Short Communication:

Study on Zinc-binding Capacity of Featherback (*Chitala ornata*) Skin Hydrolysate

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Abstract: *This study aims to valorize featherback (*Chitala ornata*) skin by-product by generating zinc-binding hydrolysate using Alcalase. Commencing with an effective assessment of hydrolysis conditions on the zinc-binding capacity (ZnBC), the hydrolysate gave the highest ZnBC. Subsequently, the hydrolysate was fractionated using ultrafiltration centrifugal devices, analyzed for its amino acid composition, and examined for the stability of its ZnBC against heat and pH. At the best hydrolysis condition, including the skin:water ratio of 1:4 (w/v), enzyme:substrate (E:S) ratio of 40 U/g protein, pH = 8, temperature of 50 °C, and hydrolysis time of 5 h, the gained hydrolysate exhibited the greatest ZnBC of 30.28 ± 0.83% (2.66 folds lower than that of ethylenediamine tetraacetic acid disodium salt (Na_2EDTA)) and contained 8 essential amino acids (making 21.12% total amino acids). Besides, the hydrolysate's ZnBC could retain above 66% after pH treatment in range 1–11 or thermal treatment at 100 °C for 180 min. The < 1 kDa fraction expressed the highest ZnBC of 38.48 ± 2.96%, 2.09 times lower than Na_2EDTA. These findings indicate that hydrolysate and/or its peptide fractions derived from featherback skin could be a natural supplement, especially when producing functional food or nutraceuticals.*

Keywords: *featherback skin; protein hydrolysate; zinc-binding capacity; peptide fraction*

■ INTRODUCTION

The yield of featherbacks reached 6,880 tons/year in Hau Giang, Vietnam, 2020 [1]. Featherback (*Chitala ornata*) skin, accounting for 17–22% of total fish weight, is a by-product of the featherback cake production line [2]. Producing protein hydrolysates/peptides with various bioactivities, including anticholesterol, anticancer, antihypertension, antidiabetics, antioxidants, and mineral-chelating capacity (Ca, Fe, Zn) were found in several studies [3-4]. Alcalase was used to form zinc-binding peptides from various sources, including tilapia skin collagen [5] and scallop adductor [6].

Zn is involved in diverse metabolisms, such as enzymatic catalysis, DNA replication, RNA transcription, cellular signal transduction, and carbohydrate conversion [7-8]. Slight Zn deficiency could lead to the impairment of wound healing process, cell growth, neurological and immune function [7]. This results from poor Zn absorption (because of the presence of phytate, fiber, and folic acid), low solubility of Zn ions in the weakly alkaline intestinal condition, and Zn loss (in certain disease states) [8-9]. Although zinc gluconate and zinc sulfate are used as the first generation of Zn supplements to address Zn shortage, they induce unpleasant metallic off-flavor for fortified foods, disordered intestinal absorption and irritation [9-10]. To meet the demand for Zn and minimize its undesirable side effects, many scientists have focused on peptide-Zn chelates, which offer high stability and bioavailability [5,11].

In this study, Alcalase hydrolysis was employed to generate the featherback skin hydrolysate and its peptide fractions with Zn-binding capacity (ZnBC), especially encompassing (i) finding the best hydrolysis condition to obtain the hydrolysate with greatest ZnBC; (ii) determining its amino acid composition; (iii) evaluating the thermal and pH ZnBC stability of the hydrolysate; and (iv) testing ZnBC for its peptide fractions.

■ EXPERIMENTAL SECTION

Materials

Featherback skin, purchased from a manufacturer in Hau Giang, Vietnam, was washed, cut into small (1 cm×1 cm) pieces, ground and stored at −20 °C until used. Its chemical composition, including 63.23 ± 0.51% moisture, 79.41 ± 1.14% crude protein, 5.76 ± 0.03% crude lipid, and 1.39 ± 0.04% ash (on dry basis), was determined according to guidelines of Nwachukwu and Aluko [12]. Alcalase® 2.4 L (with the activity of 3022.19 ± 103.19 U/mL, from Novozyme, Denmark), analytical grade chemicals (from Sigma-Aldrich and Merck), and double-distilled water were used in this study.

