Antioxidant activity of catfish (*Pangasius* sp.) protein hydrolyzed by alcalase enzyme

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Abstract. Protein hydrolysis using alcalase enzyme yields a high degree of hydrolysis, generating bioactive peptides with potential applications as nutraceuticals and functional food ingredients. Striped catfish (*Pangasius* sp.), characterized by its high protein and moderate fat content, represents a promising source of antioxidant peptides. This study aimed to evaluate the antioxidant activity of striped catfish protein hydrolysates produced by alcalase hydrolysis and subsequently fractionated using ultrafiltration. Hydrolysis was conducted with alcalase at concentrations of 1%, 2%, and 3%, followed by ultrafiltration through a 3 kDa MWCO membrane. Antioxidant activity was assessed using DPPH and ABTS assays. The highest degree of hydrolysis (43.37%) was observed at 3% enzyme concentration. The <3 kDa fraction exhibited strong antioxidant activity with IC50 values of 21.97 ppm (DPPH) and 3.28 ppm (ABTS). These findings demonstrate that ultrafiltration effectively produces smaller peptides with enhanced antioxidant activity.

1 Introduction

Protein hydrolysates are small peptides produced from the breakdown of protein polypeptides. Fish protein hydrolysates are the result of protein degradation into smaller molecules through hydrolysis by enzymes, acids, or bases. Bioactive peptides contained in protein hydrolysates function in the human body as nutraceuticals and functional foods. The advantages of producing fish protein hydrolysates include antioxidants and antibacterial activities. They also contain a complete profile of amino acids, have a protein digestibility of 87.03%, and possess good functional properties with solubility ranging from 99.84% to 99.99%, making them suitable for use as functional food ingredients. Bioactive peptides are protein fragments and amino acid derivatives with various health benefits. They act as antioxidants, anti-inflammatory, anti-obesity, anticancer, antihypertensive, antidiabetic, antimicrobial, and antithrombotic agents [1]. Antioxidants are highly beneficial in health and food sectors. In health, they prevent cancer, diabetes, cardiovascular disease, andpremature aging. In the food industry, antioxidants prevent lipid oxidation and inhibit bacterial growth in food products. The mechanisms of antioxidant activity can be categorized into five types: (1) free radical scavenging, such as 2,2′-azino-bis(3- ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), (2) reducing power such as Ferric Reducing Antioxidant Power (FRAP), (3) lipid peroxidation inhibition, (4) metal ion chelation, and (5) synergistic effects [2].

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Enzymatic hydrolysis is considered the most efficient method for producing protein hydrolysates. Enzymes promote hydrolytic changes and degradation of proteins while generating peptides that are easily degraded. A study on catfish protein hydrolysates using papain enzyme reported inhibition percentages ranging from 37.85% to 67.62% [3]. In this research, the enzyme used is alcalase. Alcalase is widely applied to obtain bioactive peptides with activities such as antioxidant, antihypertensive, and anti-inflammatory effects. The advantages of alcalase include high catalytic activity, specificity, water solubility, and eco-friendliness. Alcalase has been reported to achieve a high degree of hydrolysis, up to 78% [4].

The main source of bioactive peptides is protein, and catfish (*Pangasius* sp.) is a fish species with high protein content. Catfish contain 16.1% protein and 5.7% fat [5]. It has high demand in both domestic and international markets due to its wide availability, relatively low cost, and widespread distribution. However, its utilization is still limited. Research on fish protein hydrolysates has been widely conducted using different enzymes and fish species, yielding various bioactive peptides with potential functions, particularly antioxidants. Shorter peptides tend to exhibit higher antioxidant activity. Therefore, this study aims to determine the optimal concentration of alcalase for hydrolyzing catfish protein and to evaluate the effect of ultrafiltration on the antioxidant activity of catfish protein hydrolysates in order to enhance their bioactive potential.

