



Antioxidant Activity of Hybrid Sturgeon (*Huso dauricus* × *Acipenser schrenckii*) Protein Hydrolysate Prepared Using Bromelain, Its Fractions and Purified Peptides

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Key words: hybrid sturgeon, enzymatic hydrolysis, hydrolysis optimization, bromelain, degree of hydrolysis, radical scavenging activity, peptide purification

Protein hydrolysates could be a natural and safer source of antioxidant peptides. The purpose of this study was to optimize the hydrolysis of *Huso dauricus* × *Acipenser schrenckii* sturgeon proteins using bromelain and purify antioxidant peptides from hydrolysate. The degree of hydrolysis of 18.69% was obtained under the optimal conditions and hydrolysate had 94.76% solubility, 902 nm particle size and high antioxidant activity. The IC₅₀ for DPPH[•] and ABTS^{•+} scavenging activity were 3.14 and 3.81 mg/mL, respectively. The fraction of hydrolysate with a molecular weight of <1 kDa exhibited the highest antiradical activity against DPPH[•] with IC₅₀ of 2.10 mg/mL. In turn, the IC₅₀ of the most active fraction after the Sephadex G-15 separation was 1.77 mg/mL. The reverse phase high performance liquid chromatography (RP-HPLC) was used to purify the peptides from this fraction. The peptide with histidine, leucine and glycine (MW of 0.2955 kDa) exhibited the highest antioxidant activity (IC₅₀ of 1.33 mg/mL). The obtained fractions and peptides with antioxidant activity could be used as natural substitutes for synthetic antioxidants, especially in food and pharmaceuticals.

INTRODUCTION

Sturgeons are freshwater fishes with a high protein content in the meat ranging from 15 g/100 g to 21 g/100 g [Abraha *et al.*, 2018]. The main areas of sturgeon distribution are considered to be the estuaries of large rivers of Eurasia and North America [Billard & Lecointre, 2000]. Sturgeon aquaculture is developed in China, which is well-known for its raising large scale commercial breeding with a production of 79,638 tons (~77% of the global production) of sturgeon species in 2017 [Bronzi *et al.*, 2019]. Hybrid sturgeons are characterized by fast growth and high disease resistance [Luo *et al.*, 2015]. Among the hybrid sturgeon species, the *Huso dauricus* × *Acipenser schrenckii* is the most abundant [Jin *et al.*, 2020].

Free radicals are highly unstable molecules, which easily react with unsaturated lipids and carbohydrate polymers leading to various damages to food as a result of the oxidation process [Jang *et al.*, 2016]. Additionally, free radicals can cause a variety of serious diseases including cancer, diabetes mellitus, tissue inflammation, neurodegenerative diseases

and aging [Zhang *et al.*, 2021]. To reduce or prevent free radical-induced food oxidation, synthetic antioxidants such as propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone are required; however, due to their negative effects, the search for safe natural sources has increased [Samaranayaka & Li-Chan, 2011]. Tan *et al.* [2018] found that the peptides produced by the enzymatic hydrolysis process have antioxidant and antimicrobial properties. They can perform other physiological functions in the human body, such as antihypertensive and antithrombotic activities, in addition to their effective activity against lipid peroxidation and free radicals [Chi *et al.*, 2014; Zhang *et al.*, 2019]. The properties of hydrolysates and peptides resulting from enzymolysis make them potentially useful in the prevention, treatment, and amelioration of several diseases, in addition to extending the shelf life of food products [Famuwagun *et al.*, 2020].

Recently, many studies have found that enzymatic protein hydrolysates from different fish parts had good functional characteristics and high antioxidant activities [Elavarasan & Shamasundar, 2016; Latorres *et al.*, 2018; Wang

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Submitted: 11 October 2021

Accepted: 1 February 2022

Published on-line: 1 March 2022

Z. *et al.*, 2021]. The proper selection of enzymolysis conditions, as well as the monitoring the progress of this process, may result in a production of protein hydrolysates with exceptional sensory quality as well as desired functional and biological properties [Marson *et al.*, 2019]. The antioxidant activity of peptides (3 to 20 amino acid residues) released during hydrolysis is associated to their molecular weights and amino acid profiles [Chalamaiah *et al.*, 2015]. These peptides can be released from fish proteins through *in vitro* enzymatic hydrolysis, but also by *in vivo* digestion, or bacterial fermentation [Chi *et al.*, 2015a]. Several studies depend on protein hydrolysis to isolate antioxidant peptides using ultrafiltration and chromatography techniques from fish hydrolysates such as pacific herring [Wang *et al.*, 2019], skipjack tuna [Zhang *et al.*, 2019] and bluefin leatherjacket [Chi *et al.*, 2015b].

The objective of the current study was to find optimal hydrolysis conditions of hybrid sturgeon (*Huso dauricus* × *Acipenser schrenckii*) protein using bromelain and to purify antioxidant peptides from hydrolysate by its step-by-step fractionation using ultrafiltration membranes, gel filtration chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC).

MATERIALS AND METHODS

Chemicals and reagents

Bromelain (EC 3.4.22.33, 600 U/mg) was purchased from Beijing Solarbio Sciences and Technology Co., Ltd. (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical was obtained from Bomei Biotechnology Co., Ltd (Hefei, China), and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Sigma Chemical Co. (Shanghai, China). Standard amino acids (aspartic acid, histidine, glutamic acid, serine, glycine, threonine, alanine, arginine, tyrosine, cystine, valine, methionine, phenylalanine, isoleucine, leucine, lysine and proline) were purchased from Sigma Chemical Co. (Shanghai, China). All of the chemicals and reagents used in this study were of analytical grade and high purity.