Instrumentation

In this study, a UV-vis spectrometer (UV-vis 752, China), a water bath (Memmert WB14- Germany), and a freeze-dryer (Alpha 1–2/Ldplus, UK) were used.

Procedure

Preparation of the featherback skin hydrolysates

The skin was hydrolyzed by Alcalase, following the procedure described by Vo et al. [13]. The skin was mixed with distilled water to achieve the required skin:water ratio before heating the mixture at 95 °C for 10 min to inactivate endogenous enzymes. The mixture was then adjusted to its pH with either 1 M NaOH or 1 M HCl solution and preheated to the hydrolysis temperature. Subsequently, Alcalase was added at an appropriate E:S ratio. After hydrolysis, the sample was heated at 95 °C for 10 min to inactivate the Alcalase. Finally, the mixture was centrifuged and filtered through Whatman paper no. 3 to collect the supernatant. The soluble protein content was then determined by the Lowry method [14].

Effects of hydrolysis condition on the ZnBC of the featherback skin hydrolysate

The effect of hydrolysis parameters, including the skin:water ratio (w/v), temperature (°C), pH, E:S ratio (U/g protein), and hydrolysis time (h), on the ZnBC of the skin hydrolysate was investigated using a single factor test method, in which one factor was varied at different levels while the other factors remained constant.

Determination of ZnBC

The ZnBC was evaluated using the protocol of Vo et al. [13]. The soluble protein content of tested samples (the hydrolysates and peptide fractions) was brought to 1 mg protein/mL by 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH buffer (40 mM, pH 7.5). Then, 2 mL of the sample was mixed with 1 mL of 8 mM dithiothreitol (DTT) solution and 1 mL of 0.1 mM $ZnSO_4$ solution. Next, the mixture was incubated at 50–60 °C for 30 min before adding 0.1 mL of 2 mM 4-(2-pyridylazo)resorcinol solution and recording its absorbance at 500 nm. ZnBC of the sample was calculated using the Eq. (1);

$$\text{ZnBC } (\%) = \frac{OD_{blank} - OD_{sample}}{OD_{blank}} \times 100\% \quad (1)$$

where OD_{sample} and OD_{blank} were the absorbance at 500 nm of the sample and the blank sample, respectively. For the blank sample, 2 mL of the tested sample was replaced with 2 mL of HEPES-KOH buffer (40 mM, pH 7.5). As much as 1 mg/mL solution of Na_2EDTA in HEPES-KOH buffer (40 mM, pH 7.5) was used as a standard.

Amino acid composition analysis

The amino acid content of the featherback skin hydrolysate was estimated according to the instructions of Vo et al. [13]. The first step was the complete hydrolysis of the sample by 6 M HCl solution for 23 h at 110 ± 2 °C. Then, the amino acids in the obtained mixture were separated using ion-exchange chromatography and reacted with Ninhydrin before detected. Standard solutions of amino acids were used for the quantification of free amino acids in the sample by measuring their absorbance at 440 nm for Pro and 570 nm for the rest of the amino acids.

Determination of thermal and pH stability

The procedure presented by Vo et al. [13] was employed to measure the thermal and pH stability of the hydrolysate's ZnBC. Firstly, the hydrolysate powder was redissolved in distilled water at 40 mg/mL (stock solution). In terms of pH stability, the pH of the 5 mL stock solution was adjusted to a value within the range of 1–11 by either 6 M HCl or 6 M NaOH solution. The samples were then placed at room temperature for 30 min before their pH was readjusted to 7.0 by phosphate buffer (1 M, pH 7.0). Next, the volume of each sample was brought to 20 mL with distilled water and tested for its ZnBC. Regarding thermal stability, the 5 mL stock solution was heated at 100 °C for 0, 30, 60, 90, 120, 150 and 180 min. Subsequently, the samples were suddenly cooled to room temperature in an ice water bath before their ZnBCs were determined. The pH or thermal stability of the ZnBC was expressed as the relative activity (%), defined as the percentage of ZnBC of the treated sample compared to that of the untreated sample.

