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Antioxidant activity on protein hydrolysate peptide of mudskipper fish (*Periophthalmodon schlosseri*) using alcalase enzyme

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Abstract

Mudskipper fish is an endemic amphibian fish that has high potential to be developed into fish protein hydrolysate due to the high protein content (92% per dry weight). The aim of the research was to obtain new peptide compounds and hydrolyze the antioxidant activity of mudskipper fish (*Periophthalmodon schlosseri*). The design used in this study was a completely randomized design (CRD) with enzyme concentrations of 1.5 and 2% with three repetitions. The data obtained were analyzed using descriptive statistics and ANOVA. The results showed that mudskipper fish has the proximate content in the form of water content of 79.13% (wb), protein of 91.85% (db), fat of 1.50% (db), and ash of 4.54% (db), with glutamate amino acid being the highest (6.78%). The protein content of large molecular weight in fish protein hydrolysate obtained using an enzyme concentration of 1, 1.5, and 2% is relatively small, namely, 24.62, 4.85, and 9.47%, respectively. Several peptide compounds were identified in the hydrolysate using enzyme concentrations of 1, 1.5, and 2%, respectively: L-Arg-L-Pro, Phe-Pro, and L-Leu-L-Leu-L-Glu; L-Leu-L-Pro, L-Arg-L-Pro, and L-Leu-L-lys-L-Pro; and lys-Leu, L-Leu-L-Pro, Leu-Trp-Gln-Thr, L-Tyr-L-Gln-L-Val-L-Pro, L-Tyr-L-Gln-L-Leu-L-Pro, and L-Leu-L-Ser-L-Phe-L-Ala-L-α-Gln-L-Pro-Gly. Furthermore, the hydrolysate obtained effectively scavenged DPPH free radicals with IC_{50} values of 10.98 ± 0.57%v/v, 4.04 ± 0.79%v/v, and 8.69 ± 0.028%v/v, respectively.

Keywords: alcalase; antioxidant; DPPH; fish protein hydrolysate; mudskipper; *Periophthalmodon schlosseri*.

Practical Application: This research has many examples of practicel application, especially in discovery of new bioactive peptides type and deeper knowledge in structure and antioxidant activity of peptides.

1 INTRODUCTION

Mudskipper is an endemic amphibian fish, so it is not found in a unique habitat, namely, tidal areas with muddy beaches, especially mangroves. Mudskipper is relatively unused as a food ingredient because of its shape and habitat in muddy water. But in certain areas, it can be used as a food ingredient, is believed to be healthy, and can increase energy for the body. Even though Mudskipper is a fish that contains high protein (92% per dry weight) and has the potential to be used as a raw material for protein hydrolysate products (Edison et al., 2020), it can be developed into a food product that combines nutritional and health functions as a nutraceutical, including the ability to function as an antioxidant (Betty et al., 2014; Jeevitha et al., 2014; Wu et al., 2003). Fish protein hydrolysate is a protein that undergoes hydrolytic degradation with acids, bases, or enzymes, with the end result being a mixture of protein components, namely, amino acids and peptides (Ardiani & Rahmayanti, 2022). One of the most popular and high-performance methods of protein hydrolysis has been performed using commercial proteases such as alcalase (an alkaline serine endoprotease from *Bacillus licheniformis*) (Onsaard et al., 2022).

Protein hydrolysate, as an antioxidant, has been found in many marine protein sources. The antioxidant and ACE inhibitory activities of peptides and protein hydrolysates are influenced by factors like protein source, hydrolysis conditions, degree of hydrolysis, molecular mass, and amino acid composition, as well as the position of amino acids in the peptide sequences (Moayedi et al., 2017). The composition and main sequence of marine protein amino acids differ from one another, so marine protein can be an important protein source for a new source of protein hydrolysate antioxidants. Therefore, efforts to search for new sources of natural protein may not only lead to new types of bioactive peptides but also contribute to a better understanding of the structure and antioxidant activity of peptides.

Based on the benefits above, the process of making protein hydrolysate from mudskipper fish needs to be done so that further new peptide compounds can be obtained, as well as their potential and capacity as antioxidants.

2 MATERIALS AND METHODS

2.1 Ingredients and equipments

The main ingredients in this study were the Mudskipper (*Periophthalmodon schlosseri*) and the proteolytic enzyme alcalase. The mudskipper fish used, with a weight of 200–400 g/head, was obtained from the waters of Malacca Strait in Lima Laras

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Village, Tanjung Tiram District, Batubara Regency. Fish were immediately brought to the laboratory using an ice box to maintain freshness. After the thawing process, the head, bones, and entrails are removed, and the fish meat is used for the process of making fish protein hydrolysate. While the ingredients for protein hydrolysate preparation were alcalase as proteolytic enzyme 2.4 L (2.4 AU-A/g) obtained from Novozyme (Bagsvaerd, Denmark) and buffer phosphate 0.1 M. The chemicals used for analysis were K_2SO_4, $CuSO_4$, H_2SO_4, NaOH 40%, H_3BO_3, and HCl.

