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Characteristics of Fish Protein Hydrolysate from Mackerel (*Scomber Japonicus*) By-Products

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Abstract: Fishery processing industry produces a high number of by-products. Fortunately, some fish by-products can be reused and converted into valuable products, such as fish protein hydrolysates. Fish protein hydrolysate (FPH) is known to have a high functional value. This study aims to valorize the mackerel (*Scomber japonicus*) heads into FPH with biofunctional properties under different pH and hydrolysis duration. Hydrolysis was carried out at pH 0, 5, 7, and 9 for 12 and 24 hour. Response surface methodology was performed to determine the optimum production condition. The results showed that pH and hydrolysis duration had significant effects ($p < 0.05$) on yield, antioxidant activity, protein content, and degree of hydrolysis of mackerel FPH. The highest yield (59.89 ± 0.08%), with a protein content of 67.95 ± 0.49%, was obtained at pH 5 after 12 hour incubation. In addition, the mackerel fish protein hydrolysate had high antioxidant activity (36.95 ± 0.82%) with a degree of hydrolysis of 32.63 ± 1.12%. It was suggested that mackerel heads could be a potential resource to produce bio-functional fish protein hydrolysate. Further research on other properties of mackerel FPH and the use of different enzymes to produce better quality mackerel head FPH is recommended.

Keywords: mackerel, fish protein hydrolysate, antioxidant, fish by-products.

鯖魚（鯖魚）副產品魚蛋白水解物的特性

摘要：漁業加工業產生大量副產品。幸運的是，一些魚類副產品可以重複使用並轉化為有價值的產品，例如魚蛋白水解物。已知魚蛋白水解物具有很高的功能價值。本研究旨在將鯖魚 (鯖魚) 魚頭轉化為在不同酸鹼度值和水解持續時間下具有生物功能特性的已知魚蛋白水解物。在酸鹼度 0、5、7 和 9 下水解 12 和 24 小時。採用響應面法確定最佳生產條件。結果表明，酸碱度和水解持續時間對鯖魚已知魚蛋白水解物的產量、抗氧化活性、蛋白質含量和水解程度有顯著影響（p <0.05）。孵育 12 小時後，在酸鹼度 5 下獲得最高產率 (59.89 ± 0.08%)，蛋白質含量為 67.95 ± 0.49%。此外，鯖魚魚蛋白水解物具有高抗氧化活性（36.95 ±0.82%），水解度為 32.63±1.12%。有人提出，鯖魚頭可能是生產生物功能魚蛋白水解物的潛在資源。建議進一步研究鯖魚已知魚蛋白水解物的其他特性以及使用不同的酶來生產質量更好的鯖魚頭已知魚蛋白水解物已知魚蛋白水解物。

关键词：鯖魚、魚蛋白水解物、抗氧化劑, 鱼副产品。

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1. Introduction

Many countries worldwide make the fishery and aquaculture industries their primary economic source. Fisheries and aquaculture have also become important sources of food and nutrition for hundreds of million people worldwide [1]. Based on the current data, global food fish consumption has increased significantly over the past five decades, higher than all animal protein foods [2]. Nevertheless, the growth in the fish processing industry has resulted in an increased volume of fish by-products. Fish by-products in large quantities can be a serious environmental problem-related pollution in both developed and developing countries [3]. Fish by-products account for up to 70% of the total weight of fish processed, usually consisting of heads, skins, scales, fins, offal, and roes. Fish by-products contain about 10-20% of the total fish protein [4]. Generally, all by-products are processed into animal feed products, fish meal, and fertilizers with low market value [5]. Considering that the utilization of fish by-products is not optimal, various technologies have been developed to restore the chemical components of the by-products in the form of proteins and peptides through the hydrolysis process. The hydrolysis process converts fish protein into smaller peptides, usually containing 2-20 amino acids. Protein hydrolysate is the product of the enzymatic breakdown of proteins into smaller peptides. Protein hydrolysate is one of the available sources of amino acids for various physiological functions of the human body.

