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Preparation, Amino Acid Composition, Peptide Fractionation, Thermal and pH Activity Stability of Featherback (*Chitala Ornata*) Skin Gelatin Hydrolysate with Zinc-Binding Capacity

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This research serves as an initial exploration into a novel zinc carrier derived from gelatin obtained from the skin of featherback fish (*Chitala ornata*), which is a natural source. The skin was found to contain approximately 63.23 ± 0.51 % moisture, 71.18 ± 1.82 % crude protein, 9.13 ± 3.40 % crude lipid, and 19.69 ± 1.61 % ash (on a dry weight basis). To extract the gelatin, warm water was used, followed by hydrolysis using Alcalase. The gelatin-to-water ratio was 1:4 (w/v), with a pH of 8, temperature of 50 °C, enzyme-to-substrate (E:S) ratio of 40 U/g protein, and a hydrolysis time of 4 h. The resulting hydrolysate exhibited a degree of hydrolysis (DH) of 18.46 ± 1.35 % and a zinc-binding capacity (ZnBC) of 39.66 ± 1.56 % (which was 2.00 times lower than that of ethylenediamine tetraacetic acid disodium salt (Na_2EDTA)). The hydrolysate's activity remained above 60 % even after exposure to a wide pH range 1-11 and treatment for 180 min at 100 °C. Notably, the hydrolysate contained a significant amount of hydroxyproline (Hyp), measuring 1550 mg/L. Ultrafiltration was utilized to fractionate the hydrolysate, resulting in five peptide fractions (>30 kDa, 10-30 kDa, 3-10 kDa, 1-3 kDa, and <1 kDa), which were subsequently evaluated for their ZnBC. The <1 kDa fraction demonstrated the highest ZnBC of 53.36 ± 3.95 %, which was 1.51 folds lower than that of Na_2EDTA. These findings suggest that the gelatin hydrolysate and/or its peptide fractions derived from featherback fish skin could be utilized as a supplement in producing functional food.

1. Introduction

Zinc is an essential micronutrient in the human body, playing a crucial role in activating numerous enzymes and supporting the immune system (Peng et al., 2022). In contrast, inadequate absorption of zinc can occur due to dietary inhibitors such as tannins, phytate, and dietary fiber, as well as zinc loss in certain disease conditions, leading to zinc deficiency (Zheng et al., 2023). Zinc deficiency can manifest in symptoms like diarrhea, alopecia, hypogonadism, loss of appetite, mental lethargy, and delayed wound healing (Peng et al., 2022). To address zinc deficiency, zinc supplements in the form of zinc salts (e.g., zinc sulfate, zinc gluconate) and peptide-zinc chelates are commonly used (Zheng et al., 2023). Zinc salts can impart an unpleasant metallic taste to fortified food products and may cause irritation in the digestive tract and interfere with the absorption of other nutrients (Sun et al., 2022). Conversely, Sun et al. (2021) highlighted the advantages of peptide-zinc complexes, including their high safety profile and stability in the gastrointestinal system. Researchers have successfully generated zinc-binding peptides from various protein sources, such as tilapia skin collagen (Meng et al., 2021), scallop adductor (Sun et al., 2022), coconut cake (Zheng et al., 2023), and more.
The production of fish cakes using featherback fish (*Chitala ornata*) results in the discarding of the fish skin, which accounts for 17-22 % of the total fish weight (Kittiphattanabawon et al., 2016). But, previous study by Kittiphattanabawon et al. (2016) had demonstrated that the fish skin is a valuable source of collagen and gelatin (partially hydrolyzed collagen). Lv et al. (2019) had also emphasized the hydrophilic property and extensive use

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of gelatin hydrolysates, compared to gelatin, in producing functional foods. This study aims to convert this waste into a value-added product of zinc-binding gelatin hydrolysate. Beside estimating ZnBC of the hydrolysate under the effect of hydrolysis conditions, its amino acid (AA) composition, activity stability against pH and thermal treatment as well as bioactivity of its peptide fractions were also evaluated.