Furthermore, the chemicals used for testing the antioxidant power were 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Fenton's reagent. The equipment used in this research is equipment for the process of making protein hydrolysate and analysis equipment. The equipment used for the process includes beakers, refrigerators, analytical balances, blenders, water baths, and centrifuges. Equipment for peptide analysis includes high-performance liquid chromatography-mass spectrophotometry (HPLC-MS). While the equipment for the analysis of large molecular weight proteins and antioxidants is a mass spectrophotometer.

2.2 Methods

The study consisted of three stages, namely:

- preparation of raw material with three parameters: proportions, proximate, and amino acid;
- manufacture of the hydrolysate of the fish protein and identification of the peptide compounds in the hydrolysate of the fish with parameters of large molecular weight protein and short chain peptides;
- measurement of the antioxidant activity of the hydrolysate of the fish with antioxidant activity parameters.

The design used in this study was a completely randomized design (CRD) with 1% enzyme concentration, 1.5, and 2% with three repetitions. Data obtained were analyzed using descriptive (proportion, amino acid, proximate (water (evaporation); ash (gravimetry); protein (kjedahl); fat (soxhlet); short chain peptide) and ANOVA (large molecular weight protein (Bradford) and antioxidant activity (DPPH)).

2.2.1 Preparation of raw materials

The type of mudskipper fish studied in this study was *P. schlosseri*. Fish washed with ice water to clean the dirt attached. The meat of the body is separated from the skin, bones, and head. Fish meat that has been cleaned is tested for proportion, proximate, and amino acids. The tested proximate consisted of water, ash, protein, and fat.

2.2.2 Manufacture of fish protein hydrolysate and Identification of peptide hydrolysates

Protein hydrolysate was prepared using the Edison et al's method (2020). The fish meat was cut into small pieces and dried over a water bath at 50°C for 20 min to inactivate the endogenous enzymes present in the fish meat. Then it is cooled in an incubator and ground into flour. The dry material is then made into flour by grinding it in a mortar until it reaches a size of about 60 mesh. The preparation of fish protein hydrolysate was carried out by enzymatic hydrolysis with variations of four alcalase enzyme concentrations, starting from 0.5, 1.5, and 2.5% by weight of fish protein at hydrolysis conditions of pH 7.5; temperature of 50°C and an incubation time of 2 h. The hydrolysis of the fish protein was carried out by enzymatic hydrolysis using the alcalase enzyme. A total of 10 g of fish meat meal was mixed with 0.1 M phosphate buffer solution at pH 7.5 in a ratio of 1:2 (w/v), then homogenized. Protein hydrolysis was initiated by adding 1.5% alcalase to the weight of fish protein. The mixture was incubated for 2 h at 50°C using a water bath and temperature control. During the hydrolysis process, continuous stirring was carried out. Enzyme activity was stopped by increasing the stirring temperature to 85°C for 10 min. The hydrolysate formed was cooled to room temperature and then centrifuged at 10,000 rpm for 20 min. The supernatant was filtered using filter paper, and the degree of hydrolysis was determined using the Hoyle and Merritt (1994) method.

The content of peptide compounds present in protein hydrolysate was determined by a high-pressure liquid chromatography method equipped with a mass spectrum (HPLC-MS) of the Xevo-QTopMS type using a 1.7 μm BEH C18 column 2.1 ´ 100 mm long. The column was eluted with water and acetonitrile solution. The fractionation results obtained were identified by molecular weight using mass spectrometry.

2.2.3 Testing of antioxidant activity and reduction of DPPH radicals

Hydroxyl radicals are included in reactive oxygen species (ROS) in vivo. Testing with hydroxyl radicals can accurately reflect the intracellular ROS-scavenging activity of peptides. In vitro, hydroxyl radicals can be generated by the Fenton reaction catalyzed by iron. Hydroxyl radicals can oxidize Fe2+ to Fe3+, and only Fe2+ can react with 1,10-phenanthroline to form the red compound 1,10-Phenantroline-Fe2+, which has an absorption maximum at 536 nm. The hydroxyl radical scavenging activity (Hydroxyl Radical Scavenging Assay, HRSA) was determined based on a modified method described by Girgih et al. (2011). The protein hydrolysate sample of fish was diluted using the two-fold dilution method in a 96-well microplate with 0.1 M sodium phosphate buffer (pH 7.4) to obtain a concentration of 100–3.125% v/v each with a final volume of 50 μL. Separately, 3 mM 1,10-phenanthroline was dissolved in 0.1 M sodium phosphate buffer (pH 7.4). $FeSO_4$ (3 mM) and 0.01% hydrogen peroxide were both separately dissolved in distilled water.

Each protein hydrolysate solution (50 μL) or buffer (control) was added 50 μL 1,10-Phenanthroline, and 50 μL $FeSO_4$. Next, to start the Fenton reaction, 50 μL of hydrogen peroxide was added to the mixture, covered, and incubated at 37°C for 1 h with shaking. The absorbance was measured using a spectrophotometer at 536 nm at 10 min intervals for 1 h. The absorbance of the blank solution was also measured, which consisted of 50 μL buffer, 50 μL 1,10-Phenanthroline, and 50 μL $FeSO_4$ without the addition of hydrogen peroxide. Meanwhile, the DPPH free

radical scavenging method is based on the reduction of the colored DPPH free radical methanol solution by free radical inhibition. When the purple DPPH solution meets the electron donor material, the DPPH will be reduced, causing the purple color to fade, and be replaced by a yellow color originating from the picryl group (Wulansari, 2018).

