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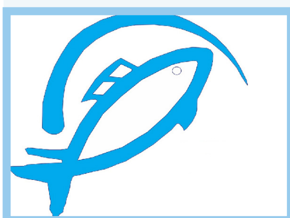

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Research Article

Impact of Enzymatic Hydrolysis on Antioxidant Activity of Snakehead Fish (*Channa striata*) Head Protein Hydrolysate

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Abstract

There is concern regarding the use of synthetic antioxidants which spurred the yearly increase of natural antioxidants to substitute synthetic ones. Fish protein hydrolysate (FPH), which has been reported to have potent antioxidant properties, could be utilized to solve this problem. This study aimed to utilize the by-product of snakehead fish (head) and determine the optimum hydrolysis conditions to obtain FPH with antioxidant activity. Two parameters were tested during the hydrolysis process: enzyme concentration (papain enzyme) and hydrolysis time. The optimum condition was evaluated by measuring dissolved protein, hydrolysis degree (DH), and antioxidant activity, including DPPH, ABTS, and FRAP. The optimal hydrolysis conditions were 5% enzyme concentration and 6 h of hydrolysis time at 55°C and pH 7.0. The DPPH, ABTS, and FRAP antioxidant activities were 50.70%, 66.67%, and 1.35 M Tr/mg, respectively. Based on the antioxidant activity, Snakehead fish head has the potential as a source of natural antioxidants.

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1. Introduction

Antioxidants are substances that can stabilize free radicals by completing the unpaired electrons of radical molecules. These compounds have been widely used to improve food quality (Zahid *et al.*, 2018; Karpińska-Tymoszczyk and Draszanowska, 2019; Tavdidishvili *et al.*, 2020) and human health quality (Villaverde *et al.*, 2019; Jideani *et al.*, 2021). There are two main categories of antioxidants, natural, and synthetic. Natural antioxidants are typically more expensive and difficult to employ than synthetic antioxidants. However, synthetic antioxidants are reported to have toxicity potential (Mbah *et al.*, 2019). The potential for toxicity is a significant concern that motivates some consumers to switch to natural antioxidants. Many natural antioxidants have been studied, such as plant-based (Memarpoor-Yazdi *et al.*, 2013; de Camargo *et al.*, 2017; Gallego *et al.*, 2020; Jideani *et al.*, 2021) and animal-based (Karnjanapratum *et al.*, 2017; Wang and Shahidi, 2018; Verma *et al.*, 2018), including protein hydrolysate from fish (Chi *et al.*, 2015; Zhang *et al.*, 2019).

Fish protein hydrolysate (FPH) is a protein-derived substance that has been hydrolyzed to produce amino acid and peptide components of varying molecular sizes. It is widely known to have bioactive peptide with functional properties such as antioxidants (Sohaib *et al.*, 2017; Nurilmala *et al.*, 2020; Shiao *et al.*, 2021), antibacterial (Trang and Pasuwan, 2018; Atef *et al.*, 2021), anti-Inflammatory (Da Rocha *et al.*, 2018), and antihypertensive (Abachi *et al.*, 2019; Naik *et al.*, 2021). Several proteases have been reported to be used for the production of FPH such as orientase, protease XXIII, trypsin, bromelain, papain, protamex, bovine pancreatin 6.0, neutrase 1.5MG, and alcalase (Ren *et al.*, 2007; Hsu, 2010; Šližytė *et al.*, 2016; Yathisha *et al.*, 2022). Among them, papain has been reported to produce FPH with good antioxidant activity (Li *et al.*, 2011; Lu *et al.*, 2014; Qiu *et al.*, 2019). Many FPH studies have been conducted using various materials such as cod (Šližytė *et al.*, 2009), jellyfish collagen (Zhuang *et al.*, 2009), *Parastromateus niger* by-product (Ganesh *et al.*, 2011), unicorn leatherjacket (Sai-Ut *et al.*, 2014), tilapia (Lin *et al.*, 2017), catfish (Abraha *et al.*, 2017), parrotfish (Prihanto *et al.*, 2019), *Pangasius* sp. (Hamzah *et al.*, 2021), including snakehead fish (Rasimi *et al.*, 2020).