2 Materials and methods

2.1 Equipment and materials

The equipment used in this study included a knife, ruler, cutting board, blender, porcelain crucible, volumetric pipette, funnel, beaker glass, micropipette, microtube, vortex, test tube (Pyrex), measuring cylinder (Pyrex), Whatman No. 41 filter paper, ultrafiltration membrane (Amicon® Ultra-15 centrifugal filters 3K MWCO, Millipore, Ireland), , digital balance (OHAUS AX224, New Jersey, USA), pH meter (Hanna Instruments, Rhode Island, USA), UV-Vis spectrophotometer (RS Spectrophotometer UV-2500), centrifuge (FC5178R 120 V), electrophoresis apparatus (PEQ Lab, Erlangen, Germany), and water bath (B-ONE, China). The main materials used in this study were fresh catfish (Pangasius sp.) fillet obtained from Dramaga Market, Bogor, and Alcalase 2.4 L enzyme from *Bacillus licheniformis* (Sigma-Aldrich, St. Louis, USA) (activity 2.4 Anson Unit/mL), as well as Phosphate Buffer Saline (PBS) pH 8. Other materials used for analysis included distilled water, trichloroacetic acid (TCA) (Merck, Darmstadt, Germany), Bradford reagent, Tris-HCl 60 mM pH 6.8, glycerol, β-mercaptoethanol, Bovine Serum Albumin (BSA), DPPH (Sigma, Missouri, USA), ABTS (Sigma, Missouri, USA), $FeCl_3\cdot 6H_2O$ (Merck, New Jersey, USA), ascorbic acid, ethanol, $K_2S_2O_8$, NaOH (Merck), SDS buffer solution (Merck, Darmstadt, Germany), protein marker (10–250 kDa, Bio-Rad, UK), acetic acid, gallic acid, Folin reagent, and Na_2CO_3.

2.2 Work procedure

This research was carried out in two stages. The first stage was the preparation of samples and characterization of catfish (*Pangasius sp.*) meat as the main raw material for producing fish protein hydrolysates. The second stage was the hydrolysis of catfish protein and the measurement of the degree of hydrolysis. The selected hydrolysate with the highest degree of hydrolysis was then subjected to ultrafiltration, followed by characterization of molecular weight, protein concentration, and antioxidant activity. In the final step, the selected catfish protein hydrolysate was further fractionated using a 3 kDa ultrafiltration membrane filter and subsequently re-characterized in terms of molecular weight, protein concentration, and antioxidant activity.

2.2.1 Preparation of catfish meat

Catfish (*Pangasius* sp.) was obtained from the Fish Market, Dramaga. Sample preparation was carried out by taking the fish meat. The catfish was first cleaned thoroughly using running water. The filleted meat was minced using a chopper or blender. The minced meat was then subjected to chemical composition analysis, including determination of moisture, ash, fat, and protein content.

2.2.2 Protein hydrolysis with alcalase enzyme

Protein hydrolysis was carried out by mixing the prepared catfish meat with buffer and alcalase enzyme. The catfish meat was homogenized with PBS buffer pH 8 (1:4) using a shaker for 2 minutes, then weighed, followed by the addition of alcalase enzyme at concentrations of 1%, 2%, and 3% (v/v). Hydrolysis was performed at 55 °C for 2 hours using a water bath shaker. Enzyme inactivation was then conducted by heating the sample at 90 °C for 15 minutes. The sample was cooled to room temperature and subsequently centrifuged at 10,000 rpm at 4 °C for 15 minutes to obtain the supernatant as the catfish protein hydrolysate. The selected protein hydrolysate was then analyzed for degree of hydrolysis, molecular weight, phenolic compounds, protein concentration, and antioxidant activity, followed by fractionation using ultrafiltration with a 3 kDa MWCO membrane.

2.3 Analysis procedure

2.3.1 Moisture Content [6]

Moisture content analysis was conducted to measure the amount of water contained in the sample. The initial step was drying the crucible in an oven at 105 °C for 1 hour. The crucible was then placed in a desiccator for 15 minutes until it reached room temperature, followed by weighing. A 3 g sample was placed into the crucible and dried in an oven at 105 °C for 5 hours. After drying, the sample was again placed in the desiccator until it reached room temperature and then weighed. The moisture content was calculated using the following formula:

$$Moisture\ content\ (\%) = \frac{B-C}{B-A} \times 100\% \qquad (1)$$

Description:
A: Mass of empty crucible (g)
B: Mass of crucible with sample before drying (g)
C: Mass of crucible with sample after drying (g)

2.3.2 Ash content [6]

Ash content analysis was conducted to measure the amount of minerals contained in the sample. One gram of the sample was placed into the crucible and ignited over a Bunsen burner flame until no smoke was observed. The crucible containing the sample was then placed in a muffle furnace at 600 °C for 6 hours. After ashing, the crucible with the sample was cooled in a desiccator to room temperature and weighed. The ash content was calculated using the following formula:

$$Ash\ Content\ (\%): \frac{B-A}{C} \times 100\% \tag{2}$$

Description:
A: Mass of empty porcelain crucible (g)
B: Mass of crucible with sample after ashing (g)
C: Mass of sample (g)