Peptide fractionation of the hydrolysate

The hydrolysate was further fractionated by ultrafiltration centrifugal devices of 30, 10, 3, and 1 kDa (Macrosep, Pall Laboratory, USA). Five peptide fractions (< 1 kDa, 1–3 kDa, 3–10 kDa, 10–30 kDa, and > 30 kDa) were gained and evaluated for their ZnBC.

Data analysis

Data were presented as means ± standard deviations of triplicate experiments. Analysis of variance (one-way ANOVA) was performed on the data using the Statgraphics Centurion 18 software.

■ RESULTS AND DISCUSSION

Effect of Hydrolysis Conditions on ZnBC of the Hydrolysate

Alcalase was used in this study since it generated high ZnBC hydrolysates/peptides from diverse sources, including tilapia skin collagen [5,15], scallop adductor [6], oyster freeze-dried powder [16], and sea cucumber [17]. Furthermore, Alcalase preferred to hydrolyze residues such as acidic (Glu), hydrophobic (Leu, Ile), sulfur-containing (Met), aromatic (Phe, Tyr), or basic (Lys, Arg) amino acids at the C-terminal [18-19], which possessed high affinities to metal ions [15], favoring the ZnBC of the obtained hydrolysates.

It was observed in Fig. 1 that the ZnBC of the hydrolysate increased as the skin:water ratio rose from 1:1 to 1:4 (w/v) and kept unchanged afterward. The lower ZnBC at the skin:water ratios before 1:4 (w/v) may be due to the high viscosity of the mixtures declined the probability of the enzyme molecules exposed to the substrates, lessening the content of bioactive peptides in the hydrolysates and their ZnBC [20]. On the other hand, a sufficient amount of solvent not only resulted in high solubility of protein but also could effectively disperse the products of hydrolysis, preventing the feedback effect and enhancing the bioactivity of the protein hydrolysate [21]. The skin:water ratio of 1:4 was applied to minimize the solvent amount for further investigations.

The E:S ratio-ZnBC profile (Fig. 2) of the hydrolysate was similar to that of the skin:water ratio-ZnBC (Fig. 1). Low enzyme amounts at E:S ratios within the range of 10–30 U/g protein may cause the excess of substrate for the hydrolysis reaction. Thus, the obtained hydrolysate did not possess the highest ZnBC [22]. Also, the statistically insignificant change in the ZnBC at high E:S ratios (50–60 U/g protein) could be caused by the limited cleavage site of the enzyme [20]. Hence, 40 U/g protein was set for the next experiments.

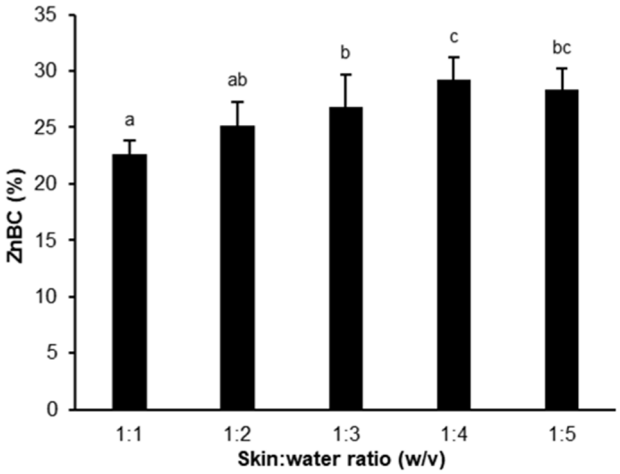

Fig 1. Effect of skin:water ratio on ZnBC of the hydrolysate. The bars with different letters indicate significant differences ($p < 0.05$)