However, few studies have used snakehead fish by-products (head) as the raw material. Snakehead fish is one of Indonesia's most popular freshwater fish, with annual production reaching 21,987 tons in 2019 (MMAF, 2020). Along with the production, by-products such as heads and viscera will be left behind. Due to the high protein content of these by-products, they can be optimized as bioactive protein-based products. Therefore, this study investigates the viability of snakehead fish by-products as materials for producing protein hydrolysate with antioxidant activity.

2. Materials and Methods

2.1 Materials

Materials used were protease Papain (Sigma-Aldrich 1.5-10 units/mg), HCl (Merck), NaOH (Merck), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich), Tris Pyridyl Triazine (TPTZ) (Sigma-Aldrich), 2,2-Azinobis 3-ethyl benzothiazoline 6-sulphonic acid (ABTS) (Sigma-Aldrich), $FeCl_3.6H_2O$ (Merck), follin-ciocalteu, NaK-tartrate, $NaCO_3$, $CuSO_4$, Bovine serum albumin (BSA) (Sigma-Aldrich), Trolox (Sigma-Aldrich), ethanol, buffer acetate, and TCA 20%.

2.2 Methods

2.2.1 Sample preparation

The heads of snakehead fish were obtained from small and medium enterprises in Palembang, South Sumatra, Indonesia. Samples were washed, packed in a polyethylene plastic zipper bag, frozen at -20°C, transported in an insulated box to the laboratory, and stored at -20°C until used.

2.2.2 By-products yield and proximate analysis

A live snakehead fish was bought and slaughtered carefully to calculate the snakehead fish by product yield. Fish heads and viscera were weighed and compared with the weight of the whole fish. Proximate analysis was conducted for moisture, fat, protein, and ash content according to AOAC (2005), while the carbohydrate was calculated by difference.

2.2.3 Hydrolysis process

The hydrolysis followed method by Souissi *et al.* (2007) with modifications on enzyme concentration and hydrolysis time. Single factor tests with enzyme concentration and hydrolysis time were conducted to determine the optimal conditions for enzymatic hydrolysis. Enzyme concentrations were 1%, 2%, 3%, 4%, 5%, and 6%, while hydrolysis times were 0, 3, 4, 5, 6, and 7 hours (each treatment was performed in triplicates). Before being used as material, snakehead fish head was minced and boiled at 90°C to deactivate indigenous protease. The optimum enzyme concentration was produced by mixing 30 grams of pretreated snakehead head with distilled water (1:1 w/v) and hydrolyzing for

3 hours. The temperature and pH were maintained at the optimum condition for papain reaction, which was 55°C and 7.0, respectively. The hydrolysis was stopped by heating at 90°C for 20 minutes, followed by supernatant collection by centrifugation at 14000 x g for 20 minutes. It was then lyophilized through freeze-drying and stored at -40 °C as a powder until used. After optimum enzyme concentration was obtained, the selected treatment was used afterward to optimize hydrolysis time in the same hydrolysis condition as mentioned before.

2.2.4 Snakehead head protein hydrolysate evaluation

2.2.4.1 Yield

The yield was calculated as a percentage of the dry protein hydrolysate powder relative to the initial raw material following Chalamaiah *et al.* (2013).

$$Yield = \frac{dry\ weight\ of\ protein\ hydrolisate\ (g)}{wet\ weight\ of\ raw\ materials\ (g)}\ x\ 100\% \quad (1)$$

2.2.4.2 Degree of hydrolysis (DH)

The degree of hydrolysis was determined according to method done by Amin *et al.* (2013). Twenty milliliters of protein hydrolysate were mixed with 20 mL of TCA 20% (w/v). The mixture was kept for 30 minutes to precipitate, then centrifuged at 7,800 x g for 15 minutes. The supernatant was analyzed for nitrogen content using the Kjeldahl method. The degree of hydrolysis was calculated as follows:

$$DH = \frac{Total\ N\ dissolved\ in\ TCA}{Total\ N\ samples}\ x\ 100\% \quad (2)$$

2.2.4.3 Protein solubility

The protein solubility was performed according to Bradford (1976). The sample was added with Bradford solutions at 1:1 (500 µl each), followed by a vortex. The mixture was incubated for 10 min and immediately measured using a spectrophotometer at λ 595 nm.