2.3.3 Fat content [6]

Fat content analysis was performed to determine the amount of fat in the sample by extracting lipids or oils from the food material using a non-polar organic solvent. The first step was cleaning, drying, and weighing an empty flask. Five grams sample was wrapped in filter paper and placed in the Soxhlet extractor, which was mounted under a condenser with the fat flask positioned below. Hexane solvent was added in sufficient volume, and reflux was carried out until the solvent returned to the fat flask. The distillation process was conducted to evaporate the solvent and collect it. The fat flask containing the lipid extract was then heated in an oven at 105 °C to evaporate the remaining solvent, while the fat remained due to its higher boiling point. After heating, the flask was cooled in a desiccator for 20–30 minutes and weighed. The weight of the extracted fat was used to calculate the fat content in the sample. The fat content was calculated using the following formula:

$$Fat\ content\ (\%): \frac{W3-W1}{W2} \times 100\% \tag{3}$$

Description:
W1: Mass of empty fat flask (g)
W2: Mass of sample (g)
W3: Mass of flask with fat extract (g)

2.3.4 Protein content [6]

Protein content analysis is carried out to determine the crude protein in a material. The Kjeldahl method is used for protein analysis, which involves three main steps: destruction, distillation, and titration. In the destruction step, a 0.3 g sample is placed into a 100 mL Kjeldahl flask, followed by the addition of 0.3 g selenium and 20 mL concentrated H2SO4. The sample is then heated at 410°C until the solution turns clear greenish-yellow, and subsequently cooled for 15 minutes. In the distillation step, 300 mL of distilled water and 100 mL of 40% NaOH are added to the clear solution, followed by distillation. The distillate is collected in a 125 mL Erlenmeyer flask containing a mixture of 10 mL 0.1 N H2SO4 with 0.1% methylene blue and 0.1% methylene red indicators in a 2:1 ratio. The resulting distillate solution appears bluish-green. The final step is titration using 0.1 N HCl until the solution in the Erlenmeyer flask changes to pink. The volume of titrant used is recorded to calculate the protein content of the sample. Protein content can then be determined using the following formulas :

$$Nitrogen\ Content\ (\%): \frac{volume\ HCl\ (mL) - volume\ blank\ (mL) \times N\ HCl \times 14}{Sample\ weight\ (mg)} \times 100\%$$

$$Protein\ content\ (\%wb) = \%N\ \times 6{,}25$$

$$Protein\ Content\ (\%wb): \frac{Protein\ content\ (\%bb)}{100 - moisture\ content\ (\%bb)} \times 100\% \qquad (4)$$

Description:
wb: Wetbasis
db: Dry basis
N: Normality of HCl solution

2.3.5 Degree of hydrolysis

Degree of hydrolysis (DH) is determined to measure the ability of protease to hydrolyze a protein. First, 20 mL of protein hydrolysate is taken and mixed with 20 mL of 20% (v/v) TCA solution. The mixture is stored for 30 minutes to allow precipitation, followed by centrifugation at 7,800 × g for 15 minutes at 4°C. The obtained supernatant is then analyzed to determine the nitrogen content using the Kjeldahl method. The degree of hydrolysis can be calculated using the following formula:

$$Degree\ of\ Hydrolysis\ (\%) = \frac{Nitrogen\ content\ in\ supernatant}{Total\ nitrogen\ cntent\ in\ sample} \times 100\% \qquad (5)$$

2.3.6 Protein concentration by Bradford method

Protein concentration assay using the Bradford method was performed with a 96-well plate. A series of Bovine Serum Albumin (BSA) standard solutions with graded concentrations (e.g., 0; 0.2; 0.4; 0.6; 0.8; 1.0; 1.2; and 1.4 mg/mL) was prepared. Protein hydrolysate samples from catfish were also prepared. A volume of 5 µL of either standard or sample solution was added into each well of the 96-well plate. Then, 250 µL of Bradford reagent was added to each well. The mixtures were gently mixed with a pipette or a shaker for 15–30 seconds to ensure homogenization. Samples were incubated at room temperature for 5 to 45 minutes. After incubation, absorbance was measured at 595 nm using a microplate reader. The absorbance values of the standards were used to construct a standard curve with absorbance (Y-axis) versus BSA concentration (X-axis). The protein concentration of the samples was determined by matching their absorbance values to the standard curve.