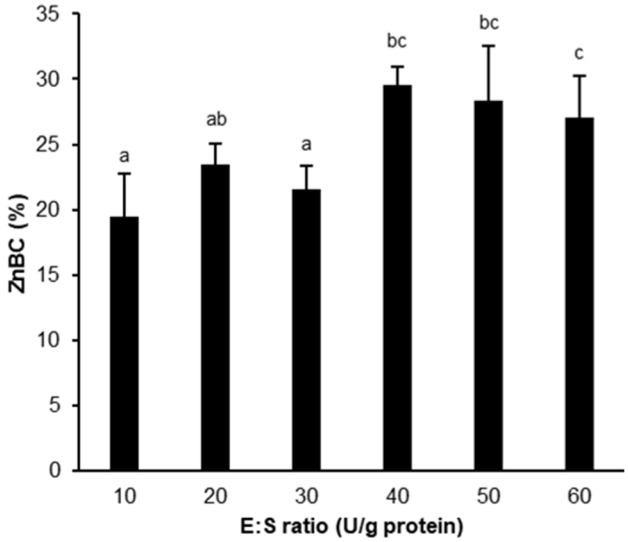

Fig 2. Effect of E:S ratio (B) on ZnBC of the hydrolysate. The bars with different letters indicate significant differences ($p < 0.05$)

The environmental pH simultaneously impacts the enzyme molecules and proteinous substrates, altering the bioactivity of the hydrolysate. pH could enable or disable the protein solubilization and enzyme-substrate assembly by changing their ionization states improving or reducing hydrolysis [21,23-24]. The enzymes displayed the greatest activity under a specific pH condition and could catalyze the reaction more quickly [25]. In this study, the pH most appropriate for the Alcalase hydrolysis of the featherback skin to obtain the hydrolysate with the highest ZnBC was pH 8 (Fig. 3).

Fig. 4 shows that the ZnBC of the hydrolysate reached the peak of 30.59 ± 1.01% at the hydrolysis temperature of 50 °C. This finding was in accordance with the Arrhenius function hypothesizing that the enzyme-catalyzed reaction rate accelerated as elevating temperature [26], releasing a high amount of bioactive peptides from intact protein, enhancing ZnBC of the hydrolysate. Therefore, 50 °C was the hydrolysis temperature for further experiments.

As presented in Fig. 5, ZnBC of the skin hydrolysate increased up to 5 h of hydrolysis and dropped afterward. This was a common result that could be found in several previous studies [5,20,27]. It was known that in the first hydrolysis period, the enzyme converted the intact proteins into high Zn affinity peptides, which would deteriorate if an extensive hydrolysis time was applied [24,28]. Thus, 5 h of hydrolysis was chosen for subsequent examinations.

ZnBC of Peptide Fractions

Fig. 6 indicated the peptide fractions' size and their ZnBC were in inverse proportion. It could be explained that small peptides, such as < 1 kDa, offer a large surface

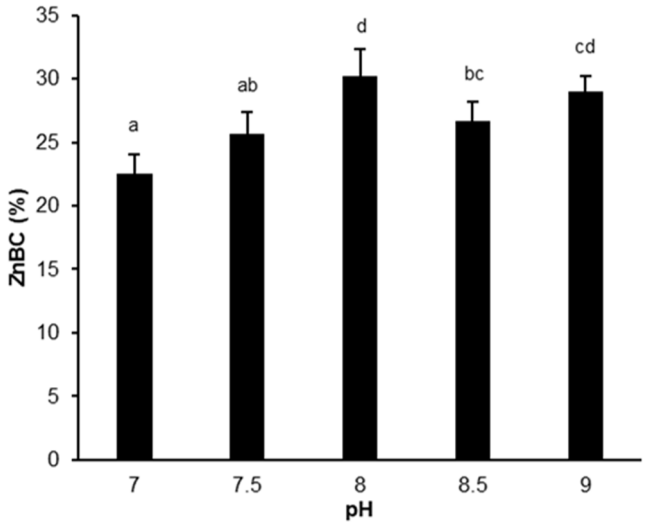

Fig 3. Effect of pH on ZnBC of the hydrolysate. The bars with different letters indicate significant differences ($p < 0.05$)