Many studies demonstrated that fish protein hydrolysate (FPH) has essential functional and physicochemical applications [6]. Henriques [7] reported protein hydrolysates with high antioxidant capacity of Atlantic horse mackerel heads. Protein hydrolysates from fish heads have also been prepared from Lophius vomerinus [8] and tilapia [9]. Mackerel by-products have been hydrolyzed using commercial enzymes. However, reports on protein hydrolysate production by utilizing endogenous enzyme is still limited, so further research is necessary.

Enzymatic hydrolysis using endogenous or commercial enzymes is generally carried out under the controlled condition of pH, temperature, and duration in order to control the degree of hydrolysis (DH) as well as allows retaining the nutritional value or functional properties of fish protein hydrolysate (FPH) [10]. Furthermore, the DH is one of the essential parameters for the production and characterization of FPH since it determines the percentage of broken peptide bonds with the original protein [4]. This study evaluates the effect of different pH and hydrolysis duration to determine the optimal hydrolysis conditions in producing high-quality mackerel head FPH using endogenous protease.

2. Materials and Methods

2.1. Materials

Heads of mackerel (*Scomber japonicus*) were obtained from PT. Mina Laut as by-products of fillet processing. The samples were kept on ice after collecting and transporting them to the laboratory. Methanol, 2.2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, trichloroacetic acid (TCA), H_2SO_4; NaOH, HCL, petroleum ether; tris base; acrylamide; N'N'-bis-methylene-acrylamide; glycine; SDS; APS; TEMED; and glacial acetic acid commasie brilliant blue G-250 were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). All other chemicals and reagents were of analytical grade.

2.2. Preparation of Fish Protein Hydrolysate (FPH)

The mackerel fish heads were washed and minced without water using a food processor (Phillips model HR7672, 650). A preliminary study was conducted to determine the best ratio of sample and distilled (w/v) for the production of FPH. At this stage, 20g of minced fish was mixed with different volumes of distilled water (w/v) to obtain fish: water ratio of 1:0 (control); 1:1; 1:2, and 1:3. The mixtures were then incubated using a shaker incubator (Biobase model BJPX-100B, Shandong China) at 150 rpm and a temperature of 30±2^{o}C for 18 hours. Afterward, the samples were centrifuged at 3000 rpm for 30 minutes. Upon centrifugation, five layers were formed in the tube, i.e.., oil layer, light lipoprotein, liquid/soluble protein, fine particles, and coarse particles at the bottom of the cuvette (Fig. 1). The ratio with the highest yield of soluble protein layer (1:2 w/v) was then used for the subsequent investigation.

The main study was carried out to determine the characteristics of mackerel head FPH and investigated the optimum pH and duration of hydrolysis for mackerel head FPH production. pH levels used were 5, 7, 9 and 6.4 (control/no pH adjustment). The hydrolysis process was conducted for 12 and 24 hours following the same procedure as the preliminary study. Soluble protein layers formed were collected and dried using a spray dryer. Dried FPH was used for further analysis and characterization.

2.3. Yield

The FPH yield (%) was determined by weighing the initial weight of the sample and final weight of FPH and calculated using the following formula:

Yield (%) = *Final weight of FPH* (g) × 100 / *Weight before incubation* (g) (1)