Fish muscle preparation

Hybrid sturgeon *Huso dauricus* × *Acipenser schrenckii* was used as experimental material. The live fish farmed in Leshan City, Sichuan Province, China were obtained from aquatic products company branch in Yibin city, Sichuan, China. The fishes (length of ~80 cm and a weight of ~2 kg) were immediately transported to the laboratory in a water basin, slaughtered, and the viscera were then removed. The hybrid sturgeon muscles (15.25 ± 0.83 g protein/100 g muscles) were separated from the by-products (head, fins, skin, tail, fat and membranes). Following that, the muscles were minced with a blender for 2 min, packaged in polyethylene bags, frozen, and stored at -18°C until use. The prepared muscles were thawed overnight in a refrigerator at 4°C before being subjected to the enzymatic hydrolysis procedure.

Hydrolysates production

To optimize the conditions of hydrolysis of hybrid sturgeon muscle proteins, the single-factor experimental design

was applied. The parameters affecting the enzymatic hydrolysis were the sample-to-buffer ratio, *i.e.* solid/liquid (S/L; 1:1, 1:2, and 1:3, w/v) with constant other parameters (E/S 1:100, pH 6.0, temperature 40°C and time 3 h), enzyme/substrate ratio (E/S; 0.5:100, 1:100, 2:100, 3:100 and 4:100, w/w) with constant other parameters (optimal S/L 1:1, pH 6.0, temperature 40°C and time 3 h), pH (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) with constant other parameters (optimal S/L 1:1, optimal E/S 1:100, temperature 40°C and time 3 h), and reaction temperature (30, 35, 40, 45, 50, 55, and 60°C) with constant other parameters (optimal S/L 1:1, optimal E/S 1:100, optimal pH 6.5, and time 3 h). To assess the impact of those parameters, the degree of hydrolysis (DH) was used as a response parameter. Finally, the hydrolysis were carried out under optimal conditions of S/L, E/S, pH and temperature for 1, 3, 6, and 9 h to determine the optimal incubation time.

The reactions were conducted in jacketed glass vessels (200 mL) heated in a circular water bath. According to the required pH, the minced muscles (100 g) were mixed with acetic acid or sodium phosphate buffer (25 mM). The bromelain was dissolved in the same buffer, and then added at required temperature. The pH was monitored during the reaction time and adjusted with NaOH or HCL (1 N). After the designated time of hydrolysis, the mixture was incubated in a water bath at 90°C for 15 min to inhibit any further enzymatic reactions [Ovissipour *et al.*, 2013], followed by a centrifugation step for 10 min at 12,000×g and 4°C (TGL-18 centrifuge, Sichuan Shuke Instrument Co., Ltd., Chengdu, China). The supernatant was freeze-dried for 48 h at -55°C with a vacuum of 0.25 mbar (BioSafer-18A freeze-dryer, Safer Co., Ltd., Nanjing, China). The lyophilized protein hydrolysates obtained using bromelain (BH) were stored at -20°C in airtight containers until they were used for final product analysis.

Degree of hydrolysis determination

DH was measured as the ratio of α-amino nitrogen (AN) content to total nitrogen (TN) content in the tested samples. The formal titration procedure proposed by Taylor [1957] with modifications was applied to determine the AN content. The homogeneous hydrolysate mixture (1.5 g) obtained from the enzymolysis process was carefully taken, and the weight was increased to 50 g by adding distilled water. The pH of the hydrolysate solution was adjusted to 7.0 using 0.1 N of NaOH solution, then 10 mL of formaldehyde 38% (v/v) was added, and the resultant mixture was left at 25 ± 2°C for 5 min. Finally, the titration process was continued to a pH of 8.5 using the same NaOH solution. The following equation was used to calculate the AN content:

$$AN = \frac{V \times C \times 14.007}{W \times 1000} \times 100 \quad (1)$$

where: V is the volume of NaOH (mL), C is the concentration of NaOH, and W is the hydrolysate weight (g).

The DH was calculated according Equation (2) after TN determination using the macro-Kjeldahl procedure [method 955.04; AOAC, 1998].

$$DH (\%) = \frac{AN}{TN} \times 100 \quad (2)$$

Amino acid composition analysis

The determination of the amino acid profile of hybrid sturgeon lyophilized protein hydrolysate and purified peptide was carried out according to the method of Noman *et al.* [2020a]. Briefly, 100 mg of BH, and 5 mg of purified peptide, which was collected during repeated batches of the purification process by RP-HPLC, were separately hydrolyzed for 22 h at 120°C under a nitrogen atmosphere using 8 mL of 6 N HCl solution. After cooling the mixtures to room temperature (25±2°C), 4.8 mL of 10 N NaOH was added. The volume of the mixtures was adjusted to 25 mL by adding distilled water, filtered through two layers of No.40 filter paper, and then centrifuged at 11,200×g for 10 min. Finally, the Agilent 1100 HPLC system (Agilent Ltd., Palo Alto, CA, USA) was utilized to separate amino acids. The pre-prepared samples (1 µL) were injected into a Zorbax 80 A, C-18 column (250×4.0 mm, 5 µm particle size; Agilent, USA), at a flow rate of 1 mL/min heated to 40°C. The UV detector was set at 338 nm. The solutions of the mobile phases A and B were prepared. The mobile phase A was sodium acetate (7.35 mM)/triethylamine/tetrahydrofuran with a mixing ratio of 500:0.12:2.5 (v/v/v) adjusted to pH 7.5 using acetic acid, whilst the mobile phase B was sodium acetate (7.35 mM)/methanol/acetonitrile (1:2:2, v/v/v) at pH 7.2. The utilized mobile phase gradient was as follows: 0 min, 8% B; 17 min, 50% B; 20.1 min, 100% B; 24 min, 0% B. Amino acids were identified based on the comparison of the retention time of an amino acid standard. External standard quantitative method was used to quantify the amino acid contents of BH and peptide, and the results were expressed as g per 100 g of hydrolysate or total amino acids (TAA) of peptide, respectively.