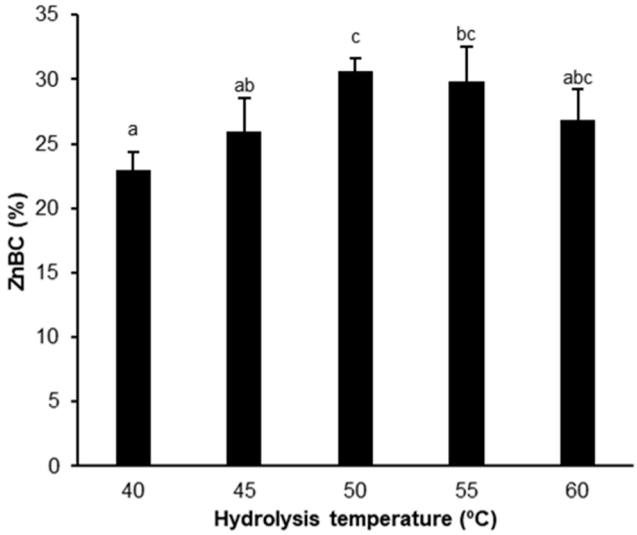

Fig 4. Effect of temperature on ZnBC of the hydrolysate. The bars with different letters indicate significant differences ($p < 0.05$)

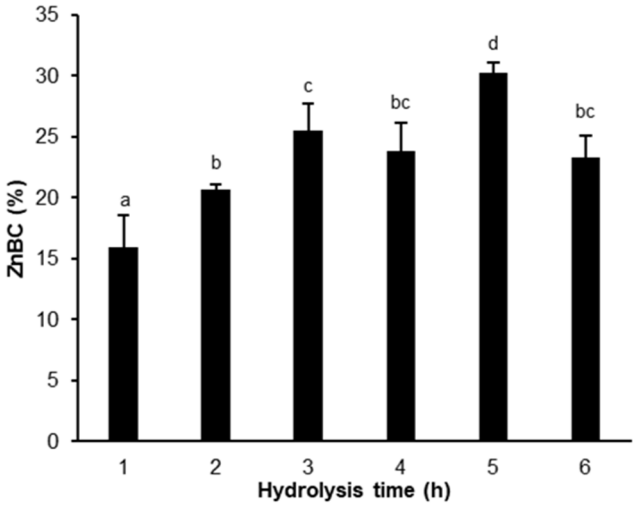

Fig 5. Effect of hydrolysis time on ZnBC of the hydrolysate. The bars with different letters indicate significant differences ($p < 0.05$)

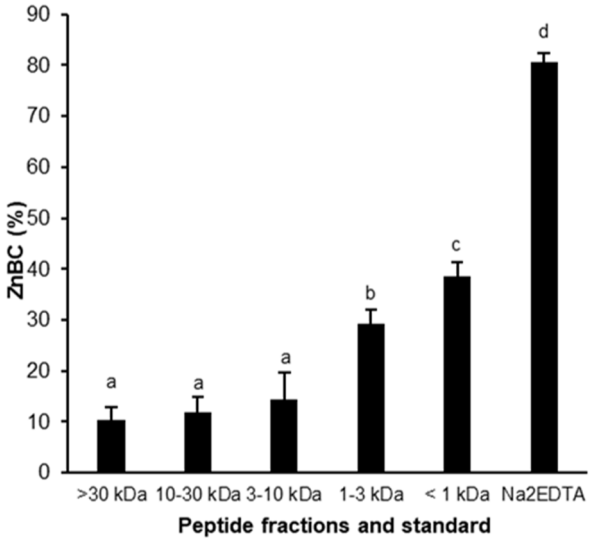

Fig 6. ZnBC of peptide fractions. The bars with different letters indicate significant differences ($p < 0.05$)

area, exposing more anchoring sites for zinc ions [8-9,15]. Conversely, the steric effect of large peptides, forming a barrier, blocks the access of Zn ions to their binding sites, lowering the ZnBC [29].