Furthermore, all data were obtained from three replications and analyzed using the SPSS 17.0 software with the design method of analysis of variance (ANOVA) from a non-factorial, completely randomized design at $\alpha = 0.05$. If the treatment has a significant effect, then an LSD (least significant difference) test is carried out to see the differences between the treatment combinations.

3 RESULT AND DISCUSSION

3.1 Preparation of raw materials

3.1.1 Proportion

The proportions of the body parts of the mudskipper fish (*P. schlosseri*) include the meat, head, viscera, and skin and bones. The average proportions of these body parts are presented in Table 1.

The proportion of meat or parts that can be consumed ranges from 25.29 to 26.86% of the total body weight of the fish. The head ranged from 36.10 to 37.01%, the viscera ranged from 6.93 to 7.71%, and the skin and bones ranged from 29.33 to 30.77%. The proportion of the head is relatively larger than the other parts. On the contrary, the proportion of meat is relatively small compared to the head, skin, and bones. Thus, the ratio of the parts of the meat, head, viscera, and skin and bones is 3.6:5.1:1:4.2. Furthermore, the fish sticks used have proportions of body parts that are not significantly different.

3.1.2 Proximate analysis in mudskipper fish

This study uses protein hydrolysate of mudskipper fish; therefore, protein and other proximate compounds need to be analyzed first. Proximate analysis was performed using fish meat as the raw material for making protein hydrolysate. The results of the analysis of the proximate content of the mudskipper fish, including water, protein, fat, and ash content are presented in Table 2.

Furthermore, the total fat content of the meat was relatively low, ranging from 1.21 to 1.72%, while the ash content was relatively high, ranging from 4.37 to 4.71%. The high ash content is inseparable from this fish, which is a filter feeder, so it can be used as a bioindicator for environmental pollution (Ansari et al., 2014). The low fat content in the fish's meat is thought to be inseparable from its habit of crawling or jumping up and down in the mud.

Table 1. Proportion of mudskipper fish.

No.	Fish part	Weight (g)	Proportion	
			(%)	Ratio
1.	Meat	34.73	26.01 ± 0.79	3.6
2.	Head	48.87	36.62 ± 0.47	5.1
3.	Viscera	9.63	7.20 ± 0.44	1
4.	Skin and bones	40.24	30.16 ± 0.75	4.2
Total		**133.47**	**100**	

3.1.3 Amino acid composition in mudskipper fish

The composition of the amino acids analyzed in the mudskipper fish meat is presented in Table 3.

The ratio of essential and non-essential amino acids is an index to determine protein quality. The ratio of essential and non-essential amino acids in this mudskipper fish is 0.7. According to Corsetti et al. (2018), a ratio of essential and non-essential amino acids < 1 can cause malnutrition.

Amino acids are the main building blocks of protein and are generally classified as essential nutrients (essential amino acids), non-essential (non-essential amino acids), or conditionally essential (conditional amino acids) (Wu, 2013). Histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, and valine are essential amino acids; arginine, cysteine, glycine, proline, and tyrosine are conditionally essential amino acids; and alanine, aspartate, glutamate, and serine are non-essential amino

Table 2. Proximate content of mudskipper fish.

No	Parameter	Concentration (% db)
1.	Water	79.13 ± 0.36*)
2.	Protein	91.85 ± 1.51
3.	Fat	1.50 ± 0.26
4.	Ash	4.54 ± 0.17

*Wet basis.

Table 3. Amino acid composition in mudskipper fish protein hydrolysate.

No.	Amino acid	Percentage (%)
Essential amino acid		
1	Histidine	1.14
2	Isoleucine	1.03
3	Leucine	4.25
4	Leucine	2.80
5	Methionine	1.55
6	Phenylalanine	0.96
7	Threonine	1.32
8	Valine	0.86
Conditionally essential amino acids		
1	Arginine	0.10
2	Cysteine	1.10
3	Glycine	2.15
4	Proline	2.29
5	Tyrosine	1.03
Non-essential amino acids		
1	Alanine	0.92
2	Aspartate	4.98
3	Glutamate	6.78
4	Serine	1.05

acids but essential for human nutrition. However, the concept of functional amino acids has emerged recently.

Functional amino acids are amino acids that participate in and regulate key metabolic pathways to promote the health, survival, growth, development, lactation, and reproduction of organisms (Wu, 2013). Functional amino acids also play a role in the prevention and treatment of metabolic diseases (such as obesity, diabetes, and cardiovascular disorders), intrauterine growth restriction, infertility, intestinal and neurological dysfunction, and infectious diseases. Arginine, cysteine, leucine, methionine, tryptophan, tyrosine, aspartate, glutamate, glycine, proline, and taurine have been classified as functional amino acids in human nutrition (Wu, 2013).