2.3.7 Molecular weight determination by SDS-PAGE

The sample was dissolved in 5% SDS solution and heated in a water bath at 85 °C for one hour. The mixture was then centrifuged at 8,500 × g for five minutes at room temperature. The separated supernatant was mixed with buffer containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol (v/v), and 10% β-mercaptoethanol at a ratio of 1:10. The mixture was subsequently heated in boiling water for two minutes. A volume of 15 µL of each sample was loaded onto a polyacrylamide gel consisting of 15% separating gel and 5% stacking gel. Electrophoresis was carried out for two hours at a constant current of 50 mA per gel. Following electrophoresis, the gel was stained with 0.05% Coomassie Brilliant Blue R-250 prepared in 15% methanol and 5% acetic acid for one hour. The gel was then rinsed with 30% methanol and 10% acetic acid for one hour, followed by a de-staining step for 30 minutes.

2.3.8 Determination of peptides containing phenolic groups

The determination of phenolic content was conducted using a modified method of Apostolidis and Lee (2010). One milliliter of sample was mixed with 1 mL of 95% ethanol and 5 mL of distilled water. Subsequently, 0.5 mL of 50% (v/v) Folin–Ciocalteu reagent was added, and the mixture was homogenized using a vortex. After incubation for 5 minutes, 1 mL of 5% Na_2CO_3 solution was added and allowed to react for 60 minutes at room temperature. The absorbance of the solution was measured at 725 nm using a UV-Vis spectrophotometer. The total phenolic content was calculated based on a gallic acid standard curve and expressed as milligrams of gallic acid equivalent per gram of fresh sample (mg GAE/g).

2.3.9 DPPH antioxidant activity

The antioxidant activity was determined using the DPPH method. A 50 ppm DPPH solution was prepared by dissolving 5 mg of DPPH in 50 mL of methanol. The stock solution of the sample was prepared by dissolving 10 mg of the sample in 10 mL of methanol. Working solutions with concentrations of 2, 5, 20, 30, 60, and 100 ppm were prepared by pipetting the appropriate volume of the stock solution into test tubes and diluting with methanol to a final volume of 10 mL. The blank solution was prepared by mixing 2 mL of methanol with 2 mL of DPPH solution, and its absorbance was measured at 510 nm using a UV-Vis spectrophotometer. For the sample analysis, 2 mL of each concentration was pipetted into vials, followed by the addition of 2 mL of DPPH solution, with additions carried out at 1- minute intervals. After the addition of DPPH, the samples were incubated for 30 minutes at room temperature. The absorbance of each concentration was then measured using a cuvette. The IC_{50} value was calculated using linear regression analysis, where the regression equation ($y = ax + b$) derived from the plot of sample concentration versus percentage of antioxidant inhibition was used to determine IC_{50}.

2.3.10 ABTS antioxidant activity

ABTS radicals were generated by mixing 7.4 mM ABTS stock solution with 2.6 mM potassium persulfate, followed by incubation for 18 hours in the dark at room temperature. After incubation, the solution was diluted with deionized water until the absorbance reached 1.1 ± 0.05 at 405 nm. A total of 200 µL of ABTS solution was mixed with 100 µL of collagen hydrolysate sample and incubated for 15 minutes at room temperature. The absorbance of the mixture was measured at 405 nm using a microplate reader. Deionized water was used as the control to replace ABTS in the sample (sample control), and for the ABTS control, water was used to replace the hydrolysate. A standard curve was prepared using vitamin C solutions at concentrations ranging from 2.5 to 15.5 µg/mL.

3 Result and discussion

3.1 Proximate analysis

The chemical composition of *Pangasius* sp. fillet was analyzed to determine its suitability as raw material for protein hydrolysate production. The chemical composition of *Pangasius* sp. is presented in Table 1.

Table 1. The proximate composition of catfish flesh (*C. gariepinus*).

Parameter (%)	Catfish (*Pangasius sp.*)
Moisture	76.10 ± 0.01
Ash	0.91± 0.04
Fat	6.03 ± 0.01
Protein	16.49 ± 0.50

The striped catfish (*Pangasius hypophthalmus*), a freshwater species introduced to Indonesia in 1972, is widely cultivated due to its economic value and nutritional quality. The fish used in this study was in fillet form, containing 16.49% protein.