Amino Acid Composition of the Hydrolysate

Table 1 indicated that Gly was the main amino acid in the hydrolysate, with a proportion of 23.22% of total amino acids. Its contribution to ZnBC of the hydrolysate via decreasing the burden in the chelate ring was highlighted by Ke et al. [5]. Aliphatic amino acids (Ile, Leu, Met, Tyr, Phe, Pro, Val, Ala), accounting for a third of total amino acids in the hydrolysate, played a role in stabilizing the peptides-Zn complexes by establishing a hydrophobic barrier that restricted the attack of water molecules [30]. Furthermore, a high proportion of Pro or Hyp in the bioactive peptide sequence would allow them to resist the gastrointestinal enzymes, a high point for the application of the hydrolysate in humans [31]. Major anchoring sites for zinc ions were identified by the amino group of Lys and Asn [5,27], imine group of His [8], carboxyl group of Glu and Asp [9], hydroxyl group of Ser and Thr [5], and S-containing group of Cys [17].

Thermal and pH Stability of the Hydrolysate

The retention of bioactivity of hydrolysates after several popular food processing operations, such as pH and heat treatment, is an important factor for their applications [32]. The pH modification can trigger crosslinking, amino acid damage, and non-specific cleavage, leading to peptide denaturation and loss of their bioactivity [33]. In this study, the hydrolysate remained

Table 1. Amino acid profile of the featherback skin hydrolysate

Amino acids	Content (mg/L)	Amino acids	Content (mg/L)
His	130	Arg	870
Ile	190	Cys	50
Leu	430	Gly	2440
Lys	480	Tyr	130
Met	100	Ala	980
Phe	260	Asp	220
Thr	240	Glu	1160
Val	390	Ser	350
Pro	1020	Hyp	1070

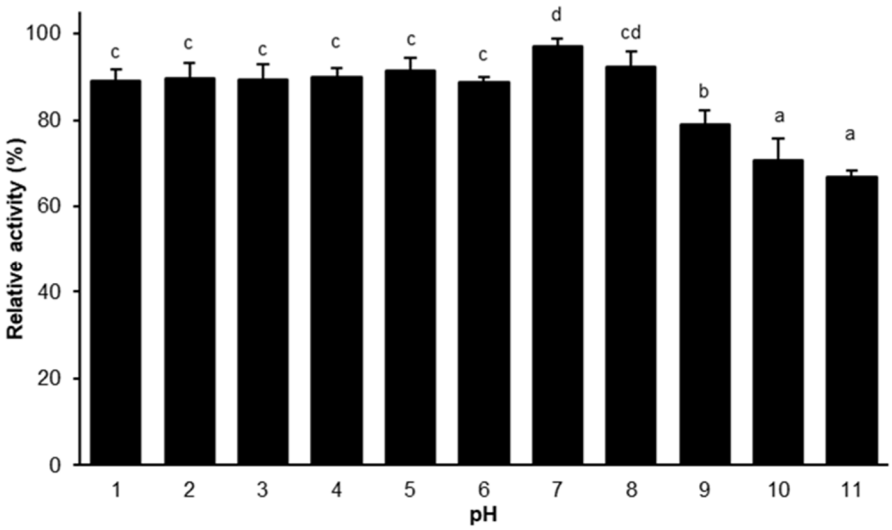

Fig 7. pH stability of ZnBC of the featherback skin hydrolysate. The bars with different letters indicate significant differences ($p < 0.05$)

