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Featherback (*Chitala ornata*) Skin Protein Hydrolysate: Antioxidant Activity, Amino Acid Composition, Foaming and Emulsifying Properties

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In this study, featherback (*Chitala ornata*) skin was used to generate an antioxidant protein hydrolysate with foaming and emulsifying properties. Hydrolysates were obtained by using Alcalase preparation, a skin:water ratio of 1:2 (w/v), a pH 7.5, 55 °C, an enzyme:substrate ratio of 80 U/g protein, and a 5 h hydrolysis time. In terms of antioxidant activity, the hydrolysate showed 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity at 3.00 mg/mL and 1.82 mg/mL as the inhibitory concentration of 50 % free radicals (IC50), which was 731.71 and 27.58 folds higher than those of vitamin C, in order. It also displayed moderate foaming property, with its foaming capacity (FC) and foaming stability (FS) being 1.08 - 1.55 and 1.19 - 2.89 times lower than those of albumin, in that order. In contrast, the hydrolysate exhibited great emulsifying properties with an emulsifying activity index (EAI) and emulsifying stability index (ESI) comparable to those of sodium caseinate at pH 3-8. The hydrolysate contained a high concentration of lipophilic amino acids (AAs) (87.28 % of total AA content). As a result, featherback skin hydrolysate may be considered as a versatile ingredient for the development of natural antioxidants with technological capabilities.

1. Introduction

The yield of featherbacks reached 6,880 t/y in 2020 in Hau Giang province (Nguyen and Le, 2022). Fish skin, which makes up 17–22 % of the total fish weight and is frequently discarded, is a common by-product in the production of featherback fish cakes in Vietnam (Karnjanapratum et al., 2021). According to Irm et al. (2020), the fish skins were typically processed into low-value goods like animal feed, fish meal, and fertilizer. In recent years, the pharmaceutical, health food, and food industries have become very interested in fish skin hydrolysates with antioxidant potential (Lv et al., 2019). The protein hydrolysates from the skins of *Cyprinus carpio* (Tkaczewska et al., 2020), sole (Viji et al., 2019), and rainbow trout skin (Yaghoubzadeh et al., 2020) have been studied extensively for their antioxidant capacity. According to Vo et al. (2022), antioxidant hydrolysates have been shown to reduce oxidative stress, which is a known contributor to many chronic diseases like cancer, cardiovascular disease, and aging. They can also be used in place of synthetic commercial preservatives like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food products to diminish the hazardous effects of these synthetic compounds (Vo et al., 2022). Furthermore, it has been reported that hydrolysates made from fish skin, such as those made from sole skin (Viji et al., 2019) and saithe skin (Casanova et al., 2020), have useful functional qualities like emulsifying and foaming properties that can help with the improvement of a variety of product properties. According to our understanding, no data on the antioxidant activity and useful qualities of hydrolysate made from *Chitala ornata* skin have been reported.

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Highly effective alkaline enzyme, obtained from *Bacillus licheniformis*, was used for the generation of antioxidant protein hydrolysates from a diversity of materials, such as turbot wastes (Vázquez et al., 2020), cobia liver (Wang et al., 2020), croaker (*Micropogonias furnieri*) and striped croaker (*Paralonchurus brasiliensis*) by-products (Rocha Camargo et al., 2021), and so on. In our study, Alcalase hydrolysis was employed to (i) recover the antioxidant protein hydrolysate from the featherback skin; (ii) supply data on amino acid composition and degree of hydrolysis (DH) of the hydrolysate; and (iii) provide information on its foaming and emulsifying properties.

2. Materials and Methods

2.1 Materials

To get rid of contaminants and any remaining blood, the featherback skin was washed in cold water after being obtained from a market in Ho Chi Minh City, Vietnam. After being removed from the water, it was chopped up and finely ground before being stored at -20 ºC. According to Nwachukwu and Aluko (2019) methods, the skin's moisture content was 63.6 ± 1.7 %, and the other components' dry basis contents were 71.2 ± 1.8 % for crude protein, 9.1 ± 3.4 % for crude lipid and 19.7 ± 1.6 % for ash.
Alcalase® 2.5 L, a commercially available enzyme from Novozyme in Denmark, at the ideal temperature and pH of 55 ºC and 7.5, in order, was utilized. Sigma-Aldrich and Merck provided analytical grade chemicals with high purity. Also, distilled water was employed in the experiments.