3.2 Characteristics of Catfish protein hydrolysate

The protein hydrolysate of striped catfish was produced through enzymatic hydrolysis using alcalase. Hydrolysis is the process of breaking chemical bonds in large molecules into smaller ones, where in protein hydrolysis, peptide bonds are cleaved into smaller peptides. The resulting hydrolysates typically exhibit a light brown to yellowish color, depending on the type of enzyme applied. Enzymes act as biocatalysts, accelerating the hydrolysis reaction and promoting more specific cleavage of peptide bonds. In this study, alcalase was used at concentrations of 1%, 2%, and 3%. The resulting supernatant represented the catfish protein hydrolysate, as shown in Figure 1.

Fig. 1. Catfish (*Pangasius* sp) protein hydrolysates obtained with Alcalase enzyme concentrations of (a) 1%, (b) 2%, and (c) 3%.

The catfish protein hydrolysate (HPI) exhibited a turbid yellowish color across enzyme concentrations of 1%, 2%, and 3%, with only minor differences observed. The darker color observed in the 3% hydrolysate can be attributed to oxidation processes and peptide bond cleavage during hydrolysis, which release amino groups that participate in Maillard reactions with reducing sugars. This reaction, together with lipid oxidation typical of medium-fat fish such as catfish, contributes to the yellowish-brown color. Extended hydrolysis time further enhances this non-enzymatic browning, leading to a more intense brownish appearance [7]. Consistently, higher enzyme concentrations have been reported to yield darker hydrolysates, which matches the trend observed in this study.

3.2.1 Degree of hydrolysis

In this study, the DH of striped catfish protein hydrolysates was determined using the SN-TCA method, which measures the total soluble nitrogen content in 20% trichloroacetic acid (TCA) solution. The results of the DH measurement of striped catfish protein hydrolysates are presented in Figure 2.

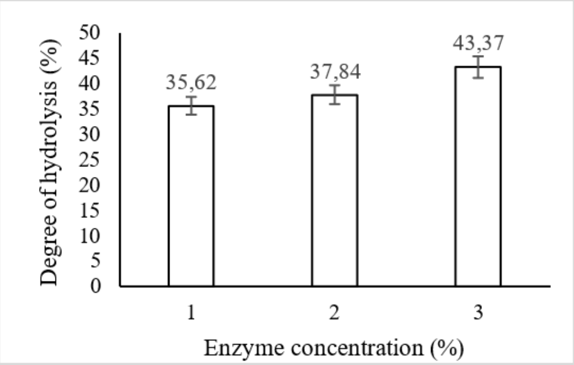

Fig. 2. Degree of hydrolysis of catfish (*Pangasius* sp) protein hydrolysates.

Figure 2 shows that the degree of hydrolysis (DH) values obtained using the SN-TCA method varied across treatments. The DH value at 1% enzyme concentration was 35.62%, which increased to 37.84% at 2% and showed a significant increase to 43.37% at 3%. This indicates that higher enzyme concentrations effectively break down proteins into peptides. A previous study reported that the DH value of shrimp head protein hydrolysates using alcalase was 30.11% [8]. The DH of striped catfish myofibrillar protein hydrolysates using alcalase at 3 mg/mL ranged from 40.60–83.6%, which was lower compared to papain hydrolysis (46.16–89.17%) [9]. Higher DH values lead to lower molecular weights, which are correlated with stronger antioxidant activity [10].

3.2.2 Soluble protein concentration

The protein hydrolysate sample of striped catfish with the highest degree of hydrolysis (DH) was further analyzed to determine protein concentration using the Bradford method. The Bradford assay is a colorimetric analysis used to quantify total protein concentration, employing Coomassie Brilliant Blue (CBB) dye, which binds to proteins and produces a blue color. The absorbance of the resulting solution is then measured spectrophotometrically at 595 nm. The measurement results of the soluble protein concentration are presented in Figure 3.

