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# Nutrient Composition and Antioxidant Properties of Fish Protein Hydrolysate from Bullet Tuna (*Auxis rochei*)

M. Zulham Efendi Sinaga[1,2], Cut Fatimah Zuhra[1,2], Sovia Lenny[1], Rini Hardiyanti[1,2], Wirda Ariyanti[1] **10.18805/ajdfr.DRF-427**

## ABSTRACT

**Background:** Bullet Tuna (*Auxis Rochei*) is commonly found in Indonesian waters and its distribution in the world includes warm waters (tropical and subtropical). The utilization of this fish is still minimal because people prefer to consume it as a daily side dish. The high protein content in Bullet Tuna has the potential to be processed into fish protein hydrolysate (FPH) which has a higher economic value for applying in the food and pharmaceutical fields.

**Methods:** This study aims to obtain FPH from Bullet Tuna using the bromelain enzyme. The method used to produce FPH was hydrolyzing muscle tissue with variations of the bromelain at 2, 3, 4, 5 and 6% (w/v) at 55ºC for 6 hours.

**Result:** Characteristics of the best FPH obtained by the addition of bromelain 6% (w/v) were observed as protein content of 77.80%, nitrogen content of 12.45%, water content of 7.32%, fat content of 0.97%, ash content of 11.88% and degree of hydrolysis of 60.46%. It had 15 types of amino acids and an antioxidant activity value of 94.05 ppm. Results suggested that the FPH of Bullet Tuna produced showed a promising additive for food and industrial applications.

**Key words:** Bromelain enzyme, Bullet tuna, DH, FPH, Hydrolysis.

## INTRODUCTION

In the last decades, researchers and industry have been paying significant attention to aquaculture and seafood processing to support the protein demands of an increasing human population. Therefore, a huge number of by-products are produced after fishery processing, such as heads, tails, spines, viscera and skin, which are accountable for 75% of the total weight of fish (Rios-Herrera *et al.,* 2021) (González-Serrano *et al.,* 2022). The total fisheries production globally is 177.8 million tonnes of which 157.4 million tonnes for human consumption and 20.8 million tonnes for non-food uses. In addition, roughly 60% of fish catch is rejected during processing without any recovery (Halim *et al.,* 2016). Fish contains mainly water (73.6%) and also consists of protein (15.8%), lipid (4.5%) and ash (6.1%), respectively (Nhi Tran *et al.,* 2023). Because of its high protein content, protein hydrolysate derived from marine by-products has become increasingly popular (Córdova Murueta *et al.,* 2007). The industry of fisheries processing increasingly developed fish products based on meat, head, viscera, skin and bone. It is a good raw material used as an ingredient in a value added product to increase the nutritional properties with a steady duration of use, contribute to protein supply and food security, reduce environmental impact and add economic value to food (Laishram *et al.,* 2023).

Many strategies have been developed for fish products including enzyme-assisted extraction (Belsare *et al.,* 2024), ultrasonic-assisted extraction (Duppeti *et al.,* 2023), microwave-assisted extraction (Rahimi *et al.,* 2017), accelerated solvent extraction (Wang *et al.,* 2021) and ultra-high-pressure extraction (Zhang *et al.,* 2021). Fermentative conversion of sardine into sauce using optimized conditions is one of the effective methods for the preservation of economically underutilized fish. However, the traditional method of fish sauce production requires fermentation for about 9-12 months to hydrolyze fish proteins into soluble peptides and amino acids using sodium chloride (NaCl). The proteolytic enzymes from halotolerant bacteria not only shorten the fermentation period in fish sauce production but also, in turn, help to reduce the formation of biogenic amines (Gowda *et al.,* 2023). Hydrolyzed protein from fish skin is an inexpensive novel method to generate bioactive peptides and a good source of gelatine (Ramakrishnan *et al.,* 2023). Acid hydrolysis breaks down proteins into smaller peptides and amino acids while destroying tryptophan. A process known as alkali hydrolysis breaks down serine and threonine. Both processes involve high temperatures and there may be issues with disposing of trash and acid

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, North Sumatra 20155, Indonesia.
[2]Centre of Excellence for Chitosan and Advanced Materials, Universitas Sumatera Utara, Medan, North Sumatra 20155, Indonesia.