Particle size distribution

A laser particle size analyzer (Brookhaven, Holtsville, NY, USA) was used to determine the particle size of BH. Briefly, the solution of hydrolysate (10 µg/mL) was diluted with deionized water, and then 3 mL of the resulting solution was placed in the measurement cell. The measurement was taken after 2 min of equilibration of sample solutions at 25°C. The particle size of the product was presented in nm.

Protein hydrolysate solubility

The solubility of BH was estimated following the method of García-Moreno *et al.* [2017] with some modifications. BH (100 mg) was mixed with 40 mL of deionized water, and the pH was adjusted with NaOH and HCL (0.1 N) solutions to 2, 4, 6, 8, and 10. The mixtures were mixed at high speed for 5 min before being centrifuged for 5 min at 1,790×g. Finally, 20 mL of the supernatant was collected and dried for 4 h at 110°C. The following equation was used to calculate the hydrolysate solubility (%):

$$\text{Hydrolysate solubility (\%)} = \left[\frac{W_2 \times 2}{W_1} \right] \times 100 \quad (3)$$

where: W_1 is the weight of the dissolved BH sample and W_2 is the weight of the dried supernatant.

DPPH radical scavenging activity

The DPPH• scavenging activity of BH, its fractions and purified peptides was estimated according to the method

mentioned by Adjimani & Asare [2015] with modifications. In short, 2 mL of BH solution, 1 mL of ultrafiltration fraction, 500 µL of gel filtration fraction or 200 µL of purified peptide solution (1, 2, 3, 4, and 5 mg/mL) was added to 2 mL of DPPH• solution in 95% ethanol (0.1 mM). These mixtures were vortexed and held for 15 min in the absence of light at 25±2°C. The absorbance of final solutions (A_{sample}) and control samples (A_{control}) at 517 nm was measured using a UV-spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China). Instead of the sample solution, distilled water was used in the control sample. The following formula was used to calculate the DPPH• scavenging activity:

$$\text{DPPH radical scavenging activity \%} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (4)$$

Next, the IC_{50} of BH, its fractions and peptide, defined as the sample concentration sufficient to inhibit 50% of DPPH radical was calculated using Excel 2010 (Microsoft, Redmond, WA, USA). A lower IC_{50} indicates a higher DPPH radical scavenging activity of the sample.

ABTS radical cation scavenging activity

The ABTS^{•+} scavenging activity of BH was determined according to Latorres *et al.* [2018] with some modifications. ABTS solution (7.4 mM) and potassium persulfate (2.6 mM) were mixed in equal quantities for 16 h at 25°C in the dark to make a stock solution. The absorbance of 1 mL of work solution was adjusted to 0.70±0.02 at 734 nm by mixing with 50 mL of ethanol (98%). Exactly, 200 µL from each BH concentration (1, 2, 3, 4 and 5 mg/mL) was mixed with 3.5 mL of ABTS^{•+} working solution and left at room temperature for 10 min in the absence of light. To prepare the control sample in this procedure, distilled water was utilized in place of the protein hydrolysate. The absorbance of solutions with BT (A_{sample}) and control (A_{control}) was measured precisely at 734 nm and the ABTS^{•+} scavenging activity was calculated using the following formula:

$$\text{ABTS (\%)} = \left[1 - \left(\frac{A_{\text{sample}}}{A_{\text{control}}} \right) \right] \times 100 \quad (5)$$

The IC_{50} , defined as the BH concentration sufficient to scavenge 50% of ABTS^{•+}, was calculated.

Ultrafiltration of hydrolysate

The BH was fractionated using an ultrafiltration unit (Millipore Minitan system, Millipore, Bedford, MA, USA) with three different molecular weight cut-off (MWCO, 1, 2 and 3 kDa) membranes. The hydrolysate was dissolved in deionized water (50 mg/mL) and four fractions were separated and collected (F1, <1 kDa), (F2, 1–2 kDa), (F3, 2–3 kDa) and (F4, >3 kDa). The fractions were lyophilized and the antioxidant activity was estimated using the DPPH assay.

Separation of peptides by gel filtration chromatography

Gel filtration was used to separate the peptides of fraction with the highest antioxidant activity (MW <1 kDa). Briefly, fraction (60 mg) was dissolved in deionized water at

a concentration of 30 mg/mL and loaded onto a Sephadex G-15 column (2.6×80 cm). The peptides were eluted from the column with deionized water at a flow rate of 1.0 mL/min, aliquots of 3 mL of the eluate were collected and monitored by measuring the absorbance at 280 nm [Chi *et al.*, 2015a] using a spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China). Six fractions were separated and freeze-dried. Hence, the fraction with the highest antioxidant activity (F4) was subjected to the following procedure.

Purification of peptide by RP-HPLC

The HPLC system (Agilent 1260, Santa Clara, CA, USA) was used to purify the peptides from F4 fraction resulting from gel filtration according to the method of Zhang *et al.* [2017] with minor modifications. Briefly, 10 mg of the fraction were diluted in 1 mL of 0.1% (v/v) trifluoroacetic acid (TFA), and 20 μ L of the resultant solution was injected into a Zorbax SB, C-18 column (4.6 mm×250 mm, 5 μ m particle size, Agilent). The mobile phase A was 0.1% (v/v) TFA and B was 30% (v/v) acetonitrile containing 0.1% (v/v) TFA under a flow rate of 0.8 mL/min using a gradient of 0–9 min, 0% B; 10–20 min, 10% B; 20.5–31.5 min, 20% B; 32–41 min, 40% B; and 41.5–43 min, 50% B. The eluate was detected at 280 nm, and six peptides (P1–P6) were isolated, collected and lyophilized.

Molecular weight determination of purified peptide

Gel permeation chromatography was applied using the HPLC system (Waters 1525, Milford, MA, USA) to determine the molecular weight of purified peptide (P2) according to the procedure described by Kong *et al.* [2008]. The TSK gel 2000SW_{XL} column (300×7.8 mm) (Tosoh, Tokyo, Japan) was equilibrated with acetonitrile:water (40:60, v/v) with TFA (0.1%, v/v). The peptides were eluted at an isocratic flow rate of 0.5 mL/min. Detection was at 220 nm and column was heated to 30°C.