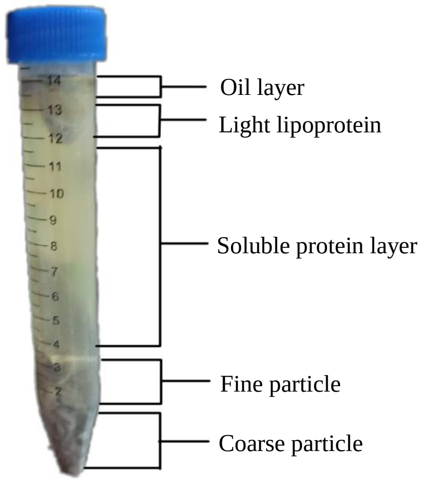

Fig. 1 FPH fractions of mackerel fish head by-products after centrifugation

2.4. Proximate Composition

Proximate analysis was conducted to determine the total N value, mineral content, and dissolved protein in protein hydrolysate samples [11]. Protein levels were determined using the Kjeldahl method by multiplying total nitrogen content by the conversion factor of 6.25 (method: 968.06), while fat content was determined gravimetrically using the Soxhlet method (method: 963.15). The moisture content was analyzed using the drying method in an oven at a temperature of 105°C for 2 hours (method: 925.10). The ash content was estimated by incinerating pre-dried samples in a muffler furnace overnight at 550°C (method: 923.03). All measurements were taken in triplicate.

2.5. Antioxidant Assay (DPPH Radical Scavenging Activity)

The antioxidant assay was done following the previously reported method [12]. Briefly, 100 µL of soluble protein extract (protein hydrolysate) was added to 3900 µL of 0.075 mM DPPH in 95% methanol. The mixture was then left in the dark for 30 minutes. After incubation, the absorbance of the resulting solution was recorded at a wavelength of 517 nm using a UV spectrophotometer. Lower absorbance values indicated higher DPPH scavenging activity. The free radical scavenging effect was expressed as shown in the following equation:

DPPH = (*blank absorbance* + *sample absorbance*) / (*blank absorbance*) (2)

2.6. Degree of Hydrolysis (DH)

The degree of hydrolysis of FPH was determined according to the published method [12]. An aliquot of 2 mL of soluble FPH was mixed with 2 mL of 20% (w/v) TCA. The sample mixture was then allowed to stand for 30 minutes for precipitation to occur, then centrifuged (5000 rpm, for 30 minutes). Next, the supernatant was analyzed for nitrogen content using the Kjeldahl method. The degree of hydrolysis was calculated using the following formula:

DH = (10% *TCA soluble nitrogen in the sample*) × 100% / (*Total nitrogen in the sample*) (3)

2.7. Molecular Weight Analysis (SDS–PAGE)

The SDS-PAGE analysis was carried out based on the Laemli [13] method. 15 µL FPH was mixed with a 15 µL sample buffer and heated at 100^{0}C for 15 minutes. A 12% gel-separating concentrate and a 4% gel stacking were used, and the running process was started at a constant current of 20 mA 100 V for three hours. Meanwhile, staining was carried out by soaking the gel in a staining solution that contained one gram of Coomassie Brilliant Blue G-250 (Merck, Catalogue No. 1154440025), 450 mL of methanol, 100 mL of glacial acetic acid, and 450 mL of distilled water. The molecular weight calculations were analyzed by comparing the bands appearing on the samples with the standard markers.

2.8. Amino Acid Composition

Analysis of amino acid composition was carried out following the method on Boogers et al. [14], using the ultra-performance liquid chromatography (UPLC) method. 0.50 mL FPH was put into a 100 ml volumetric flask, and 2.0 mL of 10mM AABA's internal standard was then added. The solution was filtered using a filter membrane of 0.22 µm, and 10 µL of the solution was taken and placed in a vial. The aliquot was added to 70 µL AccQ-fluor borate and vortexed. Similarly, approximately 20 µL of the reagent fluor A was added and vortexed. The solution stood for one minute and was further incubated for 10 minutes at a temperature of 550°C, and the sample was later injected into the UPLC system.

2.9. Statistical Analysis

Statistical analysis was performed using Minitab software version 18 (Minitab Pty Ltd., Sydney, NSW, Australia). Data were analyzed with one-way ANOVA, followed by a post-hoc test. The optimum treatment was determined using the Response Surface Methodology (RSM) presented in the Overlaid Contour Plot using the Minitab version 18 application.