2. Materials and Methods

2.1 Materials

The featherback skin, obtained from a featherback fish cake manufacturer in Hau Giang province, Vietnam, was subjected to initial preparation steps. The skin was washed thoroughly under cold running water to remove any impurities. It was cut into small pieces measuring 1x1 cm^2. The prepared skin pieces were stored at a temperature of -20 °C to maintain their freshness and quality. Alcalase® 2.4 L, a commercially available enzyme from Novozyme in Denmark, with activity of 3022.19 ± 103.19 U/mL, was utilized. The chemicals used in the research were of analytical grade and were procured from reputable suppliers such as Sigma-Aldrich and Merck. Double-distilled water was employed throughout the experiment to ensure the purity of the solutions.

2.2 Methods

The chemical composition of the featherback skin, including moisture, crude protein, lipid, and ash content, was determined following the guidelines provided by Nwachukwu and Aluko (2019).

The method described by Kittiphattanabawon et al. (2016) was employed with a slight modification to extract the gelatin from the featherback skin. The skin was immersed in a 0.1 M NaOH solution with a skin-to-solution ratio 1:10 (w/v). The mixture was stirred at 250 rpm for 2 h at a temperature ranging from 15-20 °C. This step aimed to remove noncollagenous proteins. The skin was thoroughly washed with cold running water until the water reached a neutral pH. The skin was soaked in a 0.05 M acetic acid solution with a ratio of 1:10 (w/v) for 30 min at room temperature while stirring at 250 rpm. Again, the skin was washed until the wash water reached a neutral pH. The swollen skin was then mixed with distilled water at a ratio of 1:2 (w/v) and stirred at 150 rpm for 12 h at a temperature of 45 °C. This step facilitated the extraction of gelatin from the skin. The resulting solution was filtered using Whatman no. 3 filter paper, and the supernatant was collected as the gelatin solution.

The gelatin solution was hydrolyzed using Alcalase according to the procedure described by Vo et al. (2020a). To initiate hydrolysis, the gelatin solution was blended with water to achieve the desired gelatin-to-water ratio. The pH of the mixture was then adjusted to the required value using either 1 M NaOH or 1 M HCl solution. The sample was heated to the designated hydrolysis temperature, and Alcalase was added at an appropriate ratio. After the specified hydrolysis time, the sample was treated at 95 °C for 10 min to deactivate the enzyme. The resulting mixture was centrifuged, and the supernatant was gained as the gelatin hydrolysate after filtration through Whatman paper no. 3. The impact of various hydrolysis parameters on the ZnBC of the gelatin hydrolysate was examined using a single-factor test method. In this approach, one parameter was altered at diverse levels when keeping the other factors constant as shown in Table 1.

Table 1: Hydrolysis condition of each investigation

Tested parameter	Gelatin-to-water ratio (w/v)	E:S ratio (U/g protein)	pH	Temperature (°C)	Hydrolysis time (h)
Investigation 1	Tested range	40	8	50	3
Investigation 2	Chosen level	Tested range	8	50	3
Investigation 3	Chosen level	Chosen level	Tested range	50	3
Investigation 4	Chosen level	Chosen level	Chosen level	Tested range	3
Investigation 5	Chosen level	Chosen level	Chosen level	Chosen level	Tested range

The ZnBC was determined following the protocol described by Zheng et al. (2023). 2 mL of the sample (1 mg/mL diluted in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH buffer (40 mM, pH 7.5) was mixed with 1 mL of 8 mM dithiothreitol solution and 1 mL of 0.1 mM $ZnSO_4$ solution. The mixture was incubated at 50-60 ºC for 30 min before adding 0.1 mL of 2 mM 4-(2-pyridylazo) resorcinol solution and recording its absorbance at 500 nm (ODsample). ODblank was recorded with the same procedure but 2 mL of HEPES-KOH buffer (40 mM, pH 7.5) replaced for 2 mL of tested sample. The positive control was 1 mg/mL Na_2EDTA solution prepared in HEPES-KOH buffer (40 mM, pH 7.5). ZnBC of the sample was calculated using the following equation:

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

The gelatin hydrolysate obtained under the selected hydrolysis condition was analyzed for its AA composition according to the guidelines of Nwachukwu and Aluko (2019) and thermal treatments were performed according

to protocol of Sripokar et al. (2019). In brief, in terms of pH stability, 5 mL of sample was adjusted its pH to a value within the range from 1 to 11 by either 6 M HCl or 6 M NaOH solution and placed at room temperature for 30 min. pH of the sample was then readjusted to 7.0 by phosphate buffer (1 M, pH 7.0) to minor the interference of pH on ZnBC of the samples, after that, its volume was brought to 20 mL with distilled water before testing for its ZnBC. Regarding thermal stability, 5 mL of sample was heated at 100 °C for 0, 30, 60, 90, 120, 150 and 180 min. The samples were suddenly cooled to room temperature before their ZnBC were determined. The pH or thermal stability of the hydrolysate's ZnBC was presented as the relative activity (%) that is the percentage of ZnBC of the treated sample compared to that of the untreated sample and calculated by the following equation:

$$Relative\ activity\ (\%) = \frac{ZnBC\ of\ the\ treated\ sample}{ZnBC\ of\ the\ untreated\ sample} * 100 \quad (2)$$

The gelatin hydrolysate was passed through ultrafiltration centrifugal devices with their cutoff sizes of 30 kDa, 10 kDa, 3 kDa, and 1 kDa (Macrosep, Pall Laboratory, USA), and five peptide fractions, including <1 kDa, 1-3 kDa, 3-10 kDa, 10-30 kDa, and >30 kDa, were collected and assessed for their ZnBC.

The data obtained from the experiments were expressed as means ± standard deviations, representing the average values of three independent experiments. Statistical analysis was conducted using analysis of variance (one-way ANOVA) utilizing the Statgraphics Centurion 18 software. This analysis evaluated any significant differences among the peptide fractions in terms of their ZnBC.

3. Results and Discussion

3.1 Chemical composition of the featherback skin

The featherback skin exhibited a moisture content of 63.23 ± 0.51 %. On a dry basis, the skin contained approximately 79.41 ± 1.14 % crude protein, 5.76 ± 0.03 % crude lipid and 1.39 ± 0.04 % ash. The protein content of the skin was found to be comparable to that of other protein sources such as scallop adductor (Sun et al., 2022) and tilapia skin (Meng et al., 2021), which have previously been utilized for the production of zinc-binding protein hydrolysates. Based on these findings, it can be inferred that the featherback skin holds promise as a potential raw material for the generation of bioactive protein hydrolysates or peptides.

3.2 Effect of hydrolysis conditions on ZnBC of the gelatin hydrolysate

Alcalase, an endopeptidase, is known to prefer releasing peptides with hydrophobic or aromatic amino acids at the C-terminal, which exhibit high affinities for metal ions (Guo et al., 2023). Previous studies had used Alcalase to form zinc-binding peptides from various sources, including tilapia skin collagen (Ke et al., 2021) and scallop adductor (Sun et al., 2022). In this study, Alcalase was employed as the catalytic agent for protein hydrolysis.