Statistical analysis

All chemical experiments were carried out in triplicate, and the average with the standard deviation was recorded. The results obtained were subjected to one-way analysis of variance (ANOVA) to establish the statistical differences. Fisher's least significant difference test was used to evaluate significant differences between mean values using SPSS version 20.0.0 (SPSS IBM, Chicago, IL, USA) at $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of the hydrolysis

DH is a useful parameter for measuring the efficiency of the hydrolysis process [Zheng *et al.*, 2019]. It is dependent on enzyme and substrate concentrations, temperature, pH value, and reaction time [Villamil *et al.*, 2017]. Therefore, the differences in its values may be due to one or more of factors, including enzyme specificity and the experimental conditions used, those influence the enzymatic hydrolysis mechanism [Ktari *et al.*, 2013]. Generally, DH is a valuable tool for monitoring the hydrolytic reaction, allowing end-users to obtain protein hydrolysates with distinct peptide profiles, and thus with different

functional and biological properties [Ang & Ismail-Fitry, 2019]. In the current study, the effect of conditions of hydrolysis using bromelain was tested to reach the maximum DH (Figure 1). The highest DH of 12.17% was reached for the S/L ratio of 1:1 (w/v). The DH was significantly ($p < 0.05$) reduced when the portion of liquid in the reaction mixture was increased (Figure 1A). The obtained maximal S/L ratio was consistent with previous results which indicated that the same 1:1 (w/v) ratio was optimal for hydrolysis of fish proteins using Alcalase or papain [Bhaskar *et al.*, 2008; Noman *et al.*, 2018]. As a result, this ratio was chosen for future experiments. The effect of the E/S ratio on the DH was investigated at 0.5:100, 1:100, 2:100, 3:100, and 4:100 (w/w) as shown in Figure 1B. When the bromelain concentration was increased from 0.5:100 to 1:100 (w/w), the DH increased significantly ($p < 0.05$) from 11.47 to 14.93%, respectively. However, by further increasing the concentration to 2:100 and 3:100, there was no significant ($p < 0.05$) increase in DH. Whereas, DH decreased to 14.16% as the E/S ratio was increased to 4:100. This may be attributed to enzyme aggregation, which prevents proteins from interacting with the enzymes' catalytic sites. Hence, the reaction rate is saturating and the enzymolysis process is inhibited [Gao *et al.*, 2020]. As a result, in our study, 1:100 (w/w) was selected to be the optimal E/S ratio.

The effect of pH on DH was measured over a range of six levels from 5.0 to 7.5 (Figure 1C). The DH increased from 9.79 to 16.06% by increasing the pH from 5.0 to 6.5. Increasing the pH to 7.0 and 7.5 reduced the DH insignificantly ($p \geq 0.05$) to 15.78 and 15.59%, respectively. According to the result of DH, the pH 6.5 was chosen in this stage of optimizing the hydrolysis conditions. Do Evangelho *et al.* [2017] mentioned that some differences in the DH could be attributed to the pH value. The deamination process of glutamine to glutamic acid and asparagine to aspartic acid may occur by acid hydrolysis rather than enzymatic hydrolysis. Therefore, authors observed that the acidic medium led to an increase in the DH. The optimal pH may change based on substrate, and enzyme concentration used in the reaction. However, pH may cause denaturation of the enzyme protein structure or a change in the ionic character of the substrates used in the reaction, both of which reduce the substrates' ability to bind to the enzyme [Salwanee *et al.*, 2013]. In our study, the effects of seven temperatures ranging from 30 to 60°C were investigated, and the results revealed that the maximum DH was obtained at 45°C and 50°C (Figure 1D). There was a significant decrease of DH for hydrolyses obtained at temperatures above 50°C. The reduction in DH may be due to the thermal denaturation, which renders the enzyme inactive and reduces its ability to sever the peptide bonds under high-temperature conditions [Gao *et al.*, 2020; Guérard *et al.*, 2002]. The 45°C was chosen for future experiments.

Finally, the enzymolysis was carried out for various durations under the obtained optimal conditions, and the impact of reaction time on the DH was evaluated within the time range of 1–9 h, as shown in Figure 1E. During the first hour, more than 60% of the hydrolysis occurred. This indicated that the maximum number of peptide bonds were broken during this time period. There was a significant increase ($p < 0.05$) of DH when the time was increased from 1 h (12.15%) to 6 h (18.69%). Nevertheless, increasing the hydrolysis time to