2.2 Methods

The method used to prepare the featherback skin protein hydrolysate was slightly modified from that of Vo et al. (2022). In order to inactivate endogenous enzymes, distilled water was added in the proper proportion of skin to water (w/v). The mixture was then treated at 95 °C for 15 min. Next, the pH was raised to 7.5 using either a 1 M HCl or NaOH solution. A water bath was used to maintain the hydrolysis temperature of 55 ºC while the alcalase preparation was added with the necessary E:S ratio. After the required hydrolysis time, the reaction was terminated by inactivating the alcalase in the hydrolysates for 15 min at 95 ºC. The upper fat fraction of the hydrolysate was separated using centrifugation. To remove suspended particles, the obtained supernatants underwent a second filtering process using Whatman no. 3 filter paper. Soluble protein content of the hydrolysate was determined using the Lowry method described by Nwachukwu and Aluko (2019).
The following experiments were conducted to determine how the hydrolysis condition affected the antioxidant activity of the hydrolysate: 15 g of the featherback skin was hydrolyzed using an E:S ratio of 40 U/g protein for a period of 4 h while the skin:water ratio was maintained between 1:1 and 1:10 (w/v). In order to determine the impact of the E:S ratio, 15 g of the by-product was hydrolyzed for 4 h at the skin-to-water ratio that had been previously chosen, with the E:S ratio varying from 20 to 100 U/g protein. With regard to the hydrolysis time, the hydrolysates were made using 15 g of the skin, the skin:water ratio and the E:S ratios set at the predetermined levels, determined from the previous experiments, and the hydrolysis time ranged from 3 to 7 h.
DH of the protein hydrolysate was examined using the guideline of Vo et al. (2022). In brief, 4 standard samples were prepared by mixing 0.4 mL of 0.9516 milliequivalent/L serine solution and 3 mL of reagent (0.08 %(w/v) o-phthaldialdehyde, 0.088 %(w/v) dithiothreitol, 5.08 %(w/v) sodium borate and 1.33 %(w/v) sodium dodecyl sulfate in 1.99 % (v/v) ethanol solution) in 5 s. Blank and tested samples were prepared using the same procedure, but for the blank, 0.4 ml of serine solution was replaced by 0.4 ml of distilled water, and for the tested samples, 0.4 ml of protein hydrolysate substituting 0.4 ml of serine solution. After 2 min, the absorbances of two standards were measured before reading the absorbance of the blank and tested samples. After that, the absorbances of the last 2 standard samples were recorded. The DH of the tested sample was computed via the following equation:

$$DH\ (\%) = \left(\frac{A_s - A_b}{A_{st} - A_b} * \frac{0.9516}{P} - \beta\right) * \frac{100}{\alpha * h_{tot}} \quad (1)$$

Where: A_s: absorbance of tested sample; A_b: absorbance of blank sample; A_{st}: average absorbance of 4 standard samples; P: soluble protein content of the tested sample, determined using Lowry method, mg/mL; α and β are constants, for fish, α = 1.0, β = 0.4; h_{tot}: hydrolytic equivalence at complete hydrolysis degree, for fish, h_{tot} = 8.6.
For determination of the hydrolysate's AA composition, the hydrolysate was completely hydrolyzed using 6 M HCl solution for 23 h at 110 ± 2 °C, which was then separated by ion-exchange chromatography and detected in forms of Ninhydrin-derivatives. The absorbances of standard solutions of AAs were assessed at 440 nm for Pro and 570 nm for the other AAs, to quantify the free AAs in the hydrolysate Nwachukwu and Aluko (2019).

According to the method outlined in our previous research (Vo et al., 2022), the antioxidant activity (radical scavenging activity for DPPH and ABTS) and functional properties of the hydrolysate, including foaming property (FC and FS), and emulsifying property (EAI and ESI), were assessed. Albumin, a common foaming agent from the egg white, was used in the food industry (Razi et al., 2023), and used as a positive control for foaming test (Leni et al., 2020). Sodium caseinate, a nutritional and emulsifying ingredient (Liao et al., 2022), was used as positive control for emulsifying tests Huang et al. (2023). In our study, the albumin and sodium caseinate were used as positive control as well.

Data from experiments with three replicates were presented as averages and standard deviations. The data were submitted to one-way analysis of variance (ANOVA) using Statgraphics Centurion 18 software.