2.2.4.4 DPPH radical scavenging activity

The antioxidant activity of DPPH was determined following the method utilized by Wu *et al.* (2003). As much as 1.5 mL of sample was added with 1.5 mL of 0.1 mM DPPH in 95% ethanol, followed by incubation in the dark at room temperature for 30 minutes. The solution was then measured using a spectrophotometer at λ 517 nm. The blank was prepared in the same manner by using distilled water as a sample.

$$DPPH\ value = \frac{Blank\ absorbance - sample\ absorbance}{Blank\ absorbance}\ x\ 100\% \quad (3)$$

2.2.4.5 ABTS radical scavenging activity

ABTS assay was carried out as described by Binsan *et al*. (2008). A total of 150 µl sample was mixed with 2850 µl of the working solution followed by incubation in the dark at room temperature for 2 hours. The working solution was pre-made by mixing two stock solutions, namely 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution (1:1). It was then kept at room temperature and in the dark for 12 hours to allow the reaction to occur. The absorbance was then measured at λ 734 nm. The blank was prepared in the same manner by using distilled water as a sample. The standard Trolox curve ranging from 90 – 600 µM was used as the standard, and then the reducing power was expressed in µM Trolox equivalents (TE) per mg dry sample.

$$ABTS\ value = \frac{Blank\ absorbance - sample\ absorbance}{Blank\ absorbance}\ x\ 100\% \quad (4)$$

2.2.4.6 FRAP (Ferric Reducing Antioxidant Power)

The FRAP analysis was conducted based on Benzie and Strain (1996). A total of 150 µl of the sample was mixed with 2.85 mL FRAP reagent (a mixture of 10 mM TPTZ solution in 40 mM HCL, 20 mM $FeCl_3.6H_2O$ solution, and 300 mM acetate buffer, pH 3.6 (1:1:10 v/v/v) which previously prepared (FRAP reagent was mix and at room temperature 37°C 30 minutes before use). The solution mixture was then kept in the dark place for 30 min at room temperature. The absorption was measured at λ 595 nm with distilled water as a blank. The standard Trolox curve was used, and the reducing power was expressed in µM Trolox equivalents (TE) per mg dry sample.

2.3 Statistical Analysis

The experiments were performed in a completely randomized design and the collected data were analyzed using one-way ANOVA (IBM SPSS Ver. 24). The significantly different data was further calculated with least significance different (LSD) in 95% confidence level.

3. Results and Discussion

3.1 Rendement and Proximate Analysis of Snakehead Fish

Various businesses, like the fisheries sector, have implemented the zero-waste principle by utilizing by-product resources. The head and viscera of snakehead fish were utilized in this study as a by-product of a

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small and medium-sized fish processing plant in South Sumatra, Indonesia. The snakehead fish by-products accounted for over 60% of the total weight. The highest by-product was the head (41.65%), followed by bone (9.45%), viscera (4.65%), and scale (2.98%). Therefore, the head utilization in this study contributes to utilizing 41% of total fish or 72% of total producers. The protein content of the head of the snakehead fish is high and comparable to that of the flesh (Table 1).

3.2 Optimization of Enzyme Concentration

The enzyme concentration optimization indicated that the increase of enzyme concentration affects the yield, degree of hydrolysis, and soluble protein of the resulting snakehead fish head protein hydrolysate (SFHPH) (Table 2).