3. Results and Discussion

3.1. Yield and Proximate Composition of FPH

The recovery yield is an important parameter for the practical feasibility of fish protein hydrolysate. The preliminary study results showed that the addition of different volumes of distilled water significantly affected ($p<0.05$) the yield of soluble protein (data not shown). The yield of soluble protein ranged from 36.62±0.79 to 65.13±0.19%. The lowest yield was obtained from the 1:0 ratio (36.62±0.79), while the highest yield was observed at the 1:2 ratio (65.13±0.19%). Hence, the 1:2 (sample: distilled water, w/v) ratio was employed for the main study phase.

The results showed that hydrolysis duration and pH had a significant effect on the yield of FPH ($p<0.05$), which ranged from 35.98 ± 0.13% to 59.89 ± 0.08% (Table 2). The highest yield (59.89 ± 0.08%) was obtained at pH 5 after 12-hour hydrolysis, while the lowest was obtained at pH 7 after 24 hours of hydrolysis. According to Hultin & Kelleher [15], the pH-shift mode has been known to be feasible for fish protein recovery. Several factors determined the yield of FPH, including variation in fish species, part of fish used, type of enzymes, and the hydrolysis condition applied [16]. Since there was no heating treatment before hydrolysis, endogenous enzymes in the fish substrate might contribute during the hydrolysis process and increase the soluble protein. There are two main endogenous proteases involved in post-mortem deterioration and hydrolysis, namely cathepsins and calpains [17]. Ahmed et al. (2013) [18] mentioned that cathepsin prefer acidic environment while caplains are optimally active at neutral pH

The proximate composition of mackerel FPH is presented in Table 1. The highest protein content (67.95 ± 0.49%) of the mackerel fish head by-product FPH sample was obtained at pH 5 with a hydrolysis duration of 12 hours, while obtained the lowest value in the control treatment with a hydrolysis duration of 24 hours of 49.67 ± 1.16%. The results showed that the pH treatment, hydrolysis duration, and the interaction of the two were significantly affected the FPH mackerel protein content ($P<0.05$). In previous studies, it has been reported that the protein content in fish FPH ranges from 60% to 90% of the total composition [28]. High protein content in FPH is due to protein dissolution during the hydrolysis process and the removal of the insoluble solid phase during centrifugation. Thus, the high protein content in fish FPH shows its potential as a protein supplement consumed by humans [6].

Table 1 Yield, proximate composition, DH, antioxidant activity, and mackerel FPH

Parameter (%)	5		7		9		Control	
	12	24	12	24	12	24	12	24
Yield	59.89 ± 0.08^{a}	51.65 ± 0.71^{b}	49.77 ± 0.22^{c}	35.98 ± 0.13^{f}	50.34 ± 0.29^{bc}	39.39 ± 0.59^{e}	46.66 ± 1.14^{d}	36.58 ± 0.27^{f}
DH	32.63 ± 1.12^{a}	25.89 ± 0.72d	29.70 ± 0.35	22.86 ± 0.05	30.61 ± 0.46	26.97 ± 0.17	29.09 ± 0.68	20.41 ± 0.79
Antioxidant	36.95 ± 0.82^{a}	29.45 ± 1.19^{c}	26.04 ± 1.79^{d}	20.19 ± 0.58^{e}	33.13 ± 0.85^{b}	22.84 ± 1.01^{de}	23.52 ± 0.86^{d}	15.64 ± 1.67^{f}
Protein	67.95 ± 0.49^{a}	55.83 ± 0.96^{cd}	58.64 ± 0.91^{bc}	51.7 ± 0.87	60.79 ± 0.62	53.28 ± 0.65	57.47 ± 0.87	49.67 ± 1.16
Fat	0.60 ± 0.14^{a}	0.70 ± 0.28^{a}	0.90 ± 0.14^{a}	0.85 ± 0.21^{a}	1.00 ± 0.00^{a}	0.80 ± 0.14^{a}	0.75 ± 0.21^{a}	0.95 ± 0.07a
Ash	6.50 ± 0.71^{a}	6.50 ± 0.71^{a}	5.50 ± 0.71^{a}	5.40 ± 0.57^{a}	6.56 ± 0.79^{a}	6.53 ± 0.78^{a}	6.00 ± 0.00^{a}	5.51 ± 0.71^{a}
Moisture content	7.00 ± 0.70^{ab}	7.25 ± 0.35ab	7.75 ± 0.35^{ab}	8.62 ± 0.17^{ab}	8.00 ± 0.00^{b}	9.25 ± 1.06^{a}	8.00 ± 0.70^{b}	8.25 ± 0.35^{ab}