**Corresponding Author:** M. Zulham Efendi Sinaga, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, North Sumatra 20155, Indonesia. Email: m.zulham.effendi@usu.ac.id
ORCID: 0000-0002-7111-6081

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by-products. As a mild technique, enzymatic hydrolysis has been studied by applying various enzymes to raw biomass of Rohu (*Labeo rohita*) (Kumar *et al.,* 2022).

Fish is considered as high-protein food source. To increase its value, the fish should be converted into fish protein hydrolysate (FPH), a new product with high functionality. FPH is a mixture of broken proteins consisting of smaller peptides and amino acids obtained by hydrolyzing fish protein. FPHs are valuable food ingredients because of their high nutritional and bioactive properties with complete and good amino acid balance. It was reported that FPH possesses anti-hypertensive, antimicrobial, antioxidant and antithrombotic properties (Araujo *et al.,* 2021) (Shavandi *et al.,* 2019) (Nhi *et al.,* 2023). The application of pelagic FPH in functional foods for nutritional and health benefits as well as in feed and treat products for companion animals offers many opportunities and challenges and may help to supply an excellent source of protein to the growing global population. The market of FPH is lucrative, worth $244.2 million in 2021 (Shavandi *et al.,* 2019).

The raw materials for bioactive peptides are of high protein content, quality and inexpensive (Tadesse *et al.,* 2023). Bullet Tuna (*Auxis Rochei*) is a fish most commonly found in Indonesian waters and its distribution in the world includes warm waters (tropical and subtropical), including the Mediterranean Sea and the Black Sea. Therefore, Bullet Tuna is very popular because of its relatively cheaper price, easy accessibility and good nutritional content. It has only been processed into frozen products, smoked fish products and fish dried into small pieces. A product from this fish in the form of FPH with a higher economic value has not been found yet. Therefore, we aim to hydrolyze FPH from Bullet Tuna through enzymatic hydrolysis following the antioxidant and nutritional analysis of the resulting FPH.

## MATERIALS AND METHODS

Bullet Tuna was obtained from the traditional local market, Pasar Ikan Cemara, North Sumatera, Indonesia. Distilled water was purchased from PT. Rudang Jaya, Medan, North Sumatera. Materials used such as enzyme bromelain, sodium hydroxide (NaOH), sulfuric acid ($H_2SO_4$), boric acid ($H_3BO_3$), Tashiro indicator, methanol, n-hexane, chloride acid (HCl), ortho phthalaldehyde (OPA), phenolphthalein indicator (PP), selenium, trichloroacetic acid and 2,2-diphenyl-1 -picrylhydrazyl were purchased from Sigma Aldrich.

Preparation of FPH from Bullet Tuna is described below. Firstly, Bullet Tuna was rinsed using distilled water to remove any impurities. The meat was then separated from the skin and bone and chopped into small pieces of around 1cm in length. After that, distilled water was added to the fish with a comparison of 1:4 (w/v) and stirred using a stirrer. Bromelain enzyme was added into the blend with a concentration of 2, 3, 4, 5 and 6% (w/v). After homo-genization, NaOH 2 M was added in a dropwise fashion until reaching optimum pH and hydrolyzed in an oven at 55°C for 6 hours. Afterwards, the temperature was inc-reased to 90°C for 20 minutes to deactivate the enzyme. It was filtrated and the filtrate was centrifuged at 3500 rpm for 30 minutes using a centrifugation Megafuge 16R (Hamburg, Germany, Eppendorf). The supernatant produced was dried using a ScanVac Coolsafe Touch 110-4 freeze dryer (India, ScanVac). The product was labeled as FPH and characterized.

