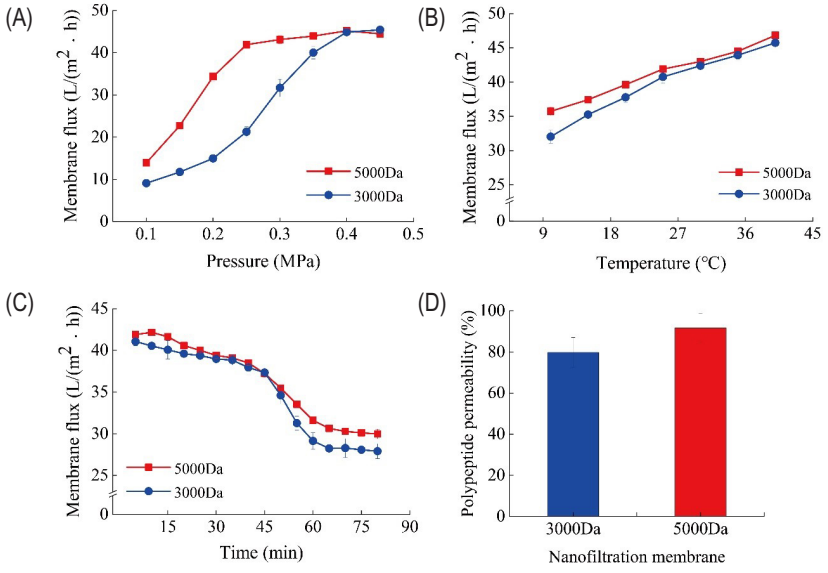

Figure 3. Effects of different operating pressures (A), temperatures (B), and times (C) on the membrane flux and peptide permeability of each component (D).

As shown in Figure 3B, membrane flux increased linearly with gradual increase in the operating temperature. The higher the temperature, the lower is the viscosity and greater the thermal movement of solute molecules, which facilitates the transport of solutes across the membrane and increases the filtrate permeability per unit membrane area (Lau and Ismail, 2009). When there is excess temperature, the solute molecules accumulate on the membrane surface and membrane pores over time, reduce the mass transfer coefficient, and cause irreversible damage to the membrane elements (Benítez *et al.*, 2009). In addition, PES was used as the membrane element material with a limit temperature of 40°C in this experiment. Herein, although a high temperature was beneficial to the increase in membrane flux, we still recommend that the temperature does not exceed 35°C during operation.

In terms of the operating time data shown in Figure 3C, the membrane flux decreased slowly in the early stage and decreased rapidly in the intermediate stage. After 65 min, the membrane flux tended to be stable, probably because a large number of macromolecular solutes were deposited on the membrane surface, forming a permeable multilayer cake (Kaya and Dayanir, 2020). Small-molecule solutes and solvent molecules can enter the membrane pores. Under pressure, solutes with large diameters are blocked by the filter cake layer and enter the boundary layer into a circulation system (Choobar *et al.*, 2019). Thus, after 65 min, the material–liquid boundary layer, filter cake layer, and inside of the membrane pores reached dynamic equilibrium.

The calculated peptide permeability results are shown in Figure 3D. The obtained peptides could penetrate through the M1 and M2 membranes, and the permeability was as high as 91.73 ± 6.96% and 79.83 ± 7.23%, respectively.

Determination of peptide molecular weight by gel permeation chromatography

As shown in Table 5, the average molecular weight (Mw) of the M1 peptides was 758 Da, accounting for 92.32% of the peptides. The Mw of the M2 peptides was 576 Da, accounting for 79.16% of the peptides, and a range of 100–576 Da accounted for 100% of the peptides. This observation was related to the damping effect of the membrane material in the filtration process, where most of the interception was of water-soluble, small-molecule substances.