The yield increased from 6.01 to 7.82% and peaked at 7.820.7% (P0.05) at an enzyme concentration of 3% before decreasing marginally. A slight decrease in yield showed that the maximum peptide cleavage process was achieved at a concentration of 3%. This study's yield is lower than those reported by Noman *et al*. (2019) with fish protein hydrolysate from Chinese Sturgeon using alcalase 0.5-4.5% and Wijayanti *et al*. (2016) with milkfish using bromelain 4-6%. This difference is believed to be caused by variances in the kind of protein in the substrate and the activity of the enzymes used during the hydrolysis process, which result in differing cleavage of peptide bonds.

The degree of hydrolysis (DH) is one of the crucial parameters for assigning protein hydrolysate functional and biological properties (Sila and Bougatef, 2016). This study showed that the hydrolysis process produced DH values ranging from 54.09 to 83.75% ($P<0.05$). The DH value increased gradually, peaked at 5% enzyme concentration, and decreased afterward. According to some research, the DH value correlated with enzyme/substrate ratio (Ramakrishnan *et al*., 2013) but not enzyme concentration (Noman *et al*., 2018). It could be described that the availability of peptides in the substrate had been hydrolyzed completely at a maximum concentration. Therefore, increasing enzyme concentrations did not give any significant results.

A significant difference ($P<0.05$) was observed in soluble protein among all the tested samples. The

Table 1. The proximate composition of snakehead fish

Sample	Water (%)	Protein (%db)	Fat (%db)	Ash (%db)	Carbohydrate (%db)
Meat	70.41±0.18[b]	20.38±0.51[c]	1.66±0.08 [b]	1.15±0.17[b]	6.4±0.58[b]
Viscera	80.05±0.25[a]	5.60±0.81[a]	3.20±0.22[c]	0.75±0.04[a]	10.4±0.29[a]
Head	72.68±0.1[c]	14.29±0.1[b]	0.62±0.17[a]	1.58±0.16[c]	10.83±0.45[a]

*Different letters within the same column in each parameter indicate significant difference at P<0.05.

Table 2. Effect of enzyme concentration on yield, degree of hydrolysis, and soluble protein of head protein hydrolysate

Protein concentration	Yield (%)	Degree of hydrolysis (%)	Soluble protein (ppm)
1%	6.31±0.7[a]	54.10±1.8[a]	111.66±6.1[a]
2%	7.01±0.5[a]	77.29±1.8[b]	212.36±22.7[b]
3%	7.82±0.7[b]	79.25±2.0[bc]	583.77±45[c]
4%	7.36±0.1[ab]	82.6±0.0[bc]	625.52±11.4[c]
5%	6.12±1.2[ab]	83.75±4.6[c]	643.24±2.1[c]
6%	6.01±0.1[a]	79.8±1.7[d]	636.75±14.9[c]

Results were obtained from three replicates ± standard deviation.
*Different letters within the same column in each parameter indicate significant difference at P<0.05.

protein solubility increased sharply along with the addition of enzyme concentration from 11166±6.1 ppm in 1% enzyme and peaked at 5% enzyme concentration with 643.24±2.1 ppm (0.643 mg/ml) (Table 2). These results agreed with Mutamimah *et al.* (2018) that increasing enzyme concentration could increase protein solubility in FPH products. During hydrolysis, insoluble protein will be converted into smaller components such as amino and peptides.