3.2 Manufacture of fish protein hydrolysate and Identification of peptide hydrolysates

3.2.1 Large molecular weight protein

Partial hydrolysis of proteins using proteolytic enzymes will produce amino acids and peptides of various sizes. Peptides with a large size are peptides in the form of large molecular weight proteins. The results of the analysis of large molecular weight protein content are presented in Figure 1.

The protein content of large molecular weight in fish protein hydrolysate obtained using enzyme concentrations of 1, 1.5, and 2% is relatively small, namely, 24.62, 4.85, and 9.47%, respectively. Several researchers reported that the protein content in fish protein hydrolysate was relatively large, ranging from 60 to 90% (Chalamaiah et al., 2012). This difference is due to the use of different test methods. The Bradford method is used here to determine proteins with large molecular weights, whereas amino acids and peptides with small molecular weights do not produce a blue color with this method (Bradford, 1976). In contrast to the Kjeldahl method, organic compounds containing nitrogen are also analyzed and measured as protein nitrogen.

The protein content of large molecular weight in the use of 1.5% alcalase enzyme is relatively smaller compared to the others. This protein content value indicates the effectiveness of enzymes in hydrolyzing proteins. The lower the large molecular weight protein found in the protein hydrolysate, the more effective the enzyme is in breaking down the protein into free amino acids and short-chain peptides. This is in line with the degree of hydrolysis, where the use of 1.5% enzyme concentration provides a relatively large degree of hydrolysis (61.07).

The results of the one-way ANOVA analysis test obtained a probability value of 0.000, which is smaller than the 0.05 significance level used. It can be stated that there is an effect of enzyme concentration on the average protein content of large molecular weight in fish protein hydrolysate. Further test results showed that there was a difference in the use of 1% alcalase enzyme concentration with 1.5 and 2% alcalase enzyme concentrations on the average value of large molecular weight protein content. This is shown from the probability value (0.000) and is smaller than the 0.05 significance level used.

3.2.2 Short chain peptides

Enzymatic hydrolysis of proteins will produce hydrolysates containing short-chain peptides and free amino acids through the breaking of peptide chain bonds. The results of the LC-MS analysis obtained 28, 21, and 25 chromatogram peaks for the hydrolysate obtained using 1, 1.5, and 2% alcalase enzymes, respectively (Figures 2, 3, and 4).

The data from Figures 1–3 show not only the result of peptide compounds but also other chemical compounds from

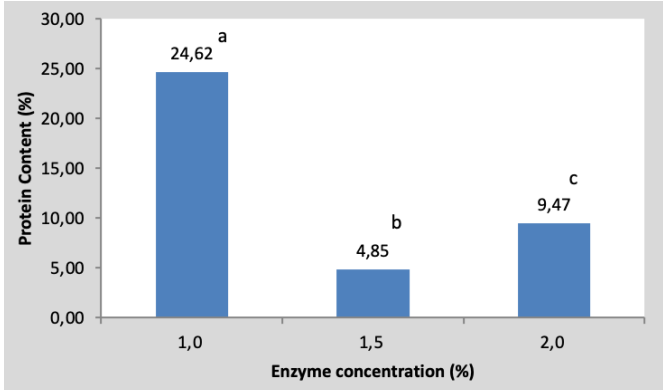

Figure 1. Percentage of large molecular weight protein content in mudskipper fish protein hydrolysate. Different lowercase letters in the figure mean significantly different according to LSD's test.

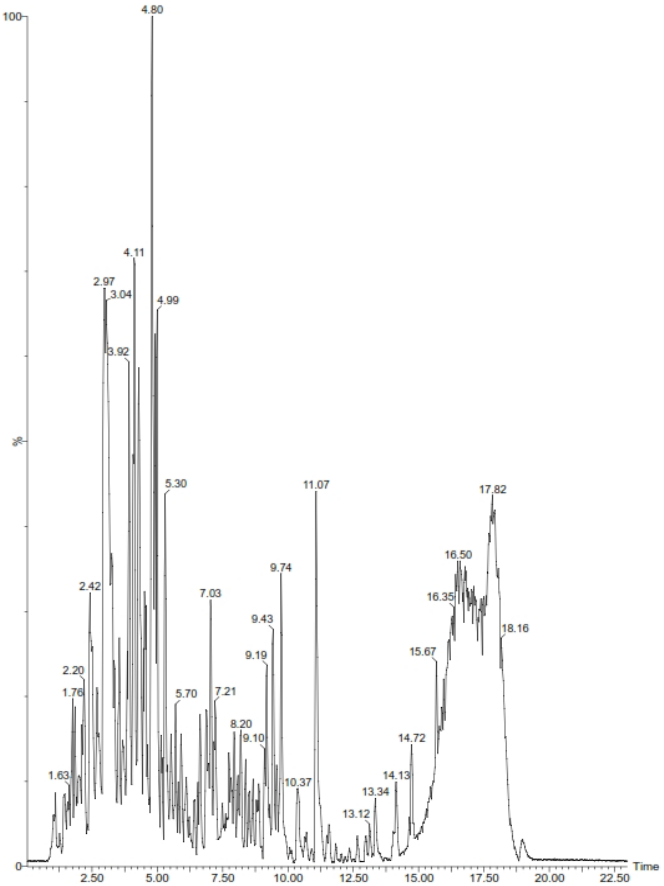

Figure 2. Mudskipper fish protein hydrolysate chromatogram using 1% alcalase.