Analysis of amino acids of different components

As presented in Table 6, M1 peptides contained more hydrophobic AAs and basic AAs, while M2 peptides contained more acidic (Glu, Asp) and aromatic AAs (Phe, Try), as well as His, Val, and Iso. The proportions of Ala, Gly, and Phe in M2 peptides were enhanced by 94.79, 860.60, and 17.95%, respectively, compared with those in the enzymatic hydrolysate. Numerous studies have shown that fish scales and skin peptides have antioxidant effects. The smaller the molecular weight, the more obvious is the antioxidant function, based on gastrointestinal absorption (Ngo *et al.*, 2010; Wu *et al.*, 2015). These functions may be mainly related to their AA compositions (Gómez-Guillén *et al.*, 2011). Studies have revealed that acidic AAs are weakly acidic due to them containing carboxylates that can dissociate H^+ and have a strong promoting effect on the scavenging of DPPH and H_2O_2 free radicals and the reduction of iron (Hernández-Ledesma *et al.*, 2010). The electron-dense side-chain groups contribute to the antioxidant properties of a peptide when there are relatively high number of Try and His residues in the peptide (Udenigwe and Aluko, 2011). In addition, Schurink *et al.* (2007) noted that Phe and Val can inhibit tyrosinase. Ishikawa *et al.* (2007) reported that Ala, Gly, L-Iso, and L-Leu play a positive role in inhibiting melanin synthesis. Based on the above studies, it was speculated that the M2 peptides from SC scales may have a strong free-radical scavenging effect and melanin inhibitory effect.

Conclusion

Referring to the peptide yield as an indicator, AP1 showed the best enzymatic hydrolysis effect on SC scales. According to theoretical and experimental results, a water:SC scale ratio of 50.6 mL/g, AP1 dose of 2313.6 U/mL, and a pH of 8.14 were determined to be the optimal process parameters, and the peptide yield was as high

Table 5. Molecular weight of filter samples.

Sample	Peak	Mn	Mw	Mp	Polymolecularity	Area %
M1 peptide	Main peak	499	758	850	1.382463	92.32
	Minor peak	67	71	69	1.062646	7.68
M2 peptide	Main peak	441	576	497	1.304956	79.16
	Minor peak	88	100	119	1.135745	20.84

Table 6. Amino acid compositions of different components.

Amino acid	Proportion (%)		
	Enzymatic hydrolysate	M1 peptides	M2 peptides
Asp	-	0.59 ± 0.01	0.72 ± 0.05
Thr	0.25 ± 0.00	0.55 ± 0.00	-
Ser	0.47 ± 0.03	1.76 ± 0.02	-
Glu	1.71 ± 0.03	-	1.69 ± 0.08
Pro	-	0.34 ± 0.00	-
Ala	1.73 ± 0.03	4.55 ± 0.08	3.37 ± 0.02
Gly	0.33 ± 0.03	1.49 ± 0.02	3.17 ± 0.06
Cys	5.99 ± 0.66	19.15 ± 1.05	1.59 ± 0.05
Val	2.74 ± 0.15	-	2.32 ± 0.04
Met	8.15 ± 0.48	14.88 ± 1.31	6.47 ± 0.04
Ile	0.57 ± 0.00	-	2.61 ± 0.03
Phe	13.15 ± 1.67	10.57 ± 1.45	15.51 ± 1.12
Orn	-	-	5.59 ± 0.08
Lys	11.98 ± 1.09	9.76 ± 1.11	7.29 ± 0.46
His	-	-	0.14 ± 0.00
Trp	49.82 ± 3.29	31.54 ± 2.07	46.55 ± 1.41
Arg	3.12 ± 0.55	4.82 ± 0.56	2.00 ± 0.00
Hydrophobic AAs	24.18 ± 1.17	34.67 ± 1.23	25.40 ± 0.83
Basic AAs	15.08 ± 1.01	14.58 ± 0.82	9.43 ± 0.45
Acidic AAs	1.71 ± 0.03	0.59 ± 0.01	2.41 ± 0.06
Aromatic AAs	62.97 ± 2.28	42.45 ± 1.43	62.06 ± 1.22

as 88.77 ± 0.32%. In membrane separation, the optimal operating pressure, temperature, and time for M1 were 0.25 MPa (0.4 MPa for M2), 35°C, and 65 min, respectively. Under these conditions, the permeability of SCSPs through the M1 (M2) was as high as 91.73 ± 6.96% (79.83 ± 7.23%). The molecular weight of M1 (M2) peptides was 797 Da (576 Da), accounting for 92.32% (79.16%) of the total peptides. M1 peptides contained more hydrophobic and basic AAs, while M2 peptides contained more acidic AAs, which can scavenge free radicals and inhibit tyrosinases and melanin. This study will provide support for the further utilization of SC scales.

Acknowledgments

The authors appreciate Prof. Xiuqin Kong of Lanzhou University of Technology for her support in this work.

Funding

The study was supported by the National Key Research and Development Program of China (2019YFD0902000), which is an environmental-friendly project.

