Properties of high-quality hydrolysate prepared from mudskipper (*Periophthalmus freycinetii*)

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Abstract. Mudskipper (*Periophthalmus freycinetii*), an amphibious fish, lives in tidal areas and is known for its ability to climb, walk and skip about out of water. It contains high protein and taurine and exhibit unique adaptation to extreme environment. Despite its potential, mudskipper is still underutilized. The aim of this research was to examine the biochemical properties and antioxidant activity of mudskipper hydrolysate. The mudskipper was hydrolyzed using endogenous enzymes under different pH (control, 5, 7, 9) and hydrolysis duration (12 and 24 hours). Hydrolysates were characterized with respect to ash, moisture, fat and protein content as well as the yield and amino acid composition. Antioxidant activity of mudskipper hydrolysate was tested using DPPH. pH 9 and 24h hydrolysis duration gave the highest yields (80.48%), degree of hydrolysis (DH) (37.22%) and protein content (52.63%). High antioxidant activity (56.12%) was also observed. The molecular weight of hydrolysate ranged from 12.55 – 117.73kDa. The hydrolysate contained 35.31% essential amino acid and 63.63% non-essential amino acids which may relate to its functional and bioactive properties. The results suggested that underutilized fish has potency as source of high-quality hydrolysate.

1. Introduction

Mudskipper fish (*Periophthalmus frycineti*) is a unique fish and has a special character as a river dweller. The behavior of mudskipper closely related to the tidal rhythm. Mudskipper has protruding eyes so that it can pass through mud and climb over trees [1]. Mudskipper has high protein and taurine content. The content of taurine in mudskipper fish reached 2.732mg/ 100g [2]. Mudskipper is a fish that has a low economic value, most people only process it as dried fish, smoked fish or used it as traditional medicine [3]. Therefore, it is necessary to process mudskipper to be more useful product, such as a protein hydrolysate which represents promising ingredients for food and industrial applications.

Fish protein hydrolysate (FPH) is a product obtained from the breakdown of proteins into simpler peptides. Basically, hydrolyzed protein is a peptide (2-20 amino acids) produced through hydrolysis by adding enzymes, acids, or bases [4]. FPH is generally used as a flavor enhancer and emulsifying agent [5]. In addition, recent studies mentioned that protein hydrolyzate have bioactive properties. Baehaki [6], reported that catfish protein hydrolysate, hydrolyzed for 6 hours using enzyme papain, had inhibitory activity against DPPH radical of 67.62%.

Based on the statement above, it is known that the mudskipper has a low economic value with a high protein content [7], so it can be utilized as a FPH. Nevertheless, production of FPH from mudskipper is still limited. This study aimed to determine the characteristics of FPH from mudskipper fish (*Periophthalmus freycinetii*) generated using different pH and hydrolysis duration.
2. Material and method

Mudskipper (Periophthalmus frycineti) were obtained from Jepara Kartini beach swamps. Distilled water, NaOH 6N, HCl 6N, and other chemicals were of analytical grade.

2.1. Preparation of Fish Protein Hydrolysate (FPH)

Fish Protein Hydrolysate was produced from minced mudskipper fish (Periophthalmus frycineti) using modified method of Prihanto and Nurdiani [8] and Nurdiani [9] with modification. Whole mudskipper fish (Periophthalmus frycineti) was analyzed for proximate composition of the raw material. The minced fish was mixed with dH2O with the ratio of 1:3 (w/v). The pH of the mixture was adjusted to 5, 7, 9 and the hydrolysis was conducted for 12 and 24 h. Sample without pH adjustment served as control (pH 6.4). Hydrolysis was conducted using orbital shaker at 150 rpm at room temperature. Afterward, the hydrolysates were centrifuged at 3000 rpm for 30 min. Upon centrifugation, all samples were segregated into 5 distinct layers (Figure 1.), which were consequently separated and distributed into separate containers for further analysis. Five layers formed after centrifugation were an oil layer, a light lipoprotein layer, a liquid (soluble) protein layer, a fine insoluble layer and a coarse insoluble layer at the bottom. All layers obtained after centrifugation were separated and liquid protein layer was analyzed for yield, % DPPH inhibition, proximate composition, degree of hydrolysis, SDS PAGE and amino acid composition. All experiments were repeated at least in triplicate and the mean values were reported.

