

Effect of ultrafiltration on the antioxidant activity of alcalase-hydrolyzed catfish (*Clarias gariepinus*) protein

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Abstract. Catfish (*Clarias* sp.) is one of Indonesia's leading aquaculture commodities with potential utilization as a source of functional protein. Protein hydrolysis using alcalase enzyme can generate bioactive peptides with antioxidant activity. These antioxidant properties are typically more prominent in low-molecular-weight peptides, thus requiring separation via ultrafiltration. This study aimed to evaluate the effect of ultrafiltration on the antioxidant activity of catfish protein hydrolysate. Hydrolysis was carried out using alcalase at concentrations of 1%, 2%, and 3%, followed by fractionation using a 3 kDa molecular weight cut-off (MWCO) ultrafiltration membrane. Antioxidant activity was assessed using the DPPH and ABTS assays. The highest degree of hydrolysis (62,42%) was obtained at 3% enzyme concentration. The <3 kDa fraction exhibited the highest antioxidant activity, with IC₅₀ values of 41,20 ppm (DPPH) and 2,80 (ABTS), both categorized as very strong antioxidants. These findings indicate that ultrafiltration is an effective method for enriching short peptides with high antioxidant potential.

1 Introduction

Fish protein hydrolysate is a product of the degradation of fish protein into short-chain peptide compounds through a hydrolysis process that can occur with the aid of enzymes, acids, or bases. Fish protein hydrolysate products have the advantages of high solubility, good emulsifying capacity, and swelling ability. Hydrolysis also increases the solubility, biological activity, and benefits of fish protein, either through a chemical process with inexpensive acids or enzymatically using proteases in low-value fish [1]. Protease enzymes for protein hydrolysis, with their proteolytic ability to specifically break down peptide chains, produce hydrolysates that maintain essential amino acid content. One enzyme commonly used in protein hydrolysate production is alcalase. The alcalase enzyme from the bacterium *Bacillus licheniformis* is known to have a broader spectrum and greater ability to break down peptides into simpler forms. Fish protein hydrolysates have been known to contain bioactive peptides that to display biological activities, including antimicrobial, antihypertensive, antithrombotic, antioxidant, and immunomodulatory activities with low toxicity [3].

Catfish (*Clarias* sp.) is a major fishery commodity in Indonesia, widely cultivated and distributed to various segments of society. The advantage of catfish over other animal

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products is high content of leucine and lysine. Leucine is known for its function in growth, nitrogen balance, and muscle formation, while lysine is important for growth, tissue repair, and child development [4]. The protein content of catfish has the potential to produce bioactive peptides through hydrolysis. Putra *et al.* [5] study on catfish gill hydrolysate (CGH) showed that a 3% increase in enzyme concentration increased the antioxidant activity of ABTS to 9.57 μ M TEAC.

Protein hydrolysates resulting from enzymatic hydrolysis are heterogeneous because they consist of peptides with varying molecular sizes. Advanced separation methods, such as ultrafiltration, are used to obtain peptide fractions based on their molecular size. Ultrafiltration aims to obtain peptide solutions with specific molecular weights, which can potentially influence their biological activity. Due to its ability to separate and concentrate bioactive compounds, ultrafiltration is widely utilized in purification and fractionation processes. Thus, this research was aimed to determine the effect of ultrafiltration on the antioxidant activity of the alcalase-hydrolyzed catfish protein.

2 Materials and methods

2.1 Equipment and materials

The tools used in this study consisted of knives, cutting boards, blenders, porcelain cups, volumetric pipettes, funnels, beakers, micropipettes, microtubes, 96-well microplates, vortex, test tubes (Pyrex), measuring cups (Pyrex), Whatman 41 filter paper, ultrafiltration membranes (Amicon® ultra-15 centrifugal filters 3K MWCO milipore, Ireland), , digital scales (OHAUS AX224 New Jersey, USA), pH meters (Hanna Instruments, Rhode Island, USA), SPECRO UV Vis (RS Spectrophotometer UV-2500), centrifugators (FC5178R 120 V), electrophoresis equipment (PEQ Lab, Erlangen, Germany), and water baths (B-ONE, China).