The biochemical parameters of the FPH samples were measured using the following methods. Nitrogen and protein measurements were accomplished using the Kjeldahl method in which 1 g of FPH was put into a 500 mL Kjeldhal flask with added 0.2 g selenium and 15 mL 98% $H_2SO_4$. The apparatus was heated until the solution was homogenous and transparent, it was done for about 2 hours. It was then allowed to cool at room temperature and 10 mL of distilled water was added followed by 30 NaOH until the solution became alkaline. After that, it was distilled and the distillate was collected in an Erlenmeyer flask containing 3 $H_3BO_3$ and Tashiro indicator until the solution turned green. The distillate was then titrated using 0,1 N HCl until turned purplish-blue (Vs). Titration was also carried out for the blank, namely titration of boric acid without the presence of NH3 (Vb). Total water content was measured using the gravimetric method in which 2 g of FPH was put in a porcelain cup. It was dried in a vacuum oven at 105°C for 3 hours. The sample was weighted several times until a constant weight was obtained. Total fat was determined using the soxhletation method by weighing 2 g of FPH in a filter paper connected between the Soxhlet and flask containing boiling stones. It was extracted using n-hexane for 6 hours at 90°C, followed by distillation at 80°C. Ash content was estimated by taking 2 g FPH in a porcelain cup and drying it in a vacuum oven at 50°C for 3 hours. It was then placed in a furnace at 600°C for 6 hours. The mass was weighed until a constant number was obtained. The degree of hydrolysis (DH) was calculated as:

$$DH = \frac{10\ \text{Trichloroacetic acid soluble } N_2 \text{ in the sample}}{\text{Total } N_2 \text{ in the sample}} \times 100$$

The total amino acid was investigated using High-Performance Liquid Chromatography - Thermo Fisher Scientific with Thermo Ultimate 3000 RS Fluorescence Detector. A 60 mg of FPH and 4 mL 6 N HCl were heated at 110°C for 24 hours. It was cooled at room temperature and neutralized using 6 N NaOH. After neutralising, it was added 10 mL of distilled water and filtered using 0.2 mm Whatmann. A 50 L of the sample was mixed with 300 mL OPA solution. It was stirred for 5 minutes and 10 ml of the solution was kept into the HPLC injector.

The amount of 10 mg FPH was dissolved with methanol in a 10 mL volumetric flask to obtain a concentration of 1000 mg/mL. The stock solution was pipetted 0.25; 0.5; 0.76; 1; and 1.27 mL into a 10 mL volumetric flask to obtain a concentration of 25; 50; 76; 100 and 127 mL and added

200 mg/mL DPPH. The solution was incubated for 30 minutes and the absorbance was measured using a spectrophotometer UV-Visible Mini 1240 Japan Shimadzu. Radical free of FPH was calculated using the formula as:

$$\text{Immersion (\%)} = \frac{\text{Control absorbance - Sample absorbance}}{\text{Control absorbance}} \times 100\%$$

The % of inhibition was plotted against concentration to calculate $IC_{50}$.

## RESULTS AND DISCUSSION

### Biochemical properties of fish protein hydrolysate from bullet tuna

In this study, the physical appearance of FPH from Bullet tuna was light yellow with the presence of 2 enzyme bromelain, the color turned pale yellow as the concentration of enzyme bromelain increased as shown in Fig 1. The biochemical properties of FPH such as yield, nitrogen, protein, water, ash and DH increased with increasing the concentration of enzyme bromelain, while fat content decreased. The data is summarized in Table 1.

### Amino acid properties of fish protein hydrolysate from Bullet tuna

Based on the analysis of protein and DH from FPH, the presence of 6 enzyme bromelain had the highest value, hence, it was used as the comparison with fresh Bullet Tuna properties as summarized in Table 2.