Note: Values with different superscripts (a–f) within the same parameter indicate a significant difference ($P < 0.05$)

The highest % fat content (1.00 ± 0.00%) of FPH was obtained with hydrolysis at pH 9 for 12 hours, and the lowest value was found at pH 5 with a hydrolysis time of 12 hours of 0.60 ± 0.14. %. Table 1 showed that the hydrolysis duration and pH did not significantly affect the FPH fat content ($P>0.05$). The FPH fat content was usually below 5% [19]. The low-fat content of FPH fish is due to the lipid removal process in the insoluble protein fraction during the centrifugation process. Abraha et al. [20] stated that the low-fat content of FPH fish is due to fat being very sensitive to auto-oxidation.

The results showed that the moisture content of mackerel FPH ranged from 7.00±0.70% to 9.25±1. 06%. The highest moisture content was obtained from hydrolysis at pH 9 for 24 hours, while the lowest was obtained from hydrolysis at pH 5 for 12 h. The pH and hydrolysis duration were significantly affected ($P<0.05$) the moisture content of mackerel head FPH. Most studies show that FPH contains moisture content below 10% [21, 22]. The low moisture content is related to higher temperatures during the evaporation or drying process – during the process, the sample loses most of its moisture [31].

The lowest ash content of mackerel FPH was obtained at pH 7 with a hydrolysis time of 24 hours of 5.40±0.57%, while the highest yield was at pH 9 with a hydrolysis time of 12 hours of 6.56±0, 79%. The ash content of FPH has been reported in many studies ranging from 0.45% to 27% of the total composition [19]. The relatively high ash content of FPH is due to the addition of acids or bases used to adjust the pH.

3.2. Degree of Hydrolysis (DH)

The results showed that the highest degree of hydrolysis of mackerel fish head by-product FPH samples was obtained at pH 5 with a hydrolysis duration of 12 hours (32.63 ± 1.12%), while the lowest value was obtained in control with hydrolysis duration of 24 hours of 20.41 ± 0.79%. Duration of hydrolysis treatment and pH were significantly affected the DH ($P<0.05$).

The increase in degree hydrolysis can be caused by the rise in peptides and amino acids dissolved in TCA due to the breaking of peptide bonds during the hydrolysis process. The longer the hydrolysis time, the higher degree hydrolysis can increase until it reaches a stationary stage [10]. Thus, degree hydrolysis is a critical parameter in the success of the hydrolysis reaction; the higher degree hydrolysis, the more influential the hydrolysis process in breaking peptide bonds [23].

3.3. Antioxidant Activity of FPH

The results showed that the highest % value of inhibition of the FPH sample of mackerel fish head by-product was obtained at pH 5 with hydrolysis duration of 12 hours of 36.95 ± 0.82%, while the lowest value was obtained in the control treatment with hydrolysis duration of 24 hours of 15.64 ± 1.67%. The hydrolysis duration and the interaction pH gave significantly different results ($P<0.05$). This result indicates that there is a relationship between antioxidant activity and treatment. The increase in pH and hydrolysis duration is in line with the rise in peptides and free amino acids. Higher antioxidant activity was reported from parrotfish head FPH (58.20%) [24]. The amino acids composition may affect the antioxidant activity of FPH.