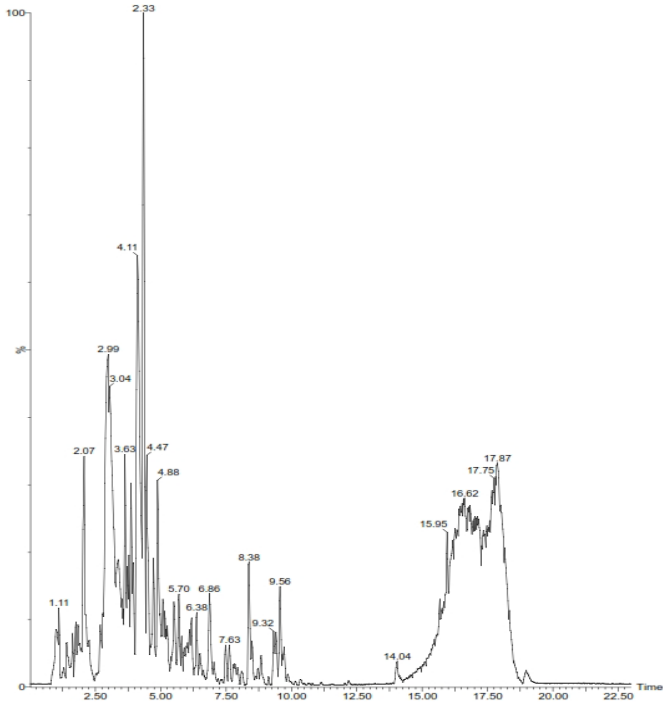

Figure 3. Mudskipper fish protein hydrolysate chromatogram using 1.5% alcalase.

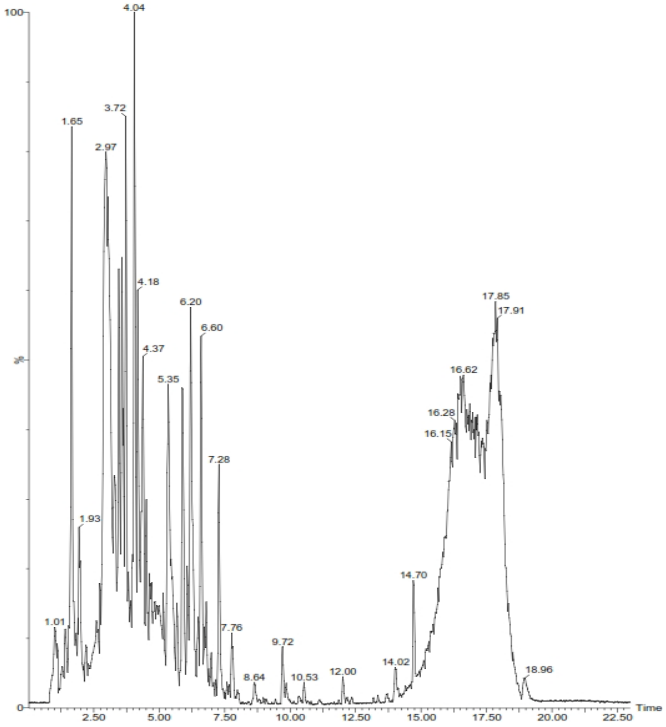

Figure 4. Mudskipper fish protein hydrolysate chromatogram using 2% alcalase.

mudskipper fish protein hydrolysate. While the results of identification using reference standards based on the mass spectral fragment pattern turned out to be only 3, 3, and 6 peptide compounds that could be identified from protein hydrolysates using alcalase enzyme concentrations of 1, 1.5, and 2%, respectively (Table 4).