### Antioxidant activity

The maximum absorbance of DPPH was performed in the 30$^{th}$ minute at a concentration of 40 mg/mL showing the wavelength at 516 nm. This result follows the previous study stating that the wavelength for DPPH is approximately 515-520 nm. Meanwhile, the $IC_{50}$ value for the FPH is summarized in Table 3.

Enzyme bromelain hydrolyzed cleavage bond to stretch chemical structure and easily dissolves hydrolyzed protein as described in the mechanism reaction (Fig 2). During the hydrolysis process, the active side of the enzyme namely histidine and cystine group break the peptide-protein chain to be a product. In this reaction, there is a structural change between the enzyme and substrate in which the cystine group bonds with the substrate to form a tetrahedral covalent enzyme substrate. Cystine also catalyzes the carbonyl group from peptides to remove amino acids. Furthermore, the histidine group is protonated and bonded with the substrate nitrogen to diffuse amine where its position is replaced by a water molecule to hydrolyze the product and the enzyme turns to its original shape (Vašková *et al.,* 2023).

The yield of FPH with the addition of enzyme bromelain from 2 to 6 slightly increased from 13.45, 13.87, 14.43, 14.57 and 14.99, respectively. The protein of FPH dissolved in water was also increased. This result is in agreement with the findings of (Permadi *et al.,* 2022). The dissolving of nutritional components such as fat, protein and minerals during the hydrolysis positively affected the yields of the product. Hence, water was added to create more water in the hydrosylate as compared to the substrate. Water easily homogenizes enzymes and substrates, increases the enzymatic reaction rate and improves the hydrolysate product. High yield improves the nutritional content of products with increased economic value of a substrate (Sharma *et al.,* 2022).

Total protein and nitrogen of FPH increased from 57% to 77.80% and from 9.12% to 12,45% with the addition of bromelain enzyme at 2 and 6, respectively. This is due to the enzyme accelerating the termination of the protein.

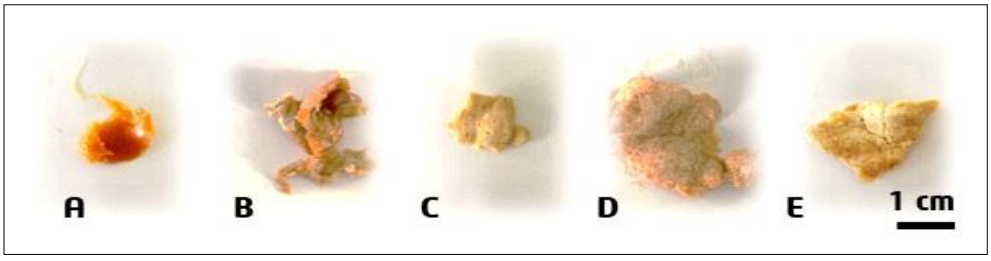



**Fig 1:** Fish protein hydrolysate from Bullet tuna with a concentration of enzyme bromelain (a) 2% (b) 3%; (c) 4%; (d) 5% and (e) 6%.

**Table 1:** Biochemical properties of fish protein hydrolysate from Bullet tuna.

| Bromelain concentration (%) | Biochemical properties | | | | | | |
|---|---|---|---|---|---|---|---|
| | Yield (%) | Nitrogen (%) | Protein (%) | Water (%) | Fat (%) | Ash (%) | Degree of hydrolysis (%) |
| 2 | 13.45 | 9.12 | 57.00 | 2.06 | 1.74 | 10.10 | 42.76 |
| 3 | 13.87 | 9.72 | 60.75 | 2.42 | 1.63 | 11.22 | 48.45 |
| 4 | 14.43 | 9.99 | 62.40 | 4.46 | 1.49 | 12.79 | 51.85 |
| 5 | 14.57 | 11.94 | 74.62 | 5.04 | 1.34 | 12.85 | 52.20 |
| 6 | 14.99 | 12.45 | 77.80 | 7.32 | 0.97 | 11.88 | 60.46 |

At 2% level of enzyme bromelain 2, the protein of FPH in this study was higher by 11.7 compared to the result reported by (Anggreini, 2022). However, the concentration of 6 bromelain, was lower compared to the result reported by Wijayanti *et al.* (2016). Increased protein content is due to the activity of the enzyme in which bromelain broke complex protein in a simple protein by breaking cleavage peptide bonds to produce soluble protein. Hence, the presence of the enzyme bromelain increased the protein content (Bustamante *et al.,* 2022). The water content also increa-ses by increasing the loading level of bromelain due to more protein breakdown.