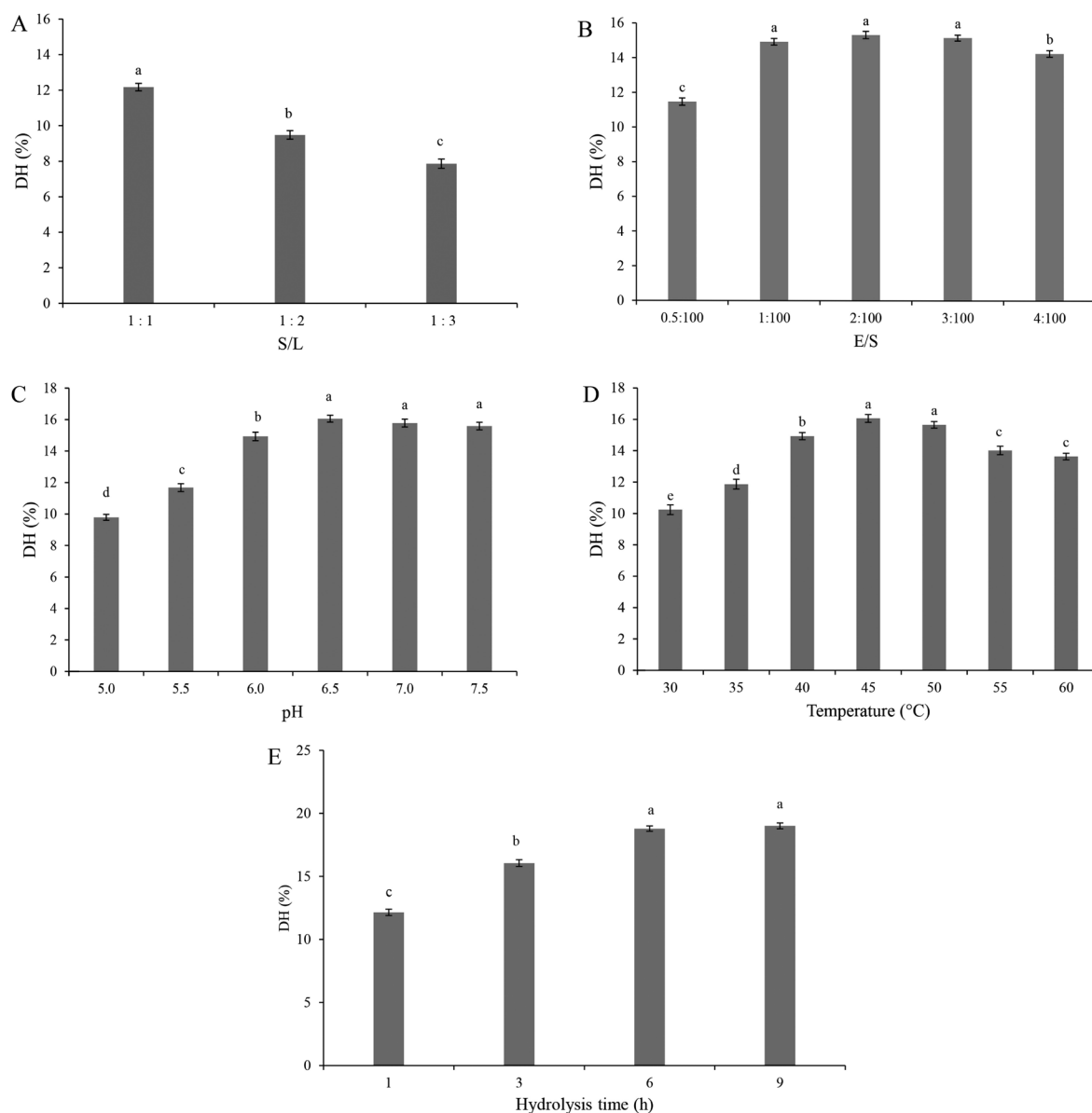


FIGURE 1. The degree of hydrolysis (DH) of hybrid sturgeon (*Huso dauricus* × *Acipenser schrenckii*) proteins using bromelain at different conditions of (A) solid to liquid ratio (S/L); (B) enzyme to substrate ratio (E/S); (C) temperature; (D) pH and (E) reaction time. Different letters above bars indicate significant differences at $p < 0.05$. Data are expressed as mean ± standard deviation of triplicate determinations.

9 h did not result in a significant increase of DH, thus 6 h was chosen as appropriate reaction time for obtaining protein hydrolysate. The DH under optimal conditions (18.69%) was close to results obtained for hydrolyses of Chinese sturgeon (*Acipenser sinensis*) muscles using Alcalase [Noman et al., 2019], and monkfish (*Lophius litulon*) muscle using trypsin [Chi et al., 2014], which were 19.1% and 19.83%, respectively. However, the DH in our study was higher than that obtained for the hydrolysis of tilapia (*Oreochromis niloticus*) frame using pepsin, trypsin, Flavourzyme, Neutrase and papain, which ranged from 5.3% to 15.1% [Fan et al., 2012].

Amino acid profile of hydrolysate

The amino acid profile of the optimized hybrid sturgeon hydrolysate is displayed in Table 1. The arginine, lysine, leucine, and valine were the most abundant among essential

amino acids, while proline, valine and leucine accounted for nearly 54% of hydrophobic amino acids. In our study (Table 1), the content of total hydrophobic amino acids was higher than that obtained by Ghanbari et al. [2015] for sea cucumber (*Actinopyga lecanora*) hydrolysates prepared with several enzymes and by Saidi et al. [2014] for tuna dark muscle by-product protein hydrolysate. On the other hand, the amino acid profile of BH showed that there were significant contents of histidine, tyrosine, and phenylalanine, which together with high contents of leucine and valine (Table 1), could be related to antioxidant activities of protein hydrolysate of hybrid sturgeon. Bahari et al. [2020] reported that the presence of the mentioned amino acids in the peptide sequences enhanced their antiradical activity.

Generally, glutamine and asparagine accounted for about 25% of the total amino acids in the obtained hydrolysate

TABLE 1. Amino acid composition of optimized hybrid sturgeon protein hydrolysate obtained using bromelain.

	Amino acids	Content (g/100 g hydrolysate)
Essential amino acid	Histidine	2.27±0.10
	Threonine	2.47±0.22
	Arginine	11.35±0.10
	Tyrosine*	2.17±0.11
	Valine*	5.70±0.14
	Methionine*	2.57±0.13
	Phenylalanine*	2.38±0.06
	Isoleucine*	3.11±0.10
	Leucine*	5.45±0.11
	Lysine	7.16±0.14
Non-essential amino acids	Asparagine	9.20±0.21
	Glutamine	16.83±0.12
	Serine	2.56±0.10
	Glycine*	3.49±0.25
	Alanine*	2.23±0.10
	Cystine*	1.65±0.06
Total	Proline*	9.46±0.30
	TAAAs	90.05±1.01
	TEAAs	44.63±0.61
	TN-EAAs	45.42±0.46
	THAAs	38.21±0.51

Results are expressed as mean ± standard deviation ($n=3$); TAAAs: total amino acids; TEAAs: total essential amino acids; TN-EAAs: total non-essential amino acids; THAAs: total hydrophobic amino acids.

*Hydrophobic amino acid.