In this study, a gelatin-to-water ratio of 1:4 (w/v) was determined as the best ratio to achieve the highest zinc-binding capacity (ZnBC) of the gelatin hydrolysate (Figure 1(a)). This ratio was selected to ensure an appropriate balance between providing sufficient water for efficient enzyme-substrate interaction and maintaining the desired viscosity for optimal enzymatic reactions (Vo et al., 2020b). The collision between enzymes and protein molecules is essential for the enzymatic reaction. Despite that, the effectiveness of this collision can be affected by the amount of water used. When the amount of water is too large, it creates a large space between the molecules, as a result, reducing the collision frequency (Vo et al., 2020b). On the other hand, when the amount of water is too low, it increases the viscosity of the reaction mixture, hindering the collision. Both scenarios can lead to a lower release of zinc-binding peptides from intact proteins, reducing the ZnBC of the hydrolysates (Fang et al., 2019). In Figure 1(b), a common correlation between the E:S ratio and the mineral-binding capacity of the hydrolysate was observed, which aligns with findings from our previous publication of Vo et al. (2021). Low E:S ratios resulted in an inadequate content of bioactive peptides in the hydrolysate, potentially reducing its overall bioactivity. On the other hand, high E:S ratios were found to possibly degrade the zinc-binding peptides generated during the early stages of hydrolysis (Vo et al., 2021). Based on these observations, an E:S ratio of 40 U/g protein was selected as the ideal ratio for further experiments. This ratio was chosen to strike a balance between ensuring a sufficient generation of bioactive peptides in the hydrolysate and preserving the integrity of the zinc-binding peptides produced during hydrolysis.In this study, the ZnBC of the gelatin hydrolysate reached its peak at pH 8 (Figure 1(c)). This finding suggests that pH 8 provides perfect condition for the formation of zinc-binding complexes within the hydrolysate, resulting in enhanced the ZnBC. The environmental pH significantly influences the charge allocation and figuration of both enzyme and substrate molecules, affecting the assembly of enzyme-substrate complexes and the metal-binding capacity of protein hydrolysates (Vo et al., 2021). pH plays a crucial role in determining the protein solubility by altering their ionizability, accordingly affecting the hydrolysis process and ultimately impacting the bioactivity of the hydrolysate (Vo et al., 2020b). The ZnBC of the gelatin hydrolysate was found to increase as the hydrolysis temperature rose from 40 to 50 °C, after which it remained unchanged (Figure 1(d)). According to the Arrhenius

function, the reaction rate of enzymatic reactions increases with higher temperatures (Vo et al., 2020b). This elevated temperature facilitates the release of a higher amount of bioactive peptides from intact proteins, thereby improving the overall zinc-binding capacity of the hydrolysate. Considering energy consumption and manufacturing feasibility, a hydrolysis temperature of 50 °C was selected for further investigations in this study. The ZnBC of the gelatin hydrolysate showed an increase up to a certain hydrolysis time (4 h) and reached a plateau, with no significant changes observed thereafter (Figure 1(e)). This phenomenon can be attributed to the initial disruption of the gelatin's quaternary, tertiary, and secondary structures by the enzyme during the early stages of hydrolysis, resulting in the release of peptides with high ZnBC into the hydrolysate (Zhu et al., 2021). As the hydrolysis progresses, the enzyme encounters a limited number of cleavage sites on the substrate, which may explain why prolonging the hydrolysis time does not significantly impact the hydrolysate's bioactivity (Fang et al., 2019). Similar patterns of ZnBC in relation to hydrolysis time had been reported by Ke et al. (2021).