The highest fat content was found in the concentration of bromelain 2 (1.74) and it was decreased with the increasing concentration of bromelain. Higher enzyme concentrations are used to increase water because the protein dissolves and insoluble fat is separated from the hydrolyzed product (Dias and de Moura Bell, 2022). It is suggested to have a low fat to hinder oxidation and durability. DH of FPH from Bullet Tuna is 60.46 with the presence of 6 bromelain. It was the highest compared to others 42.76, 48,45, 51.85 and 52.20 with the addition of bromelain 2, 3, 4 and 5 consecutively. While, DH of FPH from residues of Bullet Tuna such as head, bone and skin were 40.93, 38.13 and 37.23, respectively (Naghdi *et al.,* 2023). Enzymes accelerate enzymatic rate reaction to create more peptide bonds in hydrolyzed protein to produce simple and soluble protein. The enzymatic reaction is directly proportional to its concentration until it reaches equilibrium. The mechanism of DH by peptide bonds and soluble amino acid during the hydrolysis is presented in Fig 3. while the DH graph is shown in Fig 4.

**Table 2:** The amino acid of fish protein hydrolysate and fresh Bullet Tuna fish.

| Amino acid | Value (%) | |
|---|---|---|
| | Fresh bullet tuna fish | FPH with 6 enzyme bromelain |
| **Non-essential amino acid** | | |
| Aspartate acid | 1.96 | 2.75 |
| Glutamate acid | 2.82 | 6.06 |
| Serin | 0.91 | 0.91 |
| Glycine | 0.96 | 1.53 |
| Alanine | 1.43 | 3.20 |
| Tyrosine | 1.78 | 1.48 |
| **Essential amino acid** | | |
| Histidine | 0.82 | 1.15 |
| Threonine | 1.11 | 4.41 |
| Arginine | 5.83 | 1.22 |
| Methionine | 0.72 | 1.00 |
| Valin | 1.13 | 1.96 |
| Phenylalanine | 0.98 | 1.72 |
| Isoleucine | 1.21 | 1.72 |
| Leucine | 3.22 | 4.83 |
| Lysine | 2.27 | 3.9 |

**Table 3:** $IC_{50}$ value of fish protein hydrolysate from Bullet tuna.

| Enzyme concentration (%) | Regression equation | Correlation coefficient (R) | $IC_{50}$ (mg/mL) |
|---|---|---|---|
| 2 | Y = 0.306X - 3.611 | 0.972 | 174.992 |
| 3 | Y = 0.349X + 0.538 | 0.993 | 147.719 |
| 4 | Y = 0.494X - 2.530 | 0.977 | 106.411 |
| 5 | Y = 0.458 + 5.323 | 0.965 | 97.562 |
| 6 | Y = 0.551 - 1.813 | 0.991 | 94.058 |