![Figure 1. Layers created after centrifugation](image)

2.2. FPH yield

The yield of protein hydrolysate products is the percentage of the weight of hydrolysate produced against the weight of raw materials used before hydrolysis. The calculation of yield used the following formula:

\[
\% \text{ yield} = \frac{A}{B} \times 100\%
\]

note:

A = final weight of hydrolysate (after centrifuge) (g)
B = initial weight of the sample after mixing (before incubation) (g)

2.3. Antioxidant activity (DPPH radical scavenging activity)

Antioxidant activity of FPH was examined according to the Donkor [10] with slight modification. As much as 100 μL of liquid protein was added to 3900 μL 0.075 mM DPPH in 95% methanol. The mixture was then kept in dark for 1 h. Measured the absorbance value of the mixture of the solution at a wavelength of 517 nm using a UV / Visible Spectrophotometer spectrophotometer. Antioxidant activity was calculated using the following equation:
% antioxidant activity = \left[ \frac{\text{blank absorbance} - \text{sample absorbance}}{\text{blank absorbance}} \right] \times 100\% \quad (2)

2.4. Proximate analysis
Protein, fat, water content, and ash analysis used the method according to AOAC [11]. Protein was analyzed based on Kjeldahl method. The fat content was analyzed using Soxhlet method. Ash was determined by heating the samples in a furnace at 550°C for 8–12 h.

2.5. Degree of Hydrolysis (DH)
The method from Hoyle and Merritt [12] with slight modification was employed for DH analysis. Liquid FPH (2 ml) was added with TCA 20% (v/v). Prior centrifugation (5,000 rpm, 30 min), an aliquot was left for 30 min. The supernatant was decanted and analyzed for nitrogen content by Kjeldahl [11]. DH was calculated using the formula:

\text{Degree of Hydrolysis (DH)} = \frac{\text{TCA-soluble nitrogen}}{\text{Total Nitrogen in sample}} \times 100\% \quad (3)

2.6. Molecular weight analysis (SDS PAGE)
FPH molecular weight was determined by the SDS-PAGE method based on the Laemmli method [13]. SDS-PAGE analysis employed by 12% separating gel and 4% stacking gel. Mixed sample and loading buffers as much as 30 μL was run using 20 mA and 100 V for three hours. The gel was then stained with staining solution (Brilliant Blue R-250 1 gr, methanol 450 ml, glacial acetic acid 100 ml, and distilled water 450 ml). The stained gel was further destained using the same solution without CBB R-250.

2.7. Free Amino acid analysis
FPH-Free amino acid profiles were determined according the method of Boogers [14] with slight modification. Ultra-High Performance Liquid Chromatography (UPLC), an Acquity system (Waters), was applied for free amino acid analysis. Sample, 0.50 mL pipetted into a 100 ml volumetric flask and added 2.0 mL of AABA 10mM internal standard solution. Dilute the solution to the limit mark with 0.1N HCl then homogenized. Furthermore, the solution was filtered using a 0.22 μm membrane filter. Ten μl of the solution was added to 70 μl of AccQ-Fluor Borate. Afterward, fluorine reagent A was added as much as 20 μl and then vortexed again and let stand for one minute. One μl sample solution was injected into the UPLC system (ACCQ-Tag Ultra C18, fluid rate system of 0.7 mL per minute, the column temperature was maintained at 55°C, Photodiode array detector, with a wavelength of 260 nm).

2.8. Statistical analysis
All data and RSM optimization were analyzed using Minitab 18 Statistical software (Minitab Pty Ltd. Australia)

3. Results and discussions
3.1. Proximate composition of mudskipper
The proximate composition of minced mudskipper (Periophthalmus frycineti) sample from Jepara Kartini beach swamps is listed in Table 1. The protein content of minced mudskipper (Periophthalmus frycineti) at 32.51%, which is higher than freshwater fish species Tilapia guineensis (18.65%) and T. melanotheron (18.74%) [15]. Its fat content (0.46%), however, was lower than T. Guineensis (0.55%) [15] and T. Melanotheron (0.70%) [15]. While, the water content (62.27%) slightly lower than T. Guineensis (79.5%) [15] and T. Melanotheron (79.5%) [15]. The ash content (3.12%) was higher than T. Guineensis (1.30%) [15] and T. Melanotheron (1.06%) [15].
Table 1. Proximate composition of minced mudskipper (Periophthalmus frycineti) sample

| Composition | Unit (%) |
|-------------|----------|
| Moisture    | 62.27    |
| Protein     | 32.51    |
| Fat         | 0.46     |
| Ash         | 3.12     |

3.2. Yield

Results showed that different pH, duration of hydrolysis, and its interaction significantly affected the yield (P<0.05) (Figure 2). The results showed that the higher pH and duration of hydrolysis given would increase the amount of yield produced.