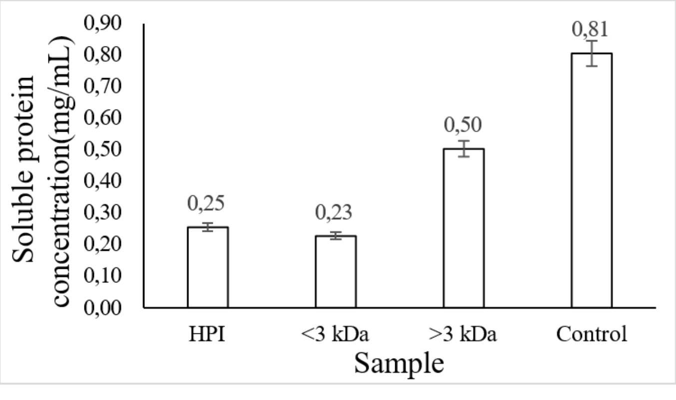

Fig. 3. Soluble protein concentration of catfish (*Pangasius* sp.) protein hydrolysate.

Figure 3 shows that the concentration of soluble protein varied across treatments. The highest protein concentration was observed in the control (0.81 ± 0.03 mg/mL), while the lowest was found in the <3 kDa hydrolysate fraction (0.23 ± 0.05 mg/mL). Analysis of variance indicated that enzyme activity and the applied method significantly affected soluble protein concentration ($P<0.05$), while Duncan's test revealed no significant difference between hydrolysates and the <3 kDa fraction, but a significant difference was observed between the >3 kDa fraction and the control. Results also showed that the <3 kDa fraction contained lower protein concentrations compared to the >3 kDa fraction.

3.2.3 Catfish protein hydrolysates after ultrafiltration

Ultrafiltration is a separation technique based on molecular weight using porous membranes, producing both filtrate and retentate fractions. In this study, crude extracts were filtered through a molecular weight cut-off (MWCO) membrane of 3 kDa (Amicon® Ultra- 15 centrifugal filters, 3K MWCO, Millipore). Ultrafiltration separates liquid components according to the size and structure of dissolved molecules under pressure. The principle of ultrafiltration is the movement of molecules across the membrane driven by concentration differences between the feed and the permeate. Smaller molecules that pass through the porous membrane form the permeate, while larger molecules retained by the membrane form the retentate [11]. The results of striped catfish protein hydrolysates fractionated by ultrafiltration are presented in Figure 4.

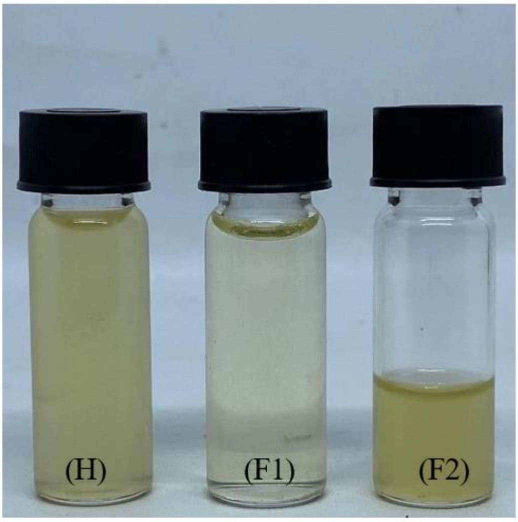

Fig. 4. Fractions of (*Pangasius* sp) protein hydrolysates: (H) protein hydrolysate, (F1) FPH <3 kDa fraction, (F2) FPH >3 kDa fraction.

Figure 4 shows that the striped catfish protein hydrolysate displayed a cloudy yellow appearance, with the <3 kDa fraction exhibiting a clearer golden-yellow color, while the >3 kDa fraction appeared darker, cloudier, and contained fine particles. This difference is attributed to the higher peptide concentration in the >3 kDa fraction compared to both the crude hydrolysate and the <3 kDa fraction. The distinct visual characteristics of each hydrolysate fraction were caused by ultrafiltration, which separates proteins based on molecular size. Larger peptides generally contain more aromatic residues, impurities, chromophores, and complex proteins, leading to a more turbid and yellowish appearance due to light scattering and higher absorbance. Ultrafiltration has been shown to enhance antioxidant activity, with lower molecular weight fractions (<5 kDa) exhibiting stronger antioxidant potential compared to larger fractions (>5 kDa) [12].