3. Results and Discussion

3.1 Effect of hydrolysis condition on antioxidant activity of the featherback skin protein hydrolysate

Figure 1(a) shows that after reaching a peak of 25.34 % for DPPH scavenging and 35.59 % for ABTS scavenging activity at the ratio of 1:2 (w/v), antioxidant activity generally decreased as skin:water ratio increased. It might be because a sufficient amount of water improved the interaction between the enzyme and the substrate, releasing more short peptide chains with high free radical scavenging activity (Fu et al., 2018). The 1:1 (w/v) ratio used significantly less water, resulting in extremely high viscosity that hinders enzyme migration and inhibits hydrolysis. The hydrolysis reaction was decelerated by the enzyme's difficulty in accessing the substrate, which resulted from the increase in water amount (Vo et al., 2020). As a result, in those instances, the hydrolysate's antioxidant activity was low.

As seen in Figure 1(b), the antioxidant activity of protein hydrolysate generally increased along with the raise in E:S ratio up to a certain threshold, after which the ABTS scavenging capacity remained stable, and the DPPH scavenging activity slightly decreased. The E:S ratio cutoffs for DPPH scavenging activity and ABTS scavenging capacity, in that order, were 80 and 50 U/g protein. This is in accordance with the observations of Martinez et al. (2023), which suggest that a high enzyme amount could produce sufficient peptide lengths for the antioxidation. The aggressive hydrolysis that might disrupt the pivotal positions for the scavenge of free radicals in the peptides, resulting in the loss of antioxidant activity, can cause a slight decrease in antioxidant activity of the skin hydrolysate along with an additional rise in the E:S ratio (Vo et al., 2018).

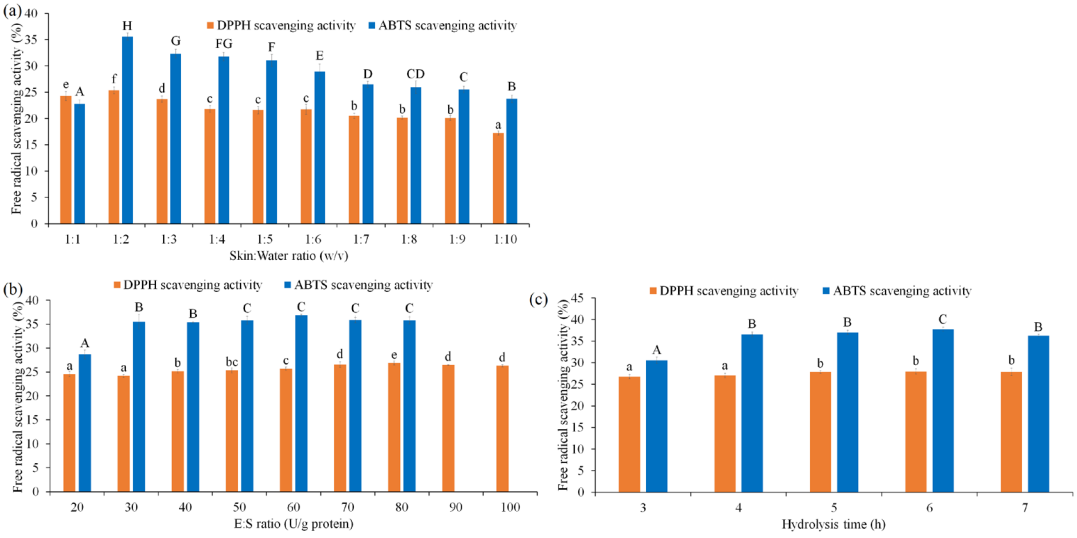

Figure 1: Effect of (a) skin:water ratio, (b) E:S ratio and (c) hydrolysis time on antioxidant activity of the featherback skin protein hydrolysate. Bars with different small letters indicate significant differences ($p<0.05$) for DPPH scavenging activity; bars with different capital letters indicate significant differences ($p<0.05$) for ABTS scavenging activity