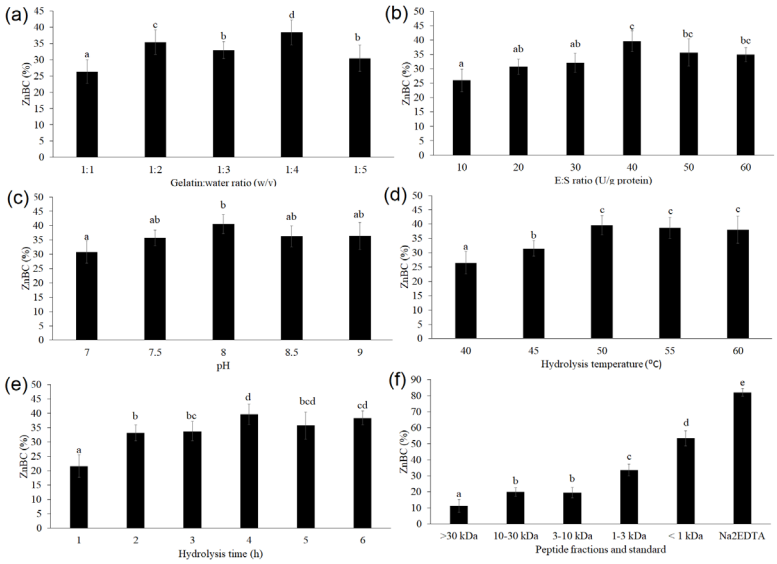

Figure 1: Effect of (a) gelatin:water ratio, (b) E:S ratio, (c) pH, (d) temperature, (e) hydrolysis time on ZnBC of the gelatin hydrolysate and (f) ZnBC of peptide fractions. The bars with various letters show significant differences ($p<0.05$)

3.3 Peptide fractionation of the gelatin hydrolysate

The relationship between molecular weight and ZnBC of the peptide fractions is evident in Figure 1(f), where it is observed that lower molecular weight peptides exhibit higher ZnBC. This can be attributed to the increased exposure of AA side chains in smaller peptides, providing more binding sites for zinc ions compared to larger peptides (Guo et al., 2023). The study by Sun et al. (2021) emphasized the significant role of peptides with molecular weight below 1 kDa in metal chelation. On the other hand, the steric effect, which is the hindrance caused by the spatial arrangement of atoms, acts as a barrier that restricts the access of transition metal ions to the anchoring sites of peptides (Vo et al., 2020b).

3.4 AA composition of the gelatin hydrolysate

The AA profile of the hydrolysate, as shown in Table 2, plays a crucial role in its ZnBC. Kong et al. (2021) reported that zinc ions utilized their blank valence d-orbitals to receive electrons from electron-rich groups presented in peptides, forming coordination bonds between peptides and Zn ions. Certain AAs contribute to the peptide-zinc chelation through their electron-rich groups such as carboxyl groups of Asp and Glu, imidazole ring of His, ε-amino group of Lys (Fu et al., 2020), sulfhydryl group of Cys, and hydroxyl groups of Thr and Ser (Sun et al., 2021). Hydrophobic AAs (Ile, Leu, Met, Phe, Val, Tyr, Pro) make up a significant proportion of the hydrolysate's total AAs (31.4 %), and they contribute to the formation of a hydrophobic barrier that protects the peptide-zinc complexes from water molecules (Vo et al., 2020b). Pro has the ability to bend the peptide chain, facilitating simultaneous binding to zinc ions by the N-terminal NH_2- group and a distant donor atom (Meng et al., 2021). A high proportion of Pro or Hyp in the peptide sequence allows them to resist digestive enzymes, making them suitable for human applications (Lv et al., 2019). The hydrolysate provides 8 of the 9 essential AAs, representing 20.9 % of the total AAs, highlighting its potential nutritional benefits for human consumption.

Table 2: AA content of the gelatin hydrolysate

AAs	Content (mg/L)	AAs	Content (mg/L)	AAs	Content (mg/L)	AAs	Content (mg/L)
His	180	Thr	340	Arg	720	Phe	110
Ile	290	Val	360	Cys	0	Pro	1350
Leu	470	Hyp	1550	Gly	2150	Asp	690
Lys	530	Glu	1170	Tyr	200	Ala	910
Met	140	Ser	400				

3.5 pH and thermal ZnBC stability of the gelatin hydrolysate

ZnBC stability of protein hydrolysate towards thermal and various pH treatments should be concerned as it is an important indicator for fortifying the hydrolysate into food products (Sripokar et al., 2019). Figure 2(a) demonstrates that the gelatin hydrolysate's ZnBC remains unchanged after treatment at pH 7. This could be due to the fact that at neutral pH, the total electrostatic repulsive energy is low, preventing protein molecules from swelling and unfolding, thus preserving their bioactivity (Damodaran and Parkin, 2017). A slight decrease in the relative activity of the hydrolysate is observed as the pH shifts to acidic conditions (Figure 2 (a)). This decrease in bioactivity can be the result of a combination of factors. Changes in peptide charges and disruption of hydrogen bonds in the amino acid side chains lead to the deformation of peptide molecules. Acidic hydrolysis can break bioactive peptides into inactive fragments, further contributing to the decrease in bioactivity under acidic conditions (Zhang et al., 2022). The relative activity of the hydrolysate significantly drops to approximately 80 % when the pH used for treatment increases from 7 to 11 (Figure 2 (a)). In addition to the aforementioned effects under acidic conditions, alkaline pH promotes deamidation and racemization of AAs in the peptide chains. This leads to changes in their structure and conformation and loss of ZnBC (Zhang et al., 2021).