3.2.4 Molecular Weight of Catfish Protein Hydrolysates

Molecular weight represents the sum of atomic masses in a molecule. Electrophoresis is the most commonly used method to analyze molecular weight. According to Liu et al. [12], peptide fractions with molecular weight <3 kDa generally exhibit higher antioxidant activity due to their smaller size, which facilitates interactions with free radicals. The SDS-PAGE method uses discontinuous polyacrylamide gel as a supporting matrix and sodium dodecyl sulfate (SDS) to denature proteins. Protein markers with molecular weights ranging from 10–250 kDa were used. The molecular weight analysis of striped catfish fillet and protein hydrolysate can be seen in Figure 5. SDS-PAGE analysis of striped catfish fillet and protein hydrolysate revealed differences in molecular weight. Fresh catfish fillet showed molecular weights ranging from 10–227 kDa, while protein hydrolysate ranged from 5.9–9.5 kDa, and the >3 kDa fraction ranged from 6.8–9.5 kDa. In contrast, hydrolysates, particularly <3 kDa and >3 kDa fractions, displayed fewer and less visible bands.

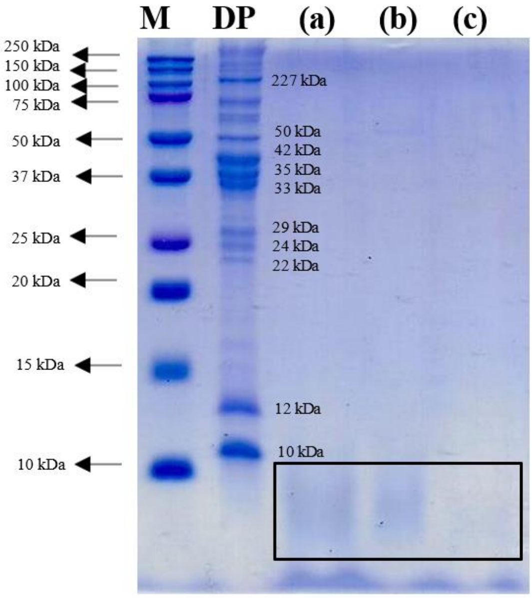

Fig. 5. Molecular weight of catfish protein hydrolysate. (M) Marker, (DP) catfish meat, (a) fish protein hydrolysate, (b) >3 kDa fraction, (c) <3 kDa fraction.

3.2.5 Phenolic-Containing Peptide Content

Phenolic compounds are characterized by hydroxyl groups attached to aromatic rings and can interact with proteins to form protein-phenolic complexes via covalent and non-covalent bonds. The total phenolic content of striped catfish fillet, protein hydrolysate, <3 kDa fraction, and >3 kDa fraction was measured to assess the contribution of phenolic compounds to the antioxidant activity of the protein hydrolysates. The results of the phenolic-containing peptides in protein hydrolysates can be seen in Figure 6.

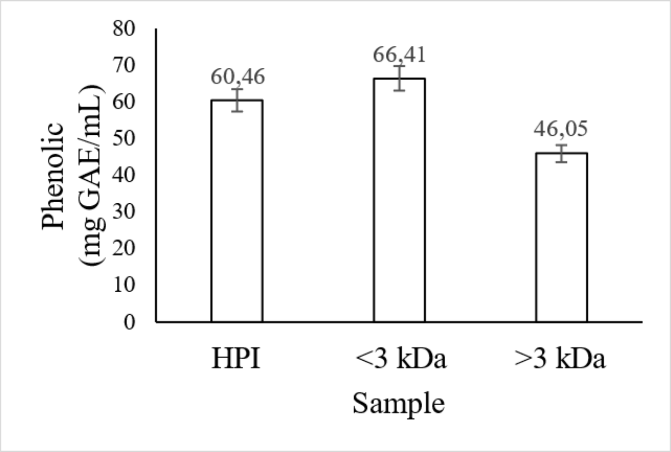

Fig. 6. Phenolic Group–Containing Peptides in Catfish (*Pangasius* sp) Protein Hydrolysates.

The determination of phenolic-containing peptides showed that striped catfish protein hydrolysates contain phenolic groups, with the highest content observed in the <3 kDa fraction. Analysis of variance indicated that molecular weight differences significantly affected the ultrafiltration results ($P<0.05$). The highest phenolic content was observed in the <3 kDa fraction (66.41 ± 6.47 mg GAE/L) and the lowest in the >3 kDa fraction (46.05 ± 9.57 mg GAE/L).