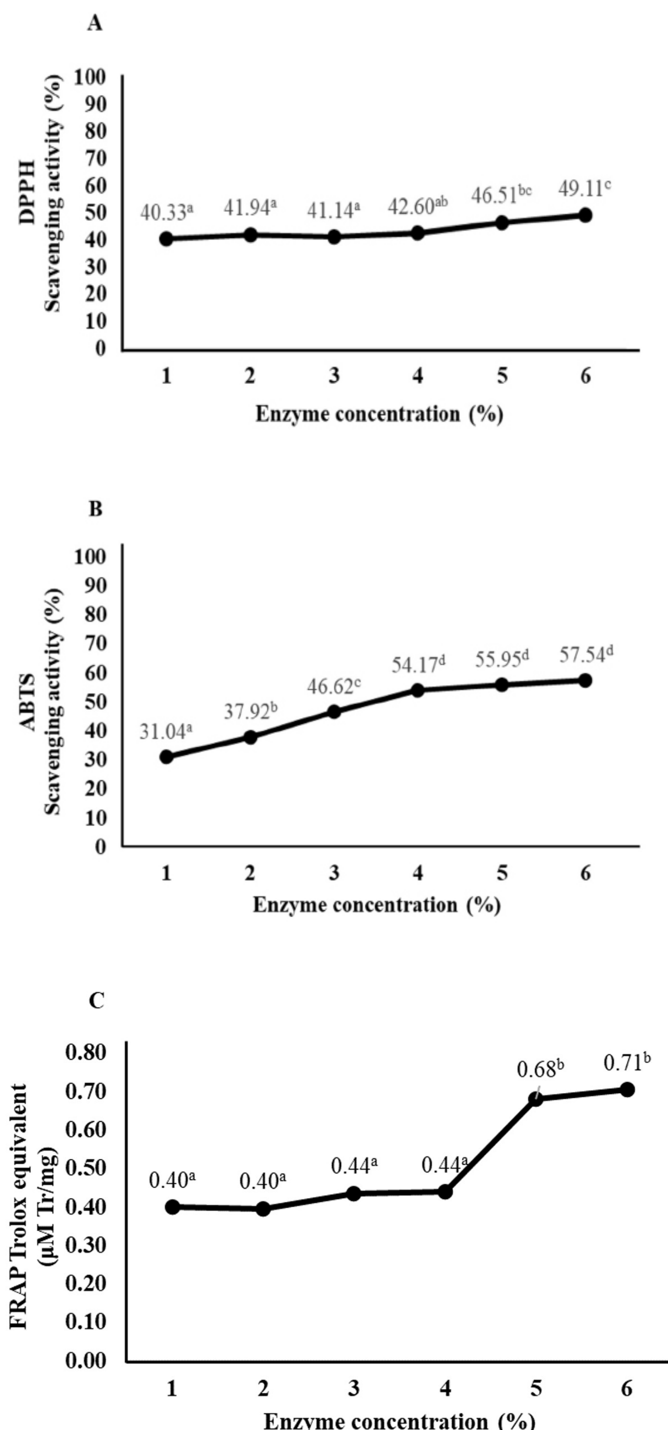

Figure 1. Antioxidant activities of protein hydrolysate at various enzyme concentration (a) DPPH; (b) ABTS; (c) FRAP.
*Different letters in each value indicate significant difference at P<0.05.