(Table 1), which was consistent with the content of these amino acids in sea cucumber *Isostichopus badionotus* hydrolysate [Pérez-Vega *et al.*, 2013], and was close to the results reported for rohu roe (*Labeo rohita*) protein hydrolysates [Chalamaiah *et al.*, 2013]. Hybrid sturgeon hydrolysate prepared using bromelain proved to be a good source of lysine with a content of 7.16 g/100 g (Table 1). Consequently, BH could be beneficial in diet formulations to alleviate protein malnutrition caused by lysine deficiency, which is limited in cereals and their products [Chalamaiah *et al.*, 2013].

Particle size of hydrolysate

The particle size of BH was 902 ± 61 nm. This result was within the particle size range (100–1500 nm) of swamp eel (*Monopterus albus*) protein hydrolysate obtained by Priatni *et al.* [2020]. The particle size distribution is affected by temperature and hydrolysis time. Noman *et al.* [2020b] reported that after 6 h of hydrolysis at 70°C the particle size was higher than after incubation at 55°C due to the high temperature-induced formation of aggregates. On the other hand, Wang Y.-Y.

et al. [2021] found that the smaller particle size was related to higher DH, due to the increasing number of small peptides or amino acids in hydrolysates with increasingly higher DH.

Solubility of hydrolysates

Solubility affects a number of other functional properties of proteins, thus increasing solubility makes protein hydrolysate a potential source suitable for application in different food systems. Figure 2A depicts the solubility of BH at various pH, and it was found that the BH exhibited a higher solubility at pH 8, representing 94.76%, followed by pH 10 (92.15%) and pH 2 (90.45%) with a significant difference ($p < 0.05$), while the minimum solubility was at the pH 4 (83.17%). These results are in line with previous studies [Latorres *et al.*, 2018; Naqash & Nazeer, 2013], which found that the protein hydrolysate solubility was affected by pH values. Solubility of sturgeon proteins increases as the pH moves away from their isoelectric points (pH 4.5–5.5) because the net charge of the original proteins is reduced at that range. Thus, protein-protein interactions increase, and the protein-water interactions decrease [Noman *et al.*, 2019]. On the other hand, increased solubility as a result of enzymatic hydrolysis may be attributed to an increase in smaller peptides content and charged groups such as $-\text{NH}_3^+$ and $-\text{COO}^-$, which reinforce protein-water interactions and cause more electrostatic repulsion between peptides in the solution [Eberhardt *et al.*, 2019].

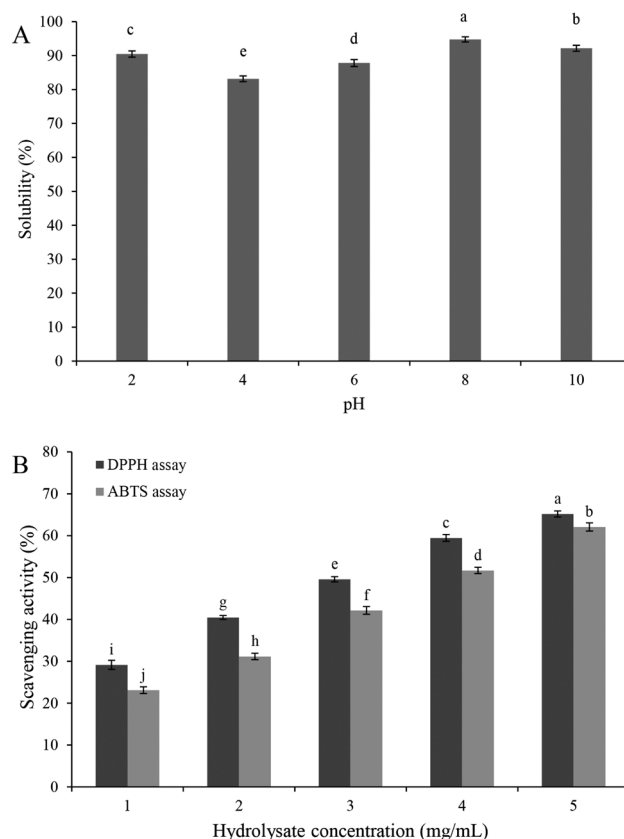


FIGURE 2. The solubility (A) and antioxidant activity (B) of hybrid sturgeon (*Huso dauricus* × *Acipenser schrenckii*) hydrolysate obtained using bromelain. Different letters above bars indicate significant differences at $p < 0.05$. Data are expressed as mean ± standard deviation.

Antioxidant activities of BH

The DPPH radical scavenging activity of hybrid sturgeon protein hydrolysate is displayed in Figure 2B. It was shown that the antiradical activity against DPPH of BH increased significantly ($p < 0.05$) from 29.15% to 65.17% as hydrolysate concentration increased from 1 to 5 mg/mL and IC_{50} was 3.14 mg/mL. The results of this study are consistent with previous studies that found an increase in antioxidant activity as protein hydrolysate concentration increased [De Quadros et al., 2019; Galla et al., 2012]. The DPPH radical scavenging activity of BH at 5 mg/mL exceeded that of shrimp protein hydrolysates prepared using Alcalase and Protamex [Latorres et al., 2018], and *Pseudosciaena crocea* protein hydrolysates obtained using neutral protease [Zhang et al., 2017] at the same concentration. The IC_{50} of BH in DPPH assay was lower than that of salmon by-product hydrolysates prepared using Alcalase, Flavorzyme, Neutrase, Protamex and trypsin, which ranged from 3.62 to 4.95 mg/mL, while it was higher than that of the hydrolysate obtained using pepsin, which was 1.63 mg/mL [Ahn et al., 2014]. The release of bioactive peptides during the hydrolysis depends on enzyme specificity [Famuwagun et al., 2020]. In turn, the DH strongly correlates with the bioactivities of peptides generated during the hydrolysis because it affects the amino acid composition, in addition to the sizes and structures of these peptides [Jang et al., 2016]. The hydrophobic amino acids are remarkably responsible for the antioxidant activities of enzymatically-hydrolyzed protein [Ghanbari et al., 2015]. Among them, leucine, valine, methionine, and alanine as well as aromatic amino acids, such as tyrosine, histidine, and phenylalanine play a key role in peptide activity in the DPPH assay [Bahari et al., 2020]. Analysis of the amino acid profile of BH indicated the presence of these amino acids with significant contents, as shown in Table 1. The type of assay employed to assess antioxidant activity can affect the results, thus, two or more systems acting with different radicals are necessary to evaluate the radical scavenging activities of the selected antioxidants [Centenaro et al., 2011]. Figure 2B displays the ability of BH with different concentrations (1–5 mg/mL) to scavenge