3.2.6 Antioxidant Activity of Catfish Protein Hydrolysate

a. DPPH Antioxidant Activity

The results of the DPPH antioxidant activity for protein hydrolysates can be seen in Figure 7. Duncan's multiple range test revealed that the <3 kDa fraction exhibited the lowest IC_{50} (21.97 ppm), significantly different from the >3 kDa fraction (62.42 ppm) and unfiltered hydrolysate (96.93 ppm). Previous studies reported that protein hydrolysates with 0–6% papain had DPPH inhibition values of 37.85–67.62% [3]. Factors such as enzyme conditions, pH, temperature, and time influence radical scavenging; extreme pH can reduce antioxidant activity by affecting protein structure [7]. Additionally, amino acid composition and peptide sequence are key determinants of hydrolysate antioxidant activity [13]. Several studies have reported that fish protein hydrolysates (FPH) exhibit varying antioxidant activities depending on the fish species, the type of enzyme used, and the hydrolysis conditions. For example, Kotson *et al.* [14] found that FPH derived from a mixture of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) rest raw material showed antioxidant activity as well as DPP-IV inhibitory effect; the molecular weight distribution and hydrolysis pretreatment (high-pressure processing) influenced the antioxidant capacity significantly.

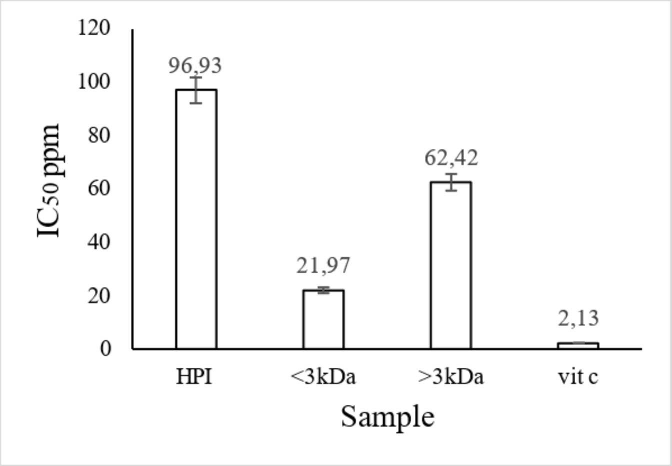

Fig. 7. DPPH antioxidant activity of catfish (*Pangasius* sp) protein hydrolysate.

b. ABTS Antioxidant Activity

The ABTS radical scavenging activity of the protein hydrolysates is shown in Figure 8. The results showed significant differences among the samples. Duncan's multiple range test revealed that the <3 kDa fraction had the lowest IC_{50} value of 3.28 ppm, indicating very strong antioxidant activity, whereas the >3 kDa fraction (5.70 ppm) and the unfiltered hydrolysate (8.35 ppm) had higher IC_{50} values. The <3 kDa fraction displayed stronger activity than the >3 kDa fraction due to its low molecular weight peptides containing hydrophobic amino acids with simpler structures that can donate hydrogen and interact effectively with free radicals [15].

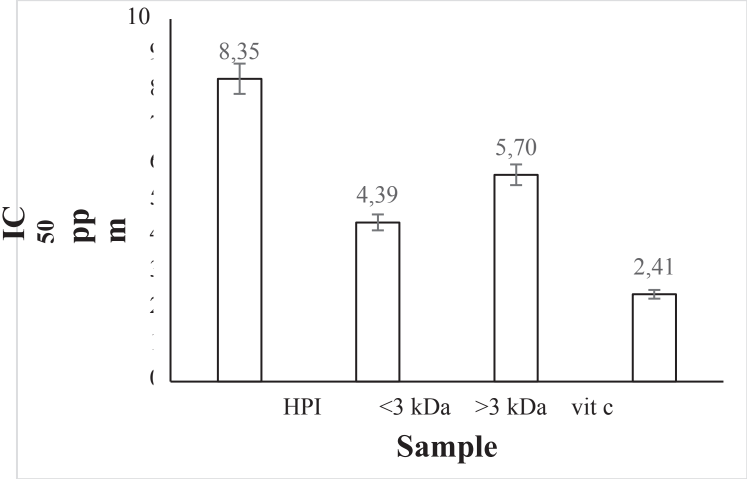

Fig. 8. ABTS antioxidant activity of catfish (*Pangasius* sp.) protein hydrolysate.

4 Conclusion

Pangasius protein hydrolysate treated with 3% alcalase enzyme concentration was the best treatment with the highest degree of hydrolysis. Ultrafiltration was able to separate small peptides <3 kDa and worked optimally on the antioxidant activity of the pangasius protein hydrolysate. The ultrafiltration results of the <3 kDa fraction had the strongest antioxidant activity compared to the *Pangasius* protein hydrolysate and the >3 kDa fraction.

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