The peptide compounds found in the use of 1% alcalase enzyme are L-Arg-L-Pro, Phe-Pro, and L-Leu-L-Leu-L-Glu. The peptide compounds found in the use of the 1.5% alcalase enzyme are L-Leu-L-Pro, L-Arg-L-Pro, and L-Leu-L-lys-L-Pro. The peptide compounds found in the use of the 2% alcalase enzyme are Lys-Leu, L-Leu-L-Pro, Leu-Trp-Gln-Thr, L-Tyr-L-Gln-L-Val-L-Pro, L-Tyr-L-Gln-L-Leu-L-Pro, and L-Leu-L-Ser-L-Phe-L-Ala-L-α-Gln-L-Pro-Gly. Here, it can be seen that the concentration of the enzyme provides diversity to the amino acid framework of the resulting peptide. The use of higher enzyme concentrations provides a more diverse peptide. Some research results show that the amino acid framework in peptides results from the breakdown of fish protein using commercial enzymes such as alcalase. Je *et al*. apud Arshad et al. (2019) obtained the peptide Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Glu-Glu-Leu-Ser on tuna backbone protein hydrolysate. Kim et al. (2007) apud Arshad et al. (2019) obtained the Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn peptides on hockey skeleton protein hydrolysates. Ren *et al*. apud Arshad et al. (2019) have obtained Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val peptides in Grass carp meat protein hydrolysate. In addition to using commercial enzymes, Je et al. apud Arshad et al. (2019) have also utilized crude mackerel digestion enzymes to obtain Leu-Pro-His-Ser-Gly-Tyr peptides in the skeletal protein hydrolysate of Alaska pollack. Bougatef et al. (2010) apud Arshad et al. (2019) have identified seven antioxidant peptides (Leu-Ala-Arg-Leu, Gly-Gly-Glu, Leu-His-Tyr, Gly-Ala-His, Gly-Ala-Trp-Ala, Pro-His-Tyr-Leu, and Gly-Ala-Leu-Ala-Aal-His) on protein hydrolysate obtained from sardine processing waste.

3.3 Antioxidant activity and DPPH radical scavenging

The commonly used free radical scavenging test by antioxidants is the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method because DPPH is a stable free radical that provides maximum absorbance at 520 nm. The antioxidant activity in this assay was shown by its ability to scavenge DPPH free radicals, thus forming colorless non-DPPH-H free radicals. The hydrolysate ability of mudskipper fish protein using 1, 1.5, and 2% alcalase enzymes to reduce DPPH free radicals was 80.94, 85.57, and 85.10%, respectively, as shown in Figure 5.

Based on Figure 5, it can be statistically stated that there is a difference in the use of alcalase enzyme concentrations of 1, 1.5, and 2% in their ability to scavenge DPPH free radicals ($p < 0.01$). In contrast, the use of alcalase enzyme concentrations of 1.5 and 2% did not show any difference. From these data, it can be seen that the use of 1.5% alcalase enzyme provides a relatively greater DPPH free radical scavenging value compared to the others. This shows that the peptides produced from protein breakdown are functional. According to Elias et al. (2008) and Ngo and Kim (2013), protein hydrolysate serves as the main source of antioxidant peptides and becomes more active after hydrolysis.

The results of testing the DPPH free radical scavenging by tobacco fish protein hydrolysate at various concentrations for each hydrolysate obtained are presented in Figure 6.

Table 4. Mudskipper fish protein hydrolysate peptide compound.

No.	HPI-Alcalase 1%		HPI-Alcalase 1,5%		HPI-Alcalase 2%	
	Retention time	Peptide	Retention time	Peptide	Retention time	Peptide
1.	2,202	Cyclo-(L-Arg-L-Prol)	1,633	L-Leu-L-Lys-L-Pro	1,654	Lys-Leu
2.	5,148	L-Leu-L-Leu-L-Glu	2,069	Cyclo-(L-Arg-L-Pro)	4,374	L-Leu-L-Ser-L-Phe-L-Ala-L-α-Gln-L-Prol-Gly
3.	6,111	Cyclo(Phe-Pro)	5,697	Cyclo(L-Leu-L-Pro)	5,345	Leu-Trp-Gln-Thr
4.	-	-	-	-	5,676	Cyclo(L-Leu-L-Pro)
5.	-	-	-	-	6,596	L-Tyr-L-Gln-L-Val-L-Pro
6.	-	-	-	-	7,279	L-Tyr-L-Gln-L-Leu-L-Pro

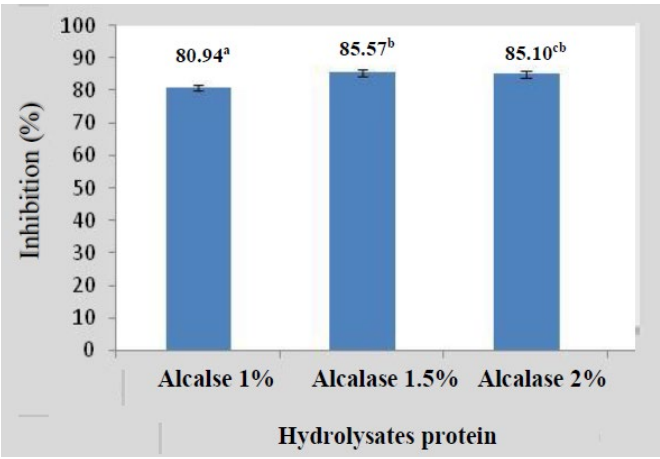

Figure 5. Percentage of antioxidant activity of fish protein hydrolysate on DPPH free radical scavenging. Different lowercase letters in the figure mean significantly different according to LSD's test.