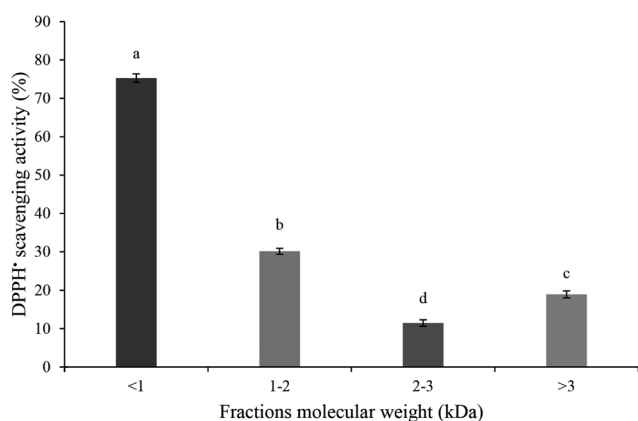


FIGURE 3. DPPH^{*} scavenging activity of membrane ultrafiltration fractions of hybrid sturgeon (*Huso dauricus* × *Acipenser schrenckii*) hydrolysate obtained using bromelain. Different letters above bars indicate significant differences at $p < 0.05$. Data are expressed as mean ± standard deviation.

the ABTS^{•+}. The results showed that the antiradical activity of BH against ABTS^{•+} ranged from 23.09% at 1 mg/mL to 62.07% at 5 mg/mL with IC_{50} of 3.81 mg/mL. Concentration-dependent activity in the ABTS assay was consistent with previous results, which found that increased fish protein hydrolysate concentrations resulted in increased antioxidant activity [Chalamaiah et al., 2013; Tian et al., 2020]. Latorres et al. [2018] reported that the ability of protein hydrolysate to scavenge the ABTS^{•+} was associated with the size of the peptide chain and the enzyme specificity to break the peptide bonds during the enzymolysis. The amino acid composition, particularly the essential and hydrophobic amino acids, may explain differences in the activities of protein hydrolysates from different sources. Histidine, methionine, cysteine, phenylalanine, and tyrosine, may lead to improved ABTS^{•+} scavenging activity [Chalamaiah et al., 2015].

Antioxidant activities of fractions and peptides

Hydrolysate fractions obtained using ultrafiltration

Ultrafiltration membranes are used to separate molecules dissolved in a solution depending on the molecular weight. In the case of protein hydrolysates, ultrafiltration is often used to extract fractions with specific biological properties [Chi et al., 2014]. Figure 3 shows the DPPH radical scavenging activity of the peptide fractions separated from BH by ultrafiltration. The results indicate that the peptide fractions had varying antioxidant activities against DPPH^{*}. The fraction of MW <1 kDa exhibited the highest antioxidant activity (75.28% and IC_{50} of 2.10 mg/mL) with significant differences compared to the other fractions at the same concentrations. This result may be attributed to the content of peptides and amino acids in this fraction that are more active as antioxidants. This finding is consistent with previous studies indicating that low molecular weight peptides were more active as antioxidants due to their ability to scavenge free radicals [Zhang et al., 2019]. According to Zaky et al. [2020], the fractions with small molecular weights resulting from the separation process contained peptides, which act as electron donors and can easily interact with free radicals to turn them into more stable products. On the other hand, not only the MW of the peptides, but also their amino acid content and sequences affect the antioxidant activity [Zou et al., 2016]. According to the result of this experiment, the MW <1 kDa fraction was subjected to further purification.

Gel filtration chromatography fractions

Gel filtration has been extensively used to separate enzymatically-hydrolyzed protein peptides and is an efficient procedure for isolating bioactive molecules with varying molecular weights [Hong et al., 2014]. As shown in Figure 4A, the fraction of MW <1 kDa was separated into six fractions (F1, F2, F3, F4, F5 and F6) using Sephadex G-15. Among these fractions, F4 fraction showed the highest DPPH radical scavenging activity ($p < 0.05$), which was 79.37% (Figure 4B) with IC_{50} of 1.77 mg/mL. The activity of F4 may be attributed to the presence of peptides that could easily scavenge DPPH radicals.