References

Anwar, A. and Saleemuddin, M., 1998. Alkaline proteases: a review. Bioresource Technology. 64: 175–183. https://doi.org/10.1016/S0960-8524(97)00182-X

Atukuri, J., Odong, B.B. and Muyonga, J.H., 2019. Multi-response optimization of extrusion conditions of grain amaranth flour by response surface methodology. Food Science and Nutrition. 7: 4147–4162. https://doi.org/10.1002/fsn3.1284

Benítez, F.J., Acero, J.L., Leal, A.I. and González, M., 2009. The use of ultrafiltration and nanofiltration membranes for the purification of cork processing wastewater. Journal of Hazardous Materials. 162: 1438–1445. https://doi.org/10.1016/j.jhazmat.2008.06.036

Cai, X.X., Chen, S.Y., Liang, J.P., Tang, M.Y. and Wang, S.Y., 2021. Protective effects of crimson snapper scales peptides against oxidative stress on *Drosophila melanogaster* and the action mechanism. Food and Chemical Toxicology. 148: 111965. https://doi.org/10.1016/j.fct.2020.111965

Chen, J.D., Li, L., Yi, R., Xu, N., Gao, R. and Hong, B., 2016. Extraction and characterization of acid-soluble collagen from scales and skin of tilapia (*Oreochromis niloticus*). LWT - Food Science and Technology. 66: 453–459. https://doi.org/10.1016/j.lwt.2015.10.070

Chen, X., Fang, F. and Wang, S.Y., 2020. Physicochemical properties and hepatoprotective effects of glycated Snapper fish scale peptides conjugated with xylose via Maillard reaction. Food and Chemical Toxicology. 137: 111115. https://doi.org/10.1016/j.fct.2020.111115

China Agricultural Press. China Fishery Statistical Yearbook. 2020. Beijing: Author. pp. 89–90.

Choobar, B.G., Shahmirzadi, M.A.A., Kargari, A. and Manouchehri, M., 2019. Fouling mechanism identification and analysis in microfiltration of laundry wastewater. Journal of Environmental Chemical Engineering. 7: 103030. https://doi.org/10.1016/j.jece.2019.103030

Davidovich-Pinhas, M., Gravelle, A.J., Barbut, S. and Marangoni, A.G., 2015. Temperature effects on the gelation of ethylcellulose oleogels. Food Hydrocolloids. 46: 76–83. https://doi.org/10.1016/j.foodhyd.2014.12.030

Fugère, R., Mameri, N., Gallot, J.E. and Comeau, Y., 2005. Treatment of pig farm effluents by ultrafiltration. Journal of Membrane Science. 255: 225–231. https://doi.org/10.1016/j.memsci.2005.01.036

Gbogouri, G.A., Linder, M., Anni, J.F. and Arementier, M.P., 2004. Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. Food and Chemical Toxicology. 69: 615–622. https://doi.org/10.1111/j.1365-2621.2004.tb09909.x

Gómez-Guillén, M.C., Giménez, B., López-Caballero, M.E. and Montero, M.P., 2011. Functional and bioactive properties of collagen and gelatin from alternative sources: a review. Food Hydrocolloids. 25: 1813–1827. https://doi.org/10.1016/j.foodhyd.2011.02.007

Hernández-Ledesma, B., Contreras, M. and Recio, I., 2010. Antihypertensive peptides: production, bioavailability and

incorporation into foods. Advances in Colloid and Interface Science. 165: 23–35. https://doi.org/10.1016/j.cis.2010.11.001

Ikoma, T., Kobayashi, H., Tanaka, J., Walsh, D. and Mann, S., 2003. Microstructure, mechanical, and biomimetic properties of fish scales from pagrus major. Journal of Structural Biology. 142: 327–333. https://doi.org/10.1016/S1047-8477(03)00053-4

Ishikawa, M., Kawase, I. and Ishii, F., 2007. Combination of amino acids reduces pigmentation in B16F0 melanoma cells. Biological and Pharmaceutical Bulletin. 30: 677–681. https://doi.org/10.1248/bpb.30.677

Jia, J.P., Zhou, Y.G., Lu, J.Z., Chen, A.Y., Li, Y.Z. and Zheng, G.L., 2010. Enzymatic hydrolysis of Alaska pollack (*Theragra chalcogramma*) skin and antioxidant activity of the resulting hydrolysate. Journal of the Science of Food and Agriculture. 90: 635–640. https://doi.org/10.1002/jsfa.3861