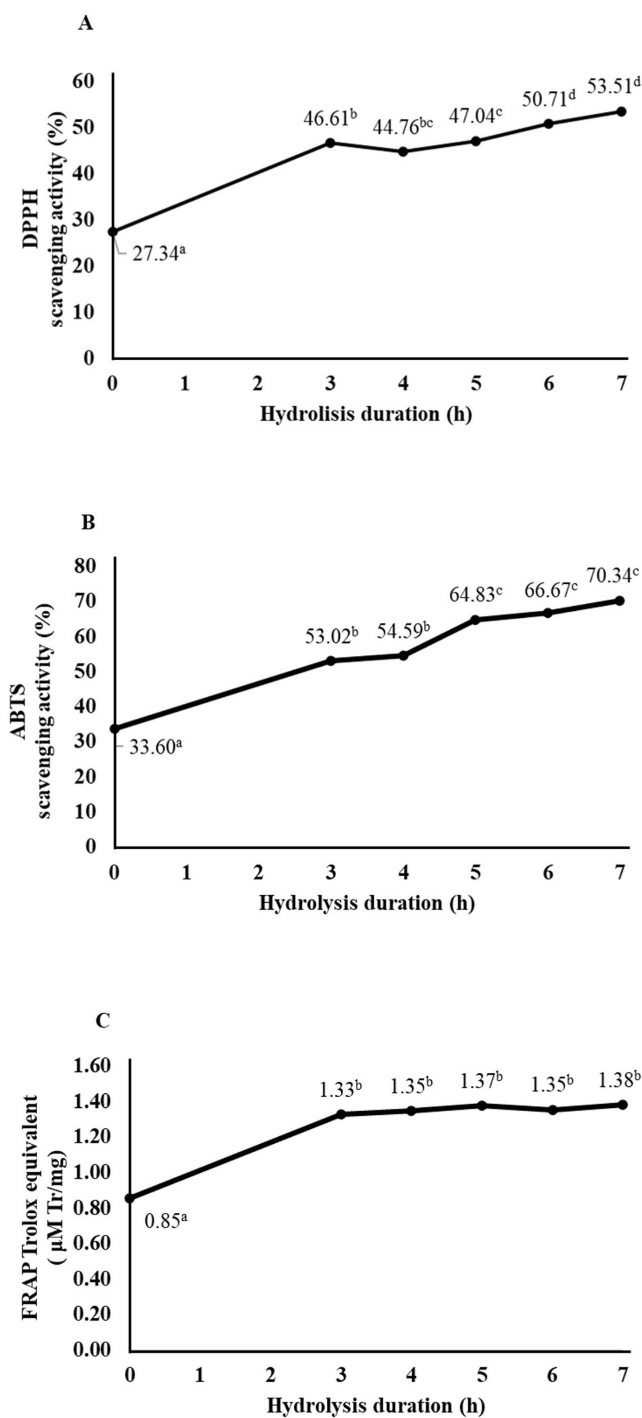

Figure 2. Antioxidant activities of protein hydrolysate at different hydrolysis time (a) DPPH; (b) ABTS; (c) FRAP.
*Different letters in each value indicate significant difference at P<0.05.

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The evaluation of the enzyme concentration on antioxidant activity by DPPH, ABTS, and FRAP on SFHPH suggested a strong effect (Figure 1). The radical scavenging activity of DPPH is widely used to evaluate the efficacy of antioxidant compounds quantitatively as hydrogen donors or free radical scavengers (Verma *et al.*, 2017). The present study showed that antioxidant activity of SFHPH based on DPPH increased following the increase in enzyme concentration (P<0.05) (Figure 1a) in the range of 40.32±0.8%-49.10±3.5% (percent inhibition) with Trolox equivalent of 3.32-13 µM Tr/mg. These findings are similar to the previous study by Puspawati *et al.* (2020), who reported that the radical inhibitory activity of DPPH increased with increasing enzyme concentration. Baehaki *et al.* (2015) investigated the catfish FPH using papain was effective in breaking peptides where a concentration of 1-6% produced four protein bands with molecular weights of 42.62 kDa, 31.46 kDa, 27.85 kDa, and 11.90 kDa. Smaller molecule weights can impede the oxidative process (Ranathunga *et al.*, 2006).

The antioxidant capacity of ABTS was between 31.04±2.3%-57.54±3.4% with Trolox equivalent of 2.10-7.7 M Tr/mg (P<0.05) (Figure 1b). A previous study by Guo *et al.* (2019) using 0.2% enzyme concentration produced lower results (148 µM Tr/g) than this study. According to Verma *et al.* (2017) amino acid composition, amino acid sequences, and DH value could affect antioxidant activity (Phanturat *et al.*, 2010).

The antioxidant activity in protein hydrolysate of SFHPH was also determined using FRAP assay. SFHPH has an antioxidant capacity ranging between 0.396 and 0.706 M Tr/mg (Figure 1c). The antioxidant capacity of SFHPH was substantially lower than protein hydrolysate from tilapia skin using bromelain, which reported an antioxidant capacity of 2,840 mM Tr/mg (Choonpicharn *et al.* 2014). These results demonstrate that utilizing different enzymes could impact the hydrolysate product’s scavenging capacity. The amino acid sequences in the peptides may also impact this distinct antioxidant effect (Ovissipour *et al.*, 2012). Proteolytic enzymes break proteins into smaller fractions during hydrolysis (Belkaaloul *et al.*, 2010). Peptides with a molecular weight of less than 100 kDa and a composition of 3 to 10 amino acids are known to have beneficial effects on the human body (Prastari *et al.*, 2017). Pan *et al.* (2010) stated that peptides with small molecular weights could be easily absorbed by the body and bind to free radicals more easily than peptides with larger molecular weights.