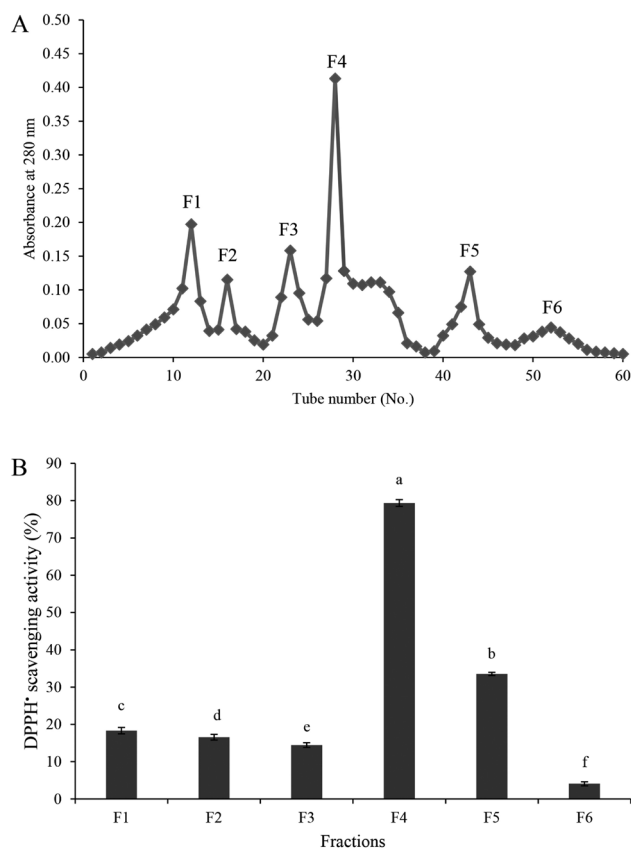


FIGURE 4. Hybrid sturgeon hydrolysate fraction with molecular weight < 1 kDa separated by gel filtration chromatography on Sephadex G-15 (A) and DPPH[•] scavenging activity of the separated F1-F6 fractions (B). Different letters above bars indicate significant differences at $p < 0.05$. Data are expressed as mean \pm standard deviation.

Peptides purified using RP-HPLC

The F4 fraction obtained by Sephadex G-15 gel filtration that displayed the highest DPPH radical scavenging activity was separated further using RP-HPLC on a Zorbax SB, C-18 column. The chromatogram shows eight peaks corresponding to the P1-P8 peptides (Figure 5A). The peak corresponding to P2 peptide was the highest. All isolated peptides were collected separately to determine DPPH radical scavenging activity. Results of the DPPH assay are shown in Figure 5B. The P2 peptide showed a superior antioxidant activity (89.65% with IC_{50} of 1.33 mg/mL) compared to the other isolated peptides. This peptide contained histidine ($28.54 \pm 0.96\%$ TAA), leucine ($16.10 \pm 1.03\%$ TAA), and glycine ($55.37 \pm 1.14\%$ TAA), and had a molecular weight of 0.2955 kDa. According literature data [Ahn *et al.*, 2014], these amino acids, especially histidine and leucine, may be responsible for the antioxidant activity of the purified peptide. The purification process of peptide leads to an increase in hydrogen at *N*-terminals, which are capable of donating hydrogen atoms and scavenging free radicals [Halim *et al.*, 2018]. On the other hand, Jang *et al.* [2016] reported that the histidine-containing peptides displayed strong radical scavenging activity due to the imidazole ring's ability to chelate and trap lipid radicals.

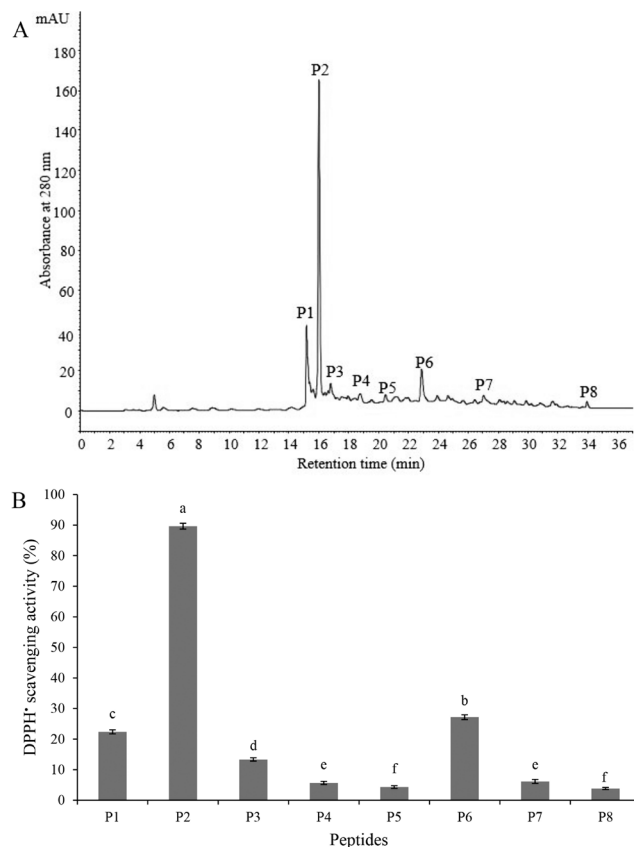


FIGURE 5. RP-HPLC chromatogram of the fraction F4 obtained using Sephadex G-15 gel filtration (A) and DPPH[•] scavenging activity of the separated P1-P8 peptides (B). Different letters above bars indicate significant differences at $p < 0.05$. Data are expressed as mean \pm standard deviation.

CONCLUSIONS

In this study, the enzymatic hydrolysis conditions were optimized to obtain a protein hydrolysate from hybrid sturgeon (*Huso dauricus* \times *Acipenser schrenckii*) using bromelain. A S/L ratio of 1:1, an E/S ratio of 1:100, a pH of 6.5, a temperature of 45°C, and a hydrolysis time of 6 h were the optimum conditions for protein hydrolysis. The bromelain hydrolysate (BH) contained 44.63 g/100 g essential amino acids and 38.21 g/100 g hydrophobic amino acids. According to the current findings, BH fraction with a MW < 1 kDa provided high DPPH[•] and ABTS^{•+} scavenging activities. The antioxidant activity of the peptides from BH could be attributed to their small size and amino acid composition. The obtained antioxidant peptide, which consisted of three amino acids and had an MW of 0.2955 kDa, exhibited a good antiradical activity against DPPH[•]. The current study provided valuable information for improving BH production and purifying its peptides, which could be used as a natural antioxidant peptide source for food preservation and as bioactive food components. However, further work should be conducted to determine the other potential activities and health benefits of BH, and its peptides by simulating the digestion conditions and applying animal models.

RESEARCH FUNDING

This work supported by Key Laboratory of Solid-state Fermentation Resource Utilization of Sichuan Province (2015GTY001), and the Scientific Research Fund of Sichuan Provincial Education Department (18TD0041).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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