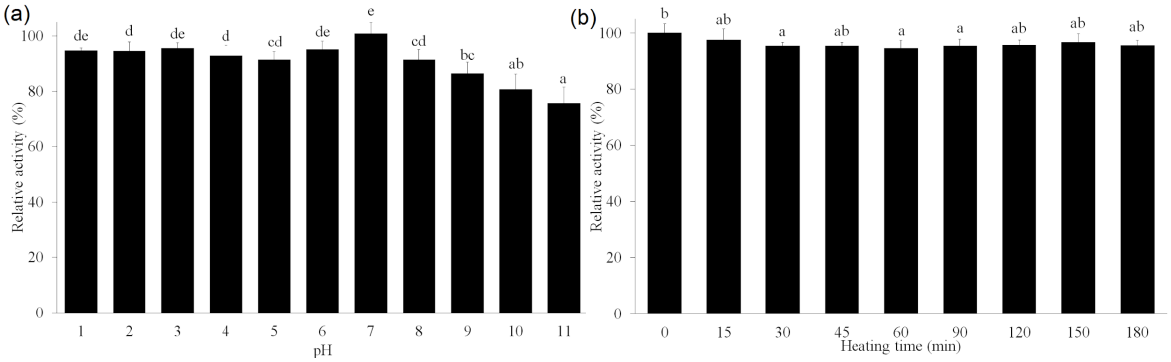

Figure 2: (a) pH and (b) thermal ZnBC stability of the gelatin hydrolysate. The bars with various letters show significant differences ($p<0.05$)

Figure 2(b) illustrates that the gelatin hydrolysate retains over 90 % of its ZnBC after being treated at 100 °C for 180 min. This remarkable thermal ZnBC stability can be owing to several factors. Peptides with a high proportion of hydrophobic AAs tend to exhibit greater heat stability (Damodaran and Parkin, 2017). In this study, the gelatin hydrolysate contained a substantial amount of hydrophobic AAs, comprising 31.4 % of the total AAs, significantly contributing to its thermal stability. The presence of AA Ile in the hydrolysate can reduce the void spaces within the interior core of peptide molecules. This reduction in void spaces decreases the configurational entropy of the peptides at high temperatures, thereby minimizing peptide denaturation (Damodaran and Parkin, 2017). The high content of Pro, especially in the loop regions of the peptide chain, confers a rigid structure to the peptides. This structural rigidity enhances their resistance to heat-induced denaturation (Zhang et al., 2021). The absence of Cys and the low content of Met in the hydrolysate help minimize oxidation reactions, preserving its ZnBC under the heating conditions (Damodaran and Parkin, 2017). These factors collectively contribute to the gelatin hydrolysate's thermal stability and ensure that it retains its ZnBC even after prolonged heat treatment.

4. Conclusion

This study is the first to produce protein hydrolysate from the featherback skin gelatin with ZnBC. This study has found a hydrolysis condition at which the obtained hydrolysate from the skin gelatin exhibited the highest ZnBC. The hydrolysate also shows promising characteristics, including its high nutritional value and resistance to pH and thermal treatments. The aim of this study was to provide preliminary data on ZnBC of the skin gelatin hydrolysates and their peptide fractions. The findings of this research could be a platform for further

investigations such as characterization of the peptides, zinc-releasing mechanism, strategies to improve the release capacity of bound zinc from the chelate, *in vivo* tests, and clinical trials

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