Kaya, Y. and Dayanir, S., 2020. Application of nanofiltration and reverse osmosis for treatment and reuse of laundry wastewater. Journal of Environmental Health Sciences. 18: 699–709. https://doi.org/10.1007/s40201-020-00496-7

Kraiem, T., Hassen-Trabelsi, A.B., Naoui, S., Belayouni, H. and Jeguirim, M., 2015. Characterization of the liquid products obtained from Tunisian waste fish fats using the pyrolysis process. Fuel Processing Technology. 138: 404–412. https://doi.org/10.1016/j.fuproc.2015.05.007

Lau, W.J. and Ismail, A.F., 2009. Theoretical studies on the morphological and electrical properties of blended PES/SPEEK nanofiltration membranes using different sulfonation degree of SPEEK. Journal of Membrane Science. 334: 30–42. https://doi.org/10.1016/j.memsci.2009.02.012

Liu, D.S., Liang, L., Regenstein, J.M. and Zhou, P., 2012. Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). Food Chemistry. 133: 1441–1448. https://doi.org/10.1016/j.foodchem.2012.02.032

Matsuo, Y., Miura, L.A., Araki, T. and Yoshie-Stark, Y., 2019. Proximate composition and profiles of free amino acids, fatty acids, minerals and aroma compounds in citrus natsudaidai peel. Food Chemistry. 279: 356–363. https://doi.org/10.1016/j.foodchem.2018.11.146

Nghiem, L.D., Schäfer, A.I. and Elimelech, M., 2005. Pharmaceutical retention mechanisms by nanofiltration membranes. Environmental Science and Technology. 39: 7698–705. https://doi.org/10.1021/es0507665

Ngo, D.H., Qian, Z.J., Ryu, B.M., Park, J.W. and Kim, S.K., 2010. In vitro antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems. Journal of Functional Foods. 2: 107–117. https://doi.org/10.1016/j.jff.2010.02.001

Okuda, M., Takeguchi, M., Tagaya, M., Tonegawa, T., Hashimoto, A., Hanagata, N. and Ikoma, T., 2009. Elemental distribution analysis of type I collagen fibrils in tilapia fish scale with energy-filtered transmission electron microscope. Micron. 40: 665–668. https://doi.org/10.1016/j.micron.2009.04.001

Puri, S., Beg, Q.K., and Gupta, R., 2002. Optimization of alkaline protease production from *Bacillusm sp.* by response surface methodology. Current Microbiology. 44: 286–290. https://doi.org/10.1007/s00284-001-0006-8

Schurink, M., van Berkel, W.J.H., Wichers, H.J. and Boeriu, C.G., 2007. Novel peptides with tyrosinase inhibitory activity. Peptides. 28: 485–495. https://doi.org/10.1016/j.peptides.2006.11.023

Sierra, L.D., Fan, H.B., Zapata, J. and Wu, J.P., 2021. Antioxidant peptides derived from hydrolysates of red tilapia (*Oreochromis sp.*) scale. LWT - Food Science and Technology. 146: 111631. https://doi.org/10.1016/j.lwt.2021.111631

Udenigwe, C.C. and Aluko R.E., 2011. Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. International Journal of Molecular Sciences. 12: 3148–3161. https://doi.org/10.3390/ijms12053148

Wu, X.S., Cai, L.Y., Zhang, Y.H. and Mi, H.B., 2015. Compositions and antioxidant properties of protein hydrolysates from the skins of four carp species. International Journal of Food Science and Technology. 50: 2589–2597. https://doi.org/10.1111/ijfs.12927

Xu, J.M., Zhang, T., Zhang, Y.Y., Yang, L.L., Nie, Y.H., Tao, N.P., Wang, X.C. and Zhong, J., 2021. Silver carp scale gelatins for the stabilization of fish oil-loaded emulsions. International Journal of Biological Macromolecules. 186: 145–154. https://doi.org/10.1016/J.IJBIOMAC.2021.07.043

Zhang, L., Shi, C.W., Xiao, K.J., Li, C.H., Mo, J.Q., Zhang, Z. and Shi, Y.Y., 2019. Improvement and application of biuret method for determination of collagen peptide from tilapia. Food Science. 40: 234–240. https://doi.org/10.7506/spkx1002-6630-20181118-206