3.4. Molecular Weight Analysis (SDS–PAGE)

The results of the FPH molecular weight of mackerel fish head by-product are presented in Fig. 2. The measurement results show the lowest value obtained at pH 5 treatment with a hydrolysis time of 12 hours (A1B1) (10.85 kDa to 90.24 kDa) with the highest antioxidant activity results (36.95 ± 0.82%).

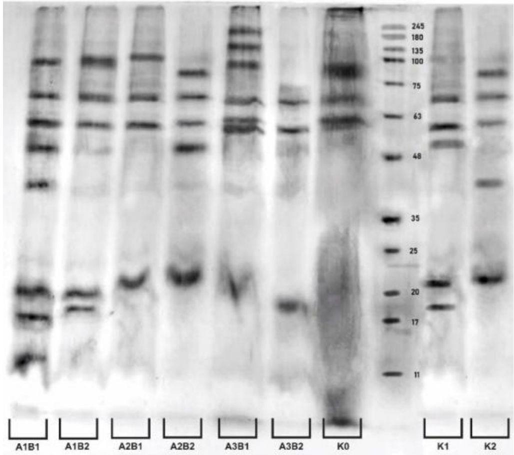

Fig. 2 Results of SDS-Page FPH analysis of mackerel fish head by-product

Table 2 Molecular weight of mackerel FPH

No.	Treatment	Molecular Weight
1	A1B1	90.24 kDa, 73,89 kDa, 58,97 kDa, 54,23 kDa, 43,83 kDa, 18,66 kDa, 16,66 kDa, and 10,85 kDa
2	A1B2	119,83 kDa, 72,24 kDa, 60,11 kDa, 18,12 kDa, and 17,15 KDa
3	A2B1	123,76 kDa, 72,01 kDa, 60,70 kDa, and 19,89 kDa
4	A2B2	89,66 kDa, 72,71 kDa, 59,92 kDa, 50,35 kDa and 21,02 kDa
5	A3B1	181,05 kDa, 137,21 kDa, 87,38 kDa, 69,28 kDa, 60,70 kDa, 58,21 kDa, and 18,90 kDa
6	A3B2	67,51 kDa, 58,40 kDa, and 16,45 kDa
7	K0	88,22 kDa, 74,37 kDa, and 61,48 kDa
8	K1	71,55 kDa, 61,50 kDa, 50,35 kDa, 21,35 kDa, and 18,47 kDa
9	K2	91,11 kDa, 70,18 kDa, 91,11 kDa, 44,98 kDa, and 22,70 kDa

Notes: A1B1 - Treatment at pH 5 with hydrolysis duration of 12 hours
A1B2 - Treatment at pH 5 with hydrolysis duration of 24 hours
A2B1 - Treatment at pH 7 with hydrolysis time of 12 hours
A2B2 - Treatment at pH 7 with hydrolysis duration of 24 hours
A3B1 - Treatment at pH 9 with hydrolysis time of 12 hours
A3B2 - Treatment at pH 9 with hydrolysis duration of 24 hours
K0 - Control treatment without adding pH and hydrolysis time
K1 - Control treatment with hydrolysis duration of 12 hours
K2 - Control treatment with 24-hour hydrolysis

The molecular weight of the FPH of mackerel head by-product is higher than that of tilapia by-product (14.4 to 116 kDa) [25]. The low molecular weight of protein hydrolysate indicates that the hydrolysis process that occurs is working effectively. This process causes the breakdown of peptide bonds (myosin, actin, troponin, and tropomyosin) by endogenous proteases and enzymes.

3.5. Optimum Conditions of Mackerel Head FPH

The optimum condition of FPH production of mackerel fish head by-product was analyzed using the RSM method based on the yield value, proximate, DH, antioxidant activity, and molecular weight. The results of Overlaid Contour Plot analysis of mackerel FPH are presented in Fig. 3. The optimum hydrolysis condition was shown as a white area. Prediction of the response surface optimization value indicates that the optimal treatment for making FPH of mackerel fish head by-product is at pH 5 with a hydrolysis time of 12 hours due to the highest desirability value (0.7238).