In contrast to the ABTS scavenging activity, which was constrained after peaking at 37.70 ± 0.52 % at 6 h, the DPPH scavenging activity increased slightly in the first 3 to 5 h before levelling off at about 27.89 ± 0.35 % (Figure 1(c)). The longer an enzyme was incubated with its substrate, the greater the content of small peptides formed, elevating the hydrolysate's bioactivity (Mongkonkamthorn et al., 2020). On the other hand, Vo et al. (2020) suggested that the enzyme may have begun to hydrolyze itself over time, which would have reduced its activity. Simultaneously, the possible intense generation of free amino acids from the bioactive peptides

released in the initial phase of hydrolysis caused the subsequent reduction of radical scavenging activity of the hydrolysate (Vo et al., 2018). Tkaczewska et al. (2020) published similar trends.
The skin hydrolysate with the highest DPPH scavenging activity, which was obtained at the hydrolysis condition including Alcalase as catalytic agent, pH 7.5, 55 ºC, the skin:water ratio of 1:2 (w/v), the E:S ratio of 80 U/g protein and 5 h of hydrolysis, was used in the subsequent experiments. Its DH was 32.25 ± 0.13 % and its IC50 values for DPPH and ABTS scavenging activity were 3.00 and 1.82 mg/mL, in order. Although these values were 731.71 and 27.58 folds higher than those of vitamin C, the skin hydrolysate still shows potential for use as a natural substitute antioxidant in the food and pharmaceutical industries.

3.2 Analysis of AA composition of the hydrolysate

As shown in Table 1, the hydrolysate from featherback skin can provide 7 out of the 9 essential AAs (aside from Met and Trp), making up roughly 73.97 % of the total, demonstrating the hydrolysate's value as an AA supplement. The hydrolysate's antioxidant potential was also influenced by the composition of the AAs. For instance, Tyr and His might have antioxidant properties because of their phenol and imidazole rings (Esfandi et al., 2019). The hydrolysate used in this study also had another noteworthy feature: a significant amount of Phe can be converted to Tyr when attacked by hydroxyl radicals or by hydroxylation *in vivo* (Csire et al., 2020), providing more phenol rings for radical scavenging. Leu, Ile, Val, and Ala are additional hydrophobic residues that may improve antioxidant activity by aiding in the solubility of peptides in the hydrophobic phase and facilitating interactions with radical species (Qoms et al., 2023).

Table 1: AA composition of the hydrolysate

AAs	Content (mg/100 mL)	AAs	Content (mg/100 mL)	AAs	Content (mg/100 mL)
His	2.69 ± 0.69	Thr	1.69 ± 0.43	Arg	0.59 ± 0.27
Ile	6.64 ± 1.70	Val	17.48 ± 2.73	Ala	32.97 ± 5.15
Leu	4.78 ± 1.22	Ser	6.37 ± 1.63	Gly	13.31 ± 2.08
Lys	1.88 ± 0.48	Glu	9.75 ± 2.50	Tyr	13.14 ± 2.05
Phe	193.69 ± 30.25	Pro	1.17 ± 0.30	Asp	3.26 ± 0.83

3.3 Determination of foaming property of the skin hydrolysate

At the air-water interface, molecules undergo three sequential processes that lead to the formation of foam: transportation, penetration, and reorganization (Vo et al., 2022). A protein must be able to move quickly to the air-water border, unfold, and rearrange itself there in order to have good FC (Fathollahy et al., 2021). According to Naghdi et al. (2023), the FC decreased with decreasing protein hydrolysate solvability because protein molecules moved to the air-water interface at a slower rate. The effect of net charge on the adsorption of proteins at the air-water boundary was also demonstrated by Qoms et al. (2023). The consistency of the protein-protein membrane is necessary for foam stability (Vo et al., 2020). In this study, the highest FC value was found at pH 3, which was 1.11 times lower than that of albumin (Figure 2 (a)), while the highest FS value was found in the hydrolysate at pH 4, which was 1.2 times lower than that of albumin (Figure 2 (b)). Those numbers were higher than those found in our earlier study using protein hydrolysates from *Acetes japonicus* (Vo et al., 2022). Accordingly, the hydrolysate of featherback skin may be served as a foaming agent in various foods, particularly those with acidic pHs.