Based on a statistical analysis of DH and antioxidant activities, 5% enzyme concentration was then used to optimize the hydrolysis time. The same evaluation parameters were conducted: yield, soluble protein, degree of hydrolysis (DH), and antioxidant activity (DPPH, ABTS, and FRAP).

3.3 Optimization of Hydrolysis Time

Table 3. Effect of hydrolysis time on yield, degree of hydrolysis, and soluble protein of Snakehead fish

Hydrolysis time	Yield (%)	Degree of hydrolysis (%)	Soluble protein (ppm)
0	4.86±0.7[a]	89±2.1[a]	415.2±61.4[a]
3	6.17±0.2[ab]	95.13±95.1[b]	454.9±115.9[a]
4	6.62±1.0[b]	95.38±95.3[b]	533.1±64.7[ab]
5	5.4±1.6[ab]	95.97±95.9[b]	488.3±49.2[ab]
6	5.59±0.8[ab]	93.34±93.3[ab]	672±51.3[c]
7	5.22±0.6[ab]	91.62±91.6[ab]	597.1±99.1[bc]

Results were obtained from three replicates ± standard deviation.
*Different letters within the same column in each property indicate significant difference at P<0.05.

3.3.1 Yield

In the stage 2, hydrolysis was conducted with 5% papain enzyme concentration with the highest yield of 6.62±1.0% (P<0.05) was achieved at 4 h hydrolysis time (Table 3). Based on the data presented in the table above, increasing hydrolysis time by more than four hours did not significantly increase yield.

3.4 Degree of Hydrolysis (DH)

The degree of hydrolysis (DH) of SFHPH were 89-95.97% at 0-6 hours of hydrolysis, respectively. The DH value was significant (P<0.05) until hydrolysis at 3 hours. After that, there was no statistically significant increase at 4-7 hours. Verma *et al.* (2017) showed a tendency similar to these results that there was no significant difference when increasing hydrolysis time for 6 hours. It probably occurred due to the rapid initial rate of enzymatic reactions, which slowed with increasing hydrolysis time and lowering the enzyme-to-substrate concentration ratio.

Klomklao and Benjakul (2016) investigated the degree of hydrolysis in protein hydrolysate from skipjack tuna viscera. They observed that the hydrolysis reaction rate was rapid for the first 20 minutes, then slowed and reached a stationary phase. According to Souissi *et al.* (2007), many peptide bonds were hydrolyzed in the initial hydrolysis phase and occurred very quickly. While the hydrolysis rate decreased, the enzymatic reaction reached a steady state. Interestingly, in this study, the DH is higher than that found by Prihanto *et al.* (2019) using Parrotfish heads at 30.65% and 49.74% using Anchovy sprats (Ovissipour *et al.*, 2012).

3.5 Soluble Protein

The protein solubility of SFHPH ranges from 415-672 ppm (Table 3). A significant difference in soluble protein among all the tested samples was observed when increasing hydrolysis time (P<0.05). It might explain hydrolysis's long duration and reflects the greater number of peptides and amino acids formed. Based on Haslaniza *et al.* (2010) that increasing enzyme concentration and hydrolysis time caused an increase in dissolved nitrogen content in fish protein hydrolysate.

3.6 DPPH (Radical Scavenging Activity)

The scavenging capacity of SFHPH increases linearly with the increase of hydrolysis time (Figure 2a). The investigation suggested that the antioxidant activity reached a maximum value of 53.50±1.2% with Trolox equivalent to 12.90 μM Tr/mg at 7 hours of hydrolysis. The statistical analysis showed a significant difference between 0, 3, 4, and 5 hours (P<0.05). However, there was no significant difference showed for 6 and 7 hours of hydrolysis time. These probably due to the presence of bioactive peptides that act as electron donors in SFHPH so that it could react with free radicals to convert them into a more stable final product. The antioxidant activity in these studies was slightly higher than snakehead fish skin which was investigated by Baehaki *et al.* (2020) and Sampath *et al.* (2011) using horse mackerel fish skin with an antioxidant capacity of 20.7% and 49.8-57.8%, respectively. However, the equivalent Trolox is smaller (94.96 μM Tr/mg) than Mirzaei *et al.* (2016). This fact could be due to the difference in peptide size and amino acid composition, Halim *et al.* (2016) stated that the amount of hydrophobic amino acids (valine, isoleucine, phenylalanine, and methionine) might increase during the hydrolysis process, where these components could contribute to the antioxidant activity of protein hydrolysates.