Based on Figure 6, it can be seen that the free radical scavenging curve is linear, so the higher the concentration of hydrolysate used, the higher the DPPH-free radical scavenging ability. Furthermore, the results of testing the absorption of protein hydrolysate antioxidants against DPPH free radicals at various concentrations mentioned above can be determined by the IC_{50} value (the effective concentration of fish protein hydrolysate needed to reduce 50% of the total DPPH). The IC_{50} value of the hydrolyzed fish protein using alcalase 1, 1.5, and 2% to reduce DPPH free radicals is as shown in Figure 7, which is 10.98 ± 0.57%v/v, 4.04 ± 0.79%v/v, and 8.69 ± 0.28%v/v, respectively.

Based on Figure 7, the protein hydrolysate of mudskipper fish has a very strong antioxidant activity with an IC_{50} value of < 50. Statistically, there was a difference in the use of alcalase enzyme concentrations of 1 and 1.5% on the ability to scavenge 50% of the total DPPH radicals ($p < 0.01$). The same thing was also seen in the use of alcalase enzymes at 1.5 and 2%. Furthermore, it was also seen that the DPPH free radical scavenging value increased significantly with increasing enzyme concentration. When the enzyme concentration increased from 1 to 1.5%, the DPPH-free radical scavenging activity increased or the IC_{50} value decreased from 10.98 to 4.04%v/v, therefore an increase in antioxidant strength occurred. Conversely, when the enzyme concentration increased even higher to 2%, the DPPH-free radical scavenging activity was relatively constant, and conversely, the IC_{50} value increased to 8.69v/v, therefore there was a decrease in its antioxidant power. Therefore, it can be stated that the use of alcalase at 1.5% has a relatively smaller IC_{50} value, so it has a very strong ability to scavenge DPPH-free radicals at a hydrolysate concentration of 4.04%v/v.

The increase in DPPH free radical scavenging was inseparable from the presence of peptide compounds in the protein hydrolysate obtained (Table 1). According to Davies (2005), the oxidative susceptibility of amino acid residues to free radical attack is largely determined by the side chains of their functional groups, such as amino acids and proteins whose side chains are aliphatic (Glu, Leu, Gly, Val, Lys, Pro, Arg, and Ile), containing sulfur (Met and Cys), and aromatic (Phe, Tyr, Try, and His).

Furthermore, according to Li et al. (2008) and Pownall et al. (2010), the hydrophobic character of amino acid and peptide residues is correlated with the magnitude of DPPH. Besides that, the magnitude of DPPH free radical scavenging can also be determined by the reaction rate constant of electrons in DPPH with amino acids. The electron reaction rate constants for the amino acids val, gly, ala, leu, thr, pro, glu, lys, ser, Arg, phe, tyr, and try are $5x10^6$, $8.8x10^6$, $9x10^6$, $1x10^7$, $1x10^7$, $2x10^7$, $2x10^7$, $2x10^7$, $3x10^7$, $5x10^7$, $1.4x10^8$, $3.4x10^8$, and $2.9x10^9$ $dm^3mol^{-1}s^{-1}$ (Buxton et al., 1988). While the protein hydrolysate obtained with a concentration of 2% alcalase enzyme contains a variety of peptide compounds (lys-Leu, L-Leu-L-Pro; Leu-Trp-Gln-Thr, L-Tyr-L-Gln-L-Val-L-Pro, L-Tyr-L-Gln-L-Leu-L-Pro, and L-Leu-L-Ser-L-Phe-L-Ala-L-α-Gln-L-Pro-Gly) when compared to alcalase enzyme concentration of 1.5% (L-Leu-L-Pro, L-Arg-L-Pro, and L-Leu-L-lys-L-Pro) and 1% (L-Arg-L-Pro, Phe-Pro, and L-Leu-L-Leu-L-Glu).

The antioxidative capacity of protein hydrolysates is known to be influenced by structural properties such as amino acid composition and peptide size or molecular weight (Ketnawa et al., 2018). The size of the peptide will determine the scavenging power of the DPPH radicals. The larger the peptide size, the less DPPH radical scavenging activity; high molecular weight peptides exhibited better radical scavenging activity (DPPH, ORAC) and reducing property (FRAP) than low molecular weight peptides (Olagunju et al., 2018). Furthermore, the peptide compounds obtained with an alcalase enzyme concentration of 1% contained the aromatic amino acid Phe, while those obtained with an enzyme concentration of 2% contained the aromatic amino acids Tyr, Phe, and Try. Statistically, the