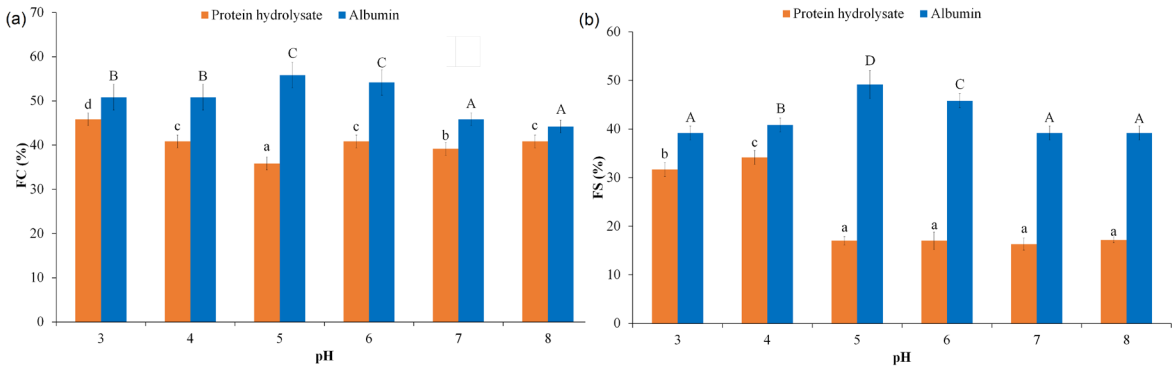

Figure 2: (a) FC and (b) FS of the featherback skin protein hydrolysate. Bars with different small letters indicate significant differences (p<0.05) for FC and FS of the hydrolysate; bars with different capital letters indicate significant differences (p<0.05) for FC and FS of the albumin

3.4 Determination of emulsifying property of the skin hydrolysate

The EAI and ESI values of the featherback skin hydrolysate peaked at 15.15 m^2/g and 14.96 min at pH 8 and 5, in order, as shown in Figures 3. According to Kumari et al. (2023), polypeptides unfold at an alkaline pH because of their negative charges, which improves interface orientation and effectively exposes hydrophilic and hydrophobic residues that encourage significant contacts at the oil-water boundary, raising the EAI of the protein hydrolysate. Conversely, the stability of the absorbed layer that covers the fat globule and the interaction between the layer and the hydrophobic component are key factors in ESI (Vo et al., 2022). According to Qoms et al. (2023), a rise in protein hydrophobicity facilitated the interaction of the surfactant with the lipid core. As a result, the hydrolysate's ESI was enhanced close to the pI. Except for the EAI values at pH 4 and 5, which were resampled to the pI value of sodium caseinate solution, the hydrolysate generally had worse emulsifying properties than those of sodium caseinate (1.06 - 1.27 times in the case of EAI and 1.21 - 1.52 folds in the case of ESI). When considered collectively, the featherback skin hydrolysate could be viewed as an emulsion-booster ingredient for some food products.

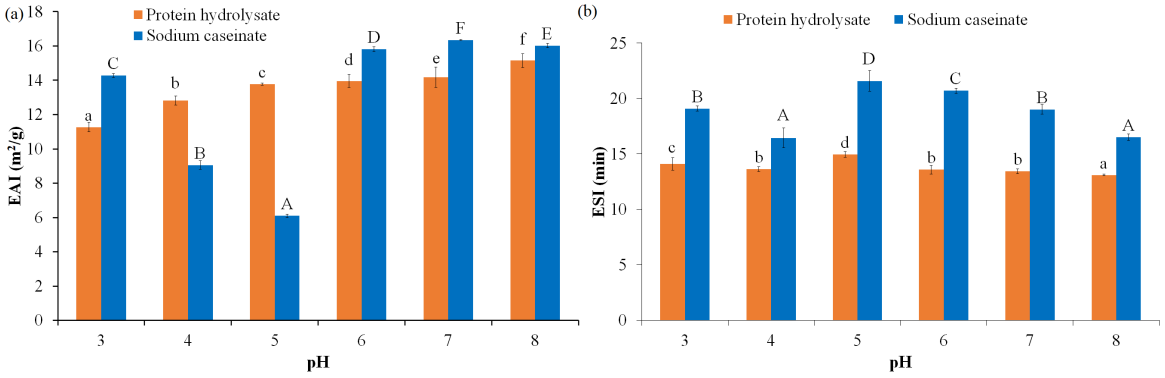

Figure 3: (a) EAI and (b) ESI of the featherback skin protein hydrolysate. Bars with different small letters indicate significant differences ($p<0.05$) for EAI and ESI of the hydrolysate; bars with different capital letters indicate significant differences ($p<0.05$) for EAI and ESI of the sodium caseinate

4. Conclusions

This research provided the featherback fish cake manufacturers with a zero-waste orientation as the first step in the valorization of the featherback skin. This was accomplished by creating a different type of natural antioxidant, skin hydrolysate, which can enhance food products' ability to foam or emulsify. It is necessary to perform more in-depth research on the antioxidant mechanism of the hydrolysate *in vivo* or its fortification into a particular food product, in which fishy taste of the hydrolysate needs to be considered.

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