3.7 ABTS Radical Scavenging Activity

SFHPH exhibited ABTS scavenging activity ranging from 33.60-70.34%, with a Trolox equivalent of 2.67 to 10.52 M Tr/mg. ABTS scavenging activity increases rapidly from 0 to 5 hours of hydrolysis time and then remains constant until 7 hours (Figure 2b). According to Le Vo *et al.* (2016), the antioxidant activity of protein hydrolysate is dependent on the size and composition of free amino acids; the longer the hydrolysis time, the freer amino acids were obtained. The hydrophobic amino acids that form during hydrolyses, such as Proline, Leucine, Alanine, Tryptophane, and Phenylalanine can increase antioxidant activity, in addition to Tyrosine, Methionine, Histidine, and Lysine, which have antioxidants activity.

According to Ovissipour *et al.* (2012), all protein hydrolysates contain peptides that can bind to free radicals to donate hydrogen atoms, stabilizing the product and terminating the radical reaction. Alemán *et al.* (2011) also stated that one of the crucial roles in antioxidant properties is the presence of different amino acids in peptides. Hydrophobic amino acid peptides are also expected to inhibit lipid peroxidation and act as metal ions and proton donor chelators to peroxyl radicals.

3.8 FRAP (Ferric Reducing Antioxidant Power)

The FRAP antioxidant assay is frequently used to assess antioxidants' ability to transfer electrons (Yildirim *et al.*, 2000). A high absorbance value indicates a high reducing power capability. FRAP activity varied significantly (P<0.05) proportional to the

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varying reaction time. The ability of protein hydrolysate from SFHPH to reduce Fe^{3+} to Fe^{2+} ranged from 0.85-1.38 µM Tr/mg, respectively. Among all FPH samples, SFHPH with hydrolysis for 7 hours showed the highest FRAP activity (Figure 2c).

The FRAP activity of the hydrolysates in this study varied in comparison to hydrolysates from other raw materials. The protein hydrolysate obtained has a higher FRAP value compared to other sources, such as shark gelatin of 0.62 µM Tr/mg (Rodríguez-Díaz *et al.*, 2011), Pacific thread herring of 0.98 µM Tr/mg (Sandoval-Gallardo *et al.*, 2020), but lower than gelatine from *Cyprinus carpio* fish of 4.18 µM Tr/mg (Tkaczewska *et al.*, 2019). According to Barkia *et al.* (2010), this difference could be due to differences in DH values, the protein type and content in the raw material, and the specificity of the enzymes used during hydrolysis. Moreover, Wu *et al.* (2003) explained that the chain length and amino acid composition of the peptides formed during hydrolysis also significantly affect the antioxidant activity of protein hydrolysates

4. Conclusion

In the present study, fish protein hydrolysate from snakehead fish heads (SFHPH) has a high inhibitory capacity at 5% papain concentration and 6 hours of hydrolysis according to DPPH, ABTS, and FRAP assay with values of 50.70% (12.11 µM Tr/mg), 66.67% (9.99 µM Tr/mg), and 1.35 µM Tr/mg, respectively. However, further research is required to identify the antioxidant peptides and to determine their application in the food system.

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Authors' Contributions

The authors' contributions are as follow, MMPP; granted the research funding. MMPP, VA, and AH; formulated the research ideas. VA; performed the field laboratory works and analyzed the data. All authors contributed to the writing, reading, and approving of the final version of this manuscript.

Conflict of Interest

The author declares that there is no conflict of interest regarding commercial or financial.

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