![Figure 2. Yield of mudskipper FPH](image)

The yield of FPH were ranged from 59.09±0.21% to 80.84±0.82%. The highest yield (80.84±0.82%) was obtained at pH 9 after 24 h of hydrolysis. The lowest yield (59.09±0.21%) was obtained at pH 5 and 12 h of hydrolysis. The results were not much different compared to the yield of catfish protein hydrolysate with the addition of commercial enzymes by 50% to 60% (wet basis) and 7.03 ± 0.83% to 9.85 ± 0.25% (dry basis) [16]. The yield value can describe the economic value of a material. The higher the yield value can increase its economic value because the higher the amount that can be utilized from the material [17].

3.3. Antioxidant activity

Results showed that different pH, duration of hydrolysis, and its interaction significantly affected the antioxidant activity (P<0.05) (Figure 3).
The antioxidant activity of FPH ranged from 24.81±0.94% to 56.12±1.35%. The highest antioxidant activity (56.12±1.35%), was obtained after 24 h hydrolysis at pH 9. FPH showed the lowest antioxidant activity (24.81±0.94%) at pH 5 and 12 h. Peptide size, solubility, amino acid composition, and the amount of free amino acids determine the DPPH radical scavenging capacity [18].

3.4. Proximate composition of FPH powder

Table 2. Proximate composition of mudskipper (Periophthalmus frcineti) protein hydrolysates powder.

| Parameter | Control | 12 | 24 | 5 | 24 | 7 | 24 | 12 | 24 |
|-----------|---------|----|----|---|----|---|----|----|----|
| Protein   | 23.01±0.22a | 28.29±0.33a | 34.51±0.58a | 46.96±0.89a | 37.41±0.71a | 47.04±0.78a | 41.51±0.85a | 52.6±0.45a |
| Fat       | 0.45±0.07a  | 0.40±0.00a  | 0.35±0.07a  | 0.27±0.04a  | 0.45±0.07a  | 0.35±0.07a  | 0.35±0.07a  | 0.23±0.04a  |
| Ash       | 6.10±0.16a  | 6.20±0.17a  | 4.75±0.35a  | 7.00±1.41a  | 5.60±0.72a  | 6.40±1.61a  | 6.18±1.34a  | 6.53±0.78a  |
| Water     | 8.10±0.70a  | 8.20±0.31a  | 7.35±0.21a  | 8.25±1.06a  | 8.00±0.70a  | 8.50±0.70a  | 8.25±0.35a  | 7.00±0.70a  |

Based on the Table 2, pH and hydrolysis time significantly affected (P <0.05) the protein content of mudskipper FPH, but not the fat, ash and water contents. The highest protein content (52.63±0.45%) was obtained at pH 9, with 24 hours of hydrolysis time. Hydrolysis duration and pH conditions can influence the conversion of peptides into simple protein [19]. The fat content of mudskipper FPH ranged from 0.23±0.04% to 0.45±0.07%. The highest fat content was obtained at pH 7 and control treatment (pH 6.4) with 12 hours hydrolysis time while the lowest fat content was obtained at pH 9 with hydrolysis time 24 hours. The low-fat content is caused by the fat content in the raw material (0.47%). FPH with low-fat content is generally more stable during storage than high-fat protein hydrolysate [20].

The ash content of the FPH of mudskipper ranged from 4.75±0.35% to 6.53±0.78%. The highest ash content was obtained at pH 9 treatment with hydrolysis time of 24 hours, while the lowest was observed in the pH 5 with a hydrolysis time of 12 hours (4.75±0.35%). The addition of alkaline or acid compounds in the process of protein hydrolysis aims to achieve the optimum pH value of the enzyme. Then this can also maintain constant condition during the hydrolysis process [21]. The water content of the mudskipper FPH ranged from 7.35±0.21% to 8.50±0.70%. The highest water content was obtained at pH 7 with hydrolysis time of 24 hours (8.50±0.70%), while the lowest water content was obtained at pH 5 with a 12 hour hydrolysis time (7.35±0.21%). Drying step can cause changes in
water content contained in the material. Water will evaporate when it comes into contact with heat, so the water content contained in food will decrease [22].