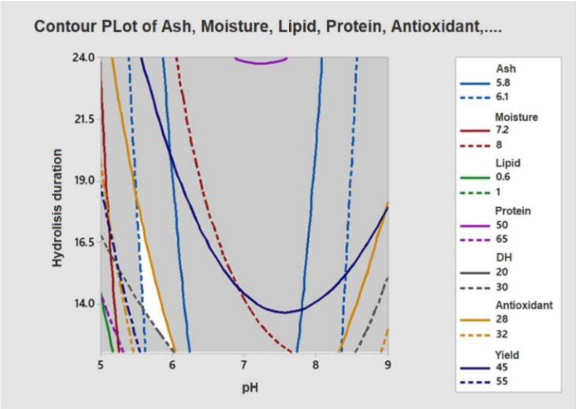

Fig. 3 Overlaid contour plot of FPH of mackerel fish head by-product

3.6. FPH Amino Acid Composition of Mackerel Fish Head By-Product

The amino acid analysis results in the FPH of mackerel fish head by-product are presented in Table 3. FPH of mackerel fish head by-product has the highest serine content at 21.16%, while the lowest was arginine at 3.01%. The highest amino acid from *Lophius vomerinus* head by-products was glutamic acid 15.42%, while histidine's lowest yield was at 1.95% [8].

Table 3 Amino acids composition of FPH from mackerel heads

Amino acids	FPH from mackerel head (%) (This study)	FPH from parrotfish head (%) [24]	FPH from yellowfin tuna (%) [27]	FPH from turbot by-products (%) [28]
Serine	21.16	1.81	5.22	5.67
Glutamic	6.17	14.43	12.69	12.86
Phenylalanine	3.88	5.53	5.36	4.16
Isoleucine	3.18	4.34	8.35	1.97
Valine	4.30	5.38	3.04	2.96
Alanine	6.74	7.41	9.14	8.38
Arginine	3.01	6. 12	8.54	6.53
Glycine	13.27	7.63	13.75	14.50
Lysine	4.28	8.3	0.49	5.57
Aspartic	6.82	11.06	10.54	8.81
Leucine	4.93	8.84	2.35	5.37
Proline	4.62	5.64	-	7.67
Tyrosine	4.93	4.22	2.78	2.90
Threonine	5.69	6.80	1.24	3.64
Histidine	7.99	2.85	1.45	1.61
Total Asam Amino Essential	37.26	41.69	30.82	31.81
Total Asam Amino Non Essential	63.71	41.00	54. 12	60.79

The amino acids Arginine, Alanine, Valine, Leucine, and Lysine, are reported to appear frequently in the amino acid sequence of antimicrobial peptides. Meanwhile, the type of protein with high cationic amino acid content has antibacterial activity against pathogenic bacteria [26]. The presence of Valine, Tyrosine, Leucine, Phenylalanine, Lysine, Histidine, and Arginine has been shown to make a significant contribution to the potential of antioxidant peptides. In addition to Moreover, the hydrophobic nature of the peptide can enhance its interaction with target lipids through the desired hydrophobic composition to achieve an antioxidant effect.

4. Conclusion

The conversion of mackerel by-products into fish protein hydrolysate may increase its economic value. This study showed that pH and hydrolysis duration significantly affected the FPH characteristics. The best mackerel FPH was obtained at pH 5 after 12h hydrolysis. The presence of essential amino acids and the high antioxidant activity of mackerel FPH demonstrated that FPH could be a potential ingredient in functional food. It was suggested to explore various hydrolysis conditions to obtain a better quality of FPH. Further investigation on the properties of mackerel FPH and its utilization for functional foods and health is necessary.

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