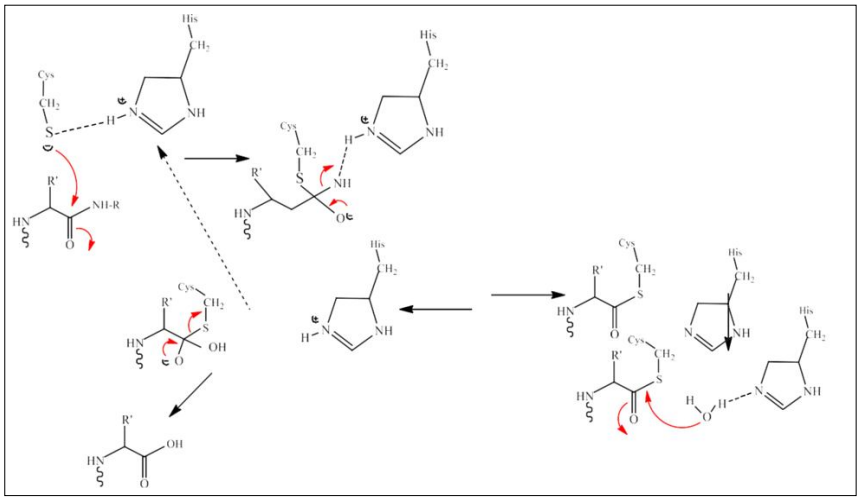


**Fig 2:** The mechanism of hydrolyzed protein with an enzyme.

FPH has a higher essential amino acid compared to fresh Bullet Tuna (Table 2). This is due to unbonded protein and free amino acid detectable before hydrolysis. In addition, the enzyme acts as a catalyst to break protein and increase amino acid after hydrolyzing (Romsuk *et al.*, 2022). FPH from Bullet Tuna had glutamic acid as 6.06