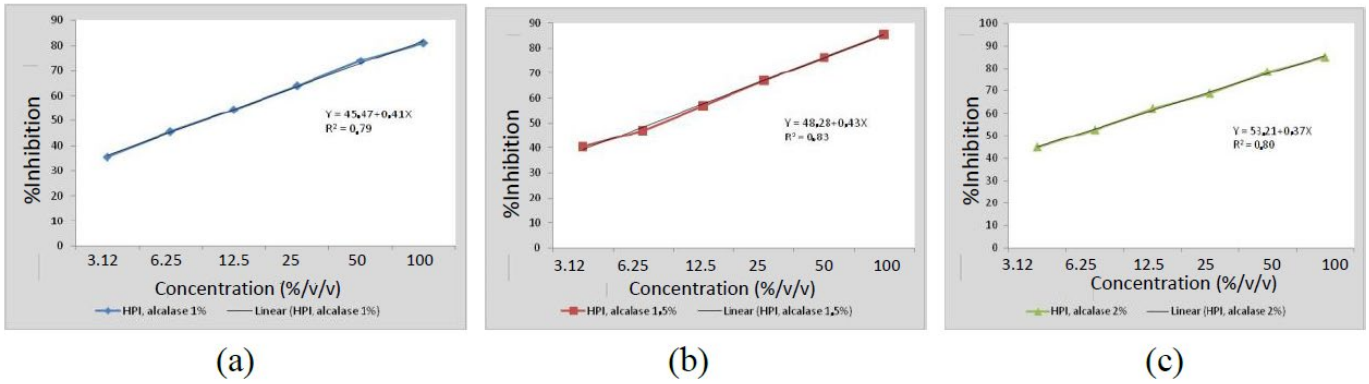

Figure 6. Correlation curve of DPPH-free radical scavenging activity to the concentration of tuna fish protein hydrolysate with various enzyme concentrations of (A) 1%, (B) 1.5%, (C) 2%.

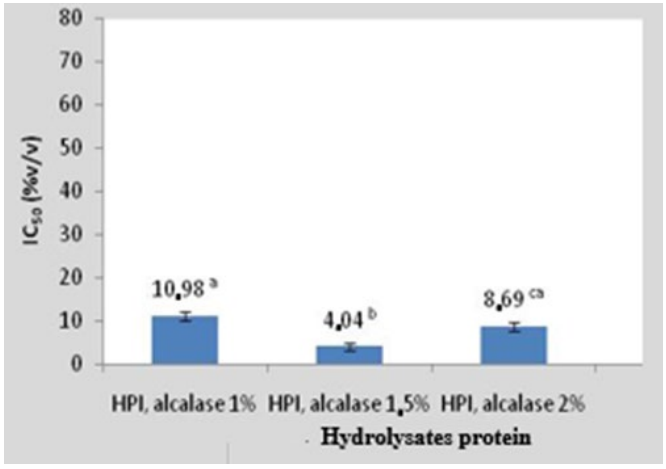

Figure 7. Activity (IC_{50}) of DPPH hydrolysate free radical scavenging of mudskipper fish. Different lowercase letters in the figure mean significantly different according to LSD's test.

damping value is not different, but the use of 2% alcalase concentration has a more optimum damping value. In general, aromatic amino acids are considered to be effective radical scavengers because they can easily donate protons to electron-deficient radicals. At the same time, the stability of the antioxidant can be maintained through the resonance structure (Ren et al., 2008). Besides that, from the description of the data, it can also be seen that the IC_{50} value of Mudskipper protein concentrate is relatively weak when compared to fish protein hydrolysate, where the IC_{50} value is >50, which is 68.26% v/v. The low IC_{50} value is inseparable from the low functional properties of the Mudskipper protein concentrate. This is because, as explained above, proteins in an intact state absorb DPPH-free radicals relatively slowly. Protein and protein hydrolysate obtained from fish have been shown to exhibit antioxidant activity in a variety of ways (Samaranayaka & Li-Chan, 2011).

From the foregoing, it can be stated that alcalase enzymes with different concentrations produce peptides with increased DPPH radical scavenging potential when compared to protein concentrates, and an alcalase enzyme concentration of 1.5% has better DPPH radical scavenging potential than the others.

4 CONCLUSION

- The proportions of the mudskipper fish meat, head, viscera, and skin and bones are 25.29–26.86, 36.10–37.01, 6.93–7.71, and 29.33–30.77%, respectively;
- The proximate content of mudskipper fish is 79.13% (wb) water, 91.85% (db) protein, 1.50% (db) fat, and 4.54% (db) ash;
- Mudskipper fish contains the highest essential amino acid (leucine, 4.25%), a conditional amino acid (proline, 2.29%), and a non-essential amino acid (glutamate, 6.78%);
- The protein content of large molecular weight in fish protein hydrolysate obtained using an enzyme concentration of 1, 1.5, and 2% are relatively small, namely, 24.62, 4.85, and 9.47%, respectively;
- The peptide compound identified in the hydrolysate produced using an alcalase concentration of 1% is L-Arg-L-Pro, Phe-Pro, and L-Leu-L-Leu-L-Glu; for the use of 1.5% alcalase enzyme is L-Leu-L-Pro, L-Arg-L-Pro, and L-Leu-L-lys-L-Pro; and for the use of 2% alcalase enzyme is Lys-Leu, L-Leu-L-Pro, Leu-Trp-Gln-Thr, L-Tyr-L-Gln-L-Val-L-Pro, L-Tyr-L-Gln-L-Leu-L-Pro, and L-Leu-L-Ser-L-Phe-L-Ala-L-α-Gln-L-Pro-Gly;
- The protein hydrolysate obtained can reduce DPPH free radicals. Increasing the alcalase enzyme concentration will increase the scavenging power of DPPH free radicals. The optimum damping power was obtained from protein hydrolysate with a concentration of 1.5% alcalase enzyme and an IC_{50} value of 4.04 ± 0.79%v/v.

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