3.5. Degree of hydrolysis (%DH)

![Figure 4. Degree of hydrolysis of mudskipper FPH](image)

Essential properties of FPH rely on the DH of the process. Degree of hydrolysis describes the extent of peptide bonds cleaved in a substrate. High DH can be used as an indicator of effective hydrolysis [23]. Both pH and duration of hydrolysis significantly affected (P<0.05) DH (Figure 4). The result of DH analysis ranged from 22.24±0.57% to 37.22±0.61%. The highest DH was observed at pH 9 after 24 h hydrolysis (22.24±0.57% to 22.24±0.57%), while the lowest DH was obtained at pH 5 after 12 h hydrolysis (22.24±0.57%). An increase in DH was caused by an increase in peptides and amino acids dissolved in TCA due to termination of peptide bonds during hydrolysis. According to Hasnaliza et al. (2010). The increase in DH value is caused by the increasing peptide and amino acids dissolved in TCA as a result of breaking the peptide bonds during hydrolysis [24].

3.6. SDS PAGE

SDS-PAGE is the most widely used method for analyzing proteins quantitatively and separating proteins according to their size [25]. SDS PAGE analysis was carried out to observe the molecular weight range of FPH obtained at optimum condition in pH 9 after 24h hydrolysis (12.55 to 16.49kDa). The results showed that molecular weight of FPH ranged from 12.45kDa to 123.85kDa (Figure. 5). The protein breaks down into smaller protein fractions during the process of protein hydrolysis by proteolytic enzymes. If a higher number of protein bands is obtained it is due to the absence of peptide bonds [26].

![Figure 5. SDS-PAGE pattern of mudskipper FPH](image)
3.7. The optimum hydrolysis condition
Response Surface Methodology (RSM) is usually used for optimization of food processing [27]. The optimum condition for parrotfish FPH production (shown as white area in the graph) was analyzed using RSM based on the yield, antioxidant activity, protein, fat, water, ash, and DH of FPH. The Overlaid Contour Plot as a result of RSM analysis is shown in Figure 6.

![Overlaid Contour Plot of mudskipper FPH](image)

**Figure 6.** Overlaid Contour Plot of mudskipper FPH

Based on Figure 6, it was suggested that pH 8-9 and 21.5-24h of hydrolysis duration were considered as the optimum condition for producing the FPH. Because the more extended time gave a better character in FPH, pH 9 and 24 h time of hydrolysis was the optimum process for generating the best characters of FPH from the mudskipper.

3.8. Amino acids composition
The amino acid composition of FPH from mudskipper FPH at pH 9 for 24-hour hydrolysis was compared to FPH from salmon fish [28] (Table 3.). Mudskipper FPH consists of essential amino acids (histidine, threonine, valine, isoleucine, leucine, phenylalanine, and lysine) and non-essential amino acids (aspartic acid, glutamic acid, serine, arginine, glycine, alanine, tyrosine, and proline). FPH enzymatic breakdown reactions can convert fish protein into smaller peptides which usually contain 2-20 amino acids [4].

| No. | Amino acids   | Mudskipper FPH (%) | Salmon FPH (%) [28] |
|-----|---------------|--------------------|---------------------|
| 1.  | L-Serine      | 6.86               | 13                  |
| 2.  | L-Glutamic acid | 12.68              | 51                  |
| 3.  | L-Phenylalanine | 6.17               | 0                   |
| 4.  | L-Isoleucine  | 3.22               | 11                  |
| 5.  | L-Valine      | 4.39               | 14                  |
| 6.  | L-Alanine     | 6.99               | 6.8                 |
| 7.  | L-Arginine    | 7.92               | 7.5                 |
| 8.  | Glycine       | 9.53               | 4.2                 |
| 9.  | L-Lysine      | 5.60               | 18                  |
| 10. | L-Aspartic Acid | 9.34               | 17                  |
11. L-Leucine 7.44 21
12. L-Tyrosine 4.99 9.8
13. L-Proline 5.2 24
14. L-Threonine 6.79 9.4
15. L-Histidine 2.71 6.4

| Essential AA | 36.31 | 79.8 |
| Non-essential AA | 63.63 | 13.3 |

The total essential amino acid of mudskipper FPH (36.31%) was lower compared to FPH from salmon (79.8%) [28]. Analysis of amino acids can determine the quality of FPH that has been made, namely from the ratio of amino acids contained in these proteins [23]. According to Peter [29], the amino acids in each fish species vary depending on internal and external factors. Internal factors includes fish species, sex, age, and reproduction phase of fish. External factors are factors that exist in the environment.

4. Conclusions
Mudskipper is a potential material for FPH production. The difference pH and hydrolysis time provided significant effect on the yield, antioxidant activity, protein and DH of mudskipper FPH. The optimum condition for hydrolysis: pH 9 after 24 h hydrolysis duration. High antioxidant activity was observed (56.12±1.35% at pH 9 after 24 h hydrolysis). Further detailed studies on physical properties as well as isolation and purification of useful compounds from mudskipper FPH are needed.

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