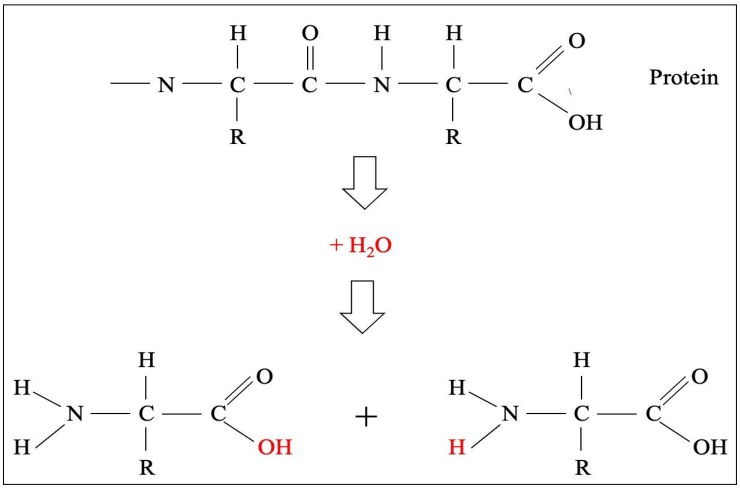



**Fig 3:** The mechanism of hydrolyzed enzymatic protein.

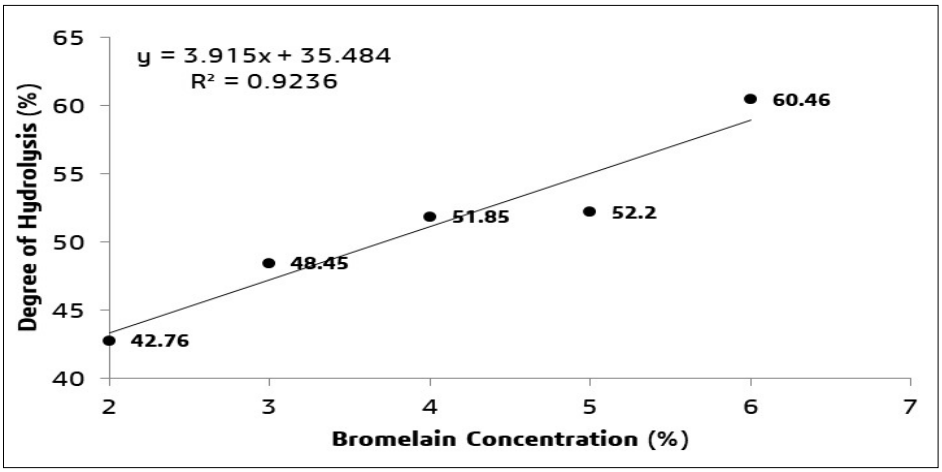



**Fig 4:** Degree of hydrolysis of FPH from Bullet Tuna (*Auxis rochei*) through bromelain hydrolysis.

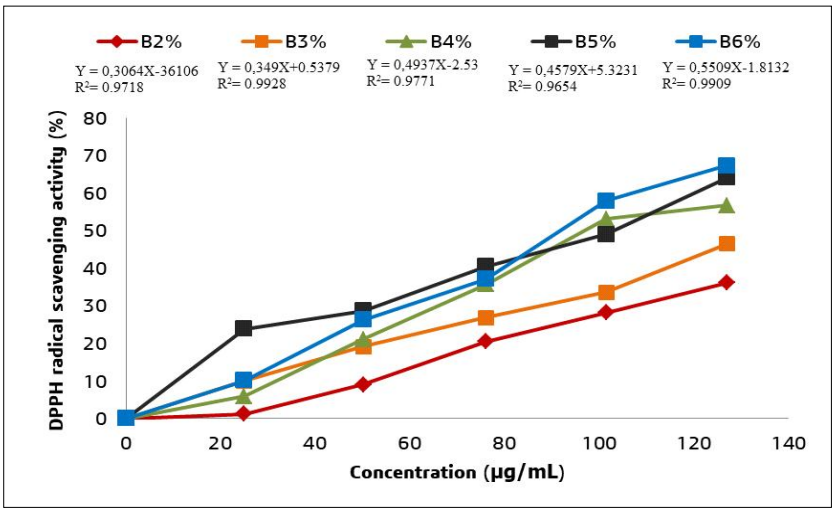



**Fig 5:** DPPH radical scavenging activity of FPHs of Bullet Tuna.

which was the higher amino acid found, followed by leucine (4.83), tyrosine (4.41), lysine (3.50) and alanine (3.20), respectively. Glutamic acid is the most common amino acid found in fish products and has a savory taste (Kos *et al.,* 2023). In contrast to cystine, glycine, alanine and valine give a sweet as well as sour flavor, whereas arginine gives bitter and sweet tastes (Fu *et al.,* 2022); (Hussen, 2021). Hence, FPH from Bullet Tuna can be used as a flavoring.

The antioxidant activity of FPH (Fig 5) increased with increasing yield since FPH having a bioactive peptide and amino acid acting as antioxidants. Free radicals in the FPH also decreased as shown by the color change from light yellow to pale yellow. This causes a decrease in absorbance which proves the DPPH free radical scavenging activity to obtain correlation values and regression equation to calculate the $IC_{50}$ value (Table 3).

The classification of antioxidants is categorized as follows: $IC_{50}$ < 50 ppm (very strong), $IC_{50}$ 50-100 ppm (strong), $IC_{50}$ 100-150 ppm (moderate), $IC_{50}$ 150-200 (weak), $IC_{50}$ > 200 ppm (very weak). In this study, the presence of bromelain at 5 and 6 levels were categorized as a strong antioxidant, while at 3 and 4 levels, it acts as a moderate antioxidant and at 2 bromelain, as a weak antioxidant. The addition of enzymes is in line with the increase in the number of peptides and free amino acids produceds in the hydrolyzed product so that the percentage of inhibition of radical activity will also increase along with the presence of hydrolyzing enzymes (Abbasi *et al.,* 2022).

## CONCLUSION

The characteristics of FPH from Bullet Tuna give the best properties at 6 bromelain such as 77.80 protein, 12.45 nitrogen, 7.32 water, 0.97 fat, 11.88 ash, with 14.99 yield and 60.46 DH. FPH from Bullet Tuna has 15 amino acids with glutamic acid as the main nutrient and is suitable for flavoring. In addition, the FPH has a strong antioxidant activity with IC50 values of 97.56 and 94.05 ppm at 5 and 6 of bromelain concentration.

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### Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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