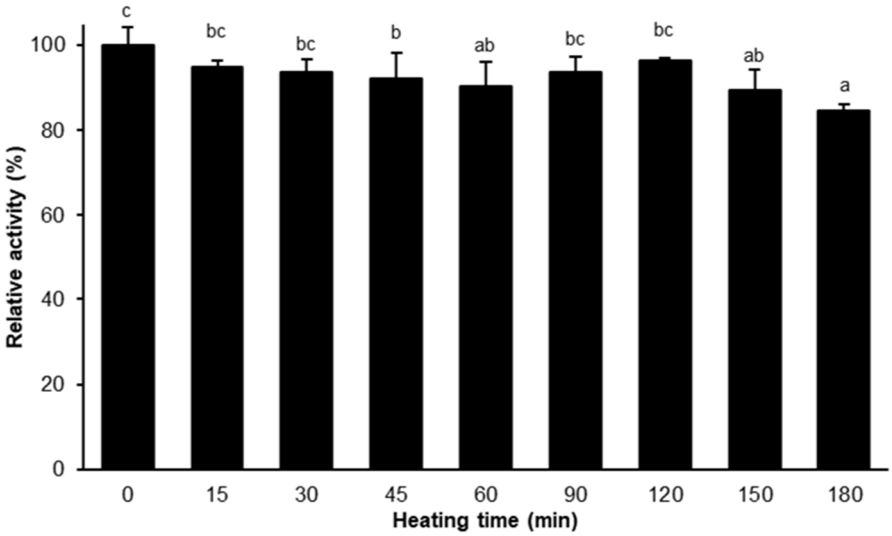

Fig 8. Thermal stability of ZnBC of the featherback skin hydrolysate. The bars with different letters indicate significant differences ($p < 0.05$)

nearly 100% ZnBC at pH = 7 and 8 (Fig. 7), at which almost all peptides were stable [34]. A slight decrease in ZnBC of the hydrolysate, observed at acid conditions (Fig. 7), may be due to the fact that bioactive peptides were cut into inactive fragments under these conditions [35]. On the other hand, alkaline pHs (9–11) may not only destroy Cys, Ser and Thr but also produce D-amino acids via racemic reaction, leading to a remarkable reduction in the relative activity of the hydrolysate [33-34].

ZnBC of the hydrolysate remained stable up to 120 min of heating and declined after that (Fig. 8). This observation was the same as that reported by Wali et al. [36]. This could be owing to the presence of a high content of Pro, which provides rigidity to the peptide structure [13]. Also, the low content of S-containing amino acids (Met and Cys) minimized the heat-induced oxidation reaction, lessening the change in the hydrolysate's bioactivity [13]. In addition, the role of

hydrophobic amino acids in the stabilization of the hydrolysate was emphasized [13]. Besides amino acid composition, a high amount of low molecular weight peptides could generate the high stability of the hydrolysate's ZnBC against heat treatment [37].

■ CONCLUSION

The skin hydrolysate exhibited the highest ZnBC of 30.28 ± 0.83% (2.66 folds lower than Na_2EDTA) at a hydrolysis condition at the skin:water ratio of 1:4 (w/v), E:S ratio of 40 U/g protein, pH = 8, temperature of 50 °C, and hydrolysis time of 5 h, comprising hydrolysis protease of Alcalase. Besides, the < 1 kDa fraction showed the greatest ZnBC of 38.48 ± 2.96%, 2.09 times lower than Na_2EDTA. The hydrolysate's ZnBC was over 66% during pH treatment in a range of 1–11 and heat treatment at 100 °C during 180 min. The hydrolysate contained 18 amino acids, of which 8 amino acids were essential. Thus, it could be used as an amino acid supplement or a Zn carrier, fortified into a broad range of functional foods or nutraceutical products, supporting amino acid or Zn nutrition. The findings of this study could be preliminary data for further investigations, such as characterization of the peptides, *in vivo* tests, action mechanisms of the peptides within a living organism, clinical trials, or their potential applications in functional foods or nutraceuticals.

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■ CONFLICT OF INTEREST

The authors declare no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Conceptualization: Tam Dinh Le Vo; Funding acquisition: Tam Dinh Le Vo; Project administration: Tam Dinh Le Vo; Supervision: Tam Dinh Le Vo; Visualization: Bao Chi Vo; Writing-review and editing: Tam Dinh Le Vo; Writing-original draft preparation: Bao Chi Vo; Data curation: Bao Chi Vo; Resources: Tam Dinh Le Vo; Investigation: Tam Dinh Le Vo, Thinh Ngoc Tran, Bao Chi Vo, Hieu Trung Ma, Hoa Gia Tran, Son Manh Nguyen, Quyen Phuong Hoang, Van Thi Tuyet Nguyen, Mai Thi Ngoc Nguyen, Thao Huynh Ngoc Nguyen, Vy Thuy Pham, Khang Tran Gia Cao, Cuong Viet Pham; Formal analysis: Tam Dinh Le Vo, Bao Chi Vo; Validation: Tam Dinh Le Vo, Bao Chi Vo; Methodology: Tam Dinh Le Vo, Bao Chi Vo. All authors agreed to the final version of this manuscript.

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