The main ingredients in this study were catfish (*Clarias gariepinus*) and alcalase with an enzyme activity of 2,4 U/g (Sigma-Aldrich, St. Louis, USA). Other additional materials used in the analysis were distilled water, Phosphate Buffer Saline (PBS) buffer pH 8, trichloroacetic acid (TCA) (Merck, Darmstadt, Germany), Bradford Reagent, Tris HCl 60 mM pH 6,8, glycerol, β -Mercaptoethanol, Bovine Serum Albumin (BSA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma, Missouri, USA), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Sigma, Missouri, USA), FeCL₃.6H₂O (Merck, New Jersey, USA), ascorbic acid, methanol, K₂S₂O₈, NaOH (Merck), SDS buffer solution (Merck, Darmstadt, Germany), marker (10-250 kDa, BioRad, UK), acetic acid, gallic acid, follin, and Na₂CO₃.

2.2 Work procedure

The research consisted of two stages. The first stage involved the preparation and characterization of catfish meat, which would then be used to produce fish protein hydrolysate (FPH). The catfish protein was hydrolyzed using the alcalase enzyme to measure the degree of hydrolysis. The selected catfish protein hydrolysate was characterized by its degree of hydrolysis. In the second stage, the selected fish protein hydrolysate was subjected to ultrafiltration testing using a membrane filter to characterize its molecular weight and antioxidant activity.

2.2.1 Preparation of catfish meat

The raw materials were purchased at the local fish market as fresh fish. Preparation of the catfish began with cleaning it under running water. The skin and internal organs were thoroughly removed. The catfish meat was separated from the head, skin, bones, and internal organs. The separated meat was then ground using a grinder or blender. The softened meat was then tested to determine its contents, such as moisture, ash, fat, and protein.

2.2.2 Protein hydrolysis with alcalase enzyme

The protein breakdown process was carried out by adding the alcalase enzyme to the previously prepared catfish meat. The fish meat was mixed with PBS buffer solution at a ratio of 1:4, then homogenized for 2 minutes and weighed. Alcalase enzyme was added at concentrations of 1%, 2%, and 3%, then the mixture was heated at 55°C for 2 hours to break down the protein. Enzyme inactivation by heating the mixture at 90°C for 15 minutes. The mixture was then cooled and spun with a centrifuge to separate the liquid containing the protein breakdown products. The supernatant was filtered again using Whatman 41 filter paper. The best hydrolysate was selected for further analysis, such as how far the protein was broken down, its molecular size, and how strong its antioxidant activity was (DPPH and ABTS methods). 12 mL of the best hydrolysate was taken from the best results to be filtered again using a special filter tool, namely an ultrafiltration membrane using a centrifuge. The results of this filtration were divided into two parts, namely molecules larger than 3 kDa and smaller than 3 kDa.

2.3 Analysis procedure

2.3.1 Moisture content

Moisture content analysis was conducted to determine the amount of water contained in the sample. The initial step involved 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. Approximately 3 g of sample was placed in the crucible and dried in the oven at 105°C for 5 hours. After drying, the crucible containing the sample was placed back into the desiccator until it reached room temperature and then weighed. The moisture content was calculated using the following formula:

$$\text{Moisture content (\%)}: \frac{B-C}{B-A} \times 100\% \quad (1)$$

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

Ash content analysis was performed to determine the total mineral content in the sample. The procedure began with drying the crucible in an oven at 105°C for 1 hour. The crucible was cooled in a desiccator for 15 minutes and weighed. One gram of sample was placed in the crucible and charred over a Bunsen flame until no smoke appeared. The crucible containing the sample was then placed in a muffle furnace at 600°C for 6 hours. After ashing, the

crucible was cooled in a desiccator to room temperature and weighed. The ash content was calculated using the following formula:

$$\text{Ash content (\%)}: \frac{B-A}{C} \times 100\% \quad (2)$$

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

Fat content analysis was carried out to determine the amount of fat in the sample by extracting fat or oil using a non-polar organic solvent. The procedure began with cleaning, drying, and weighing an empty flask. Approximately 5 g of sample was wrapped in filter paper and placed in a Soxhlet extraction apparatus, fitted with a condenser on top and a fat flask at the bottom. Hexane solvent was added in sufficient volume, and reflux extraction was carried out until the solvent returned to the flask. Distillation was then performed to evaporate and recover the solvent. The fat flask containing the extract was dried in an oven at 105°C to remove residual solvent, as the boiling point of fat is higher. The flask was cooled in a desiccator for 20–30 minutes and then weighed. The fat content was calculated using the following formula:

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

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

Protein content analysis was performed to determine the crude protein in the sample using the Kjeldahl method. In the digestion stage, a 0.3 g sample was placed into a 100 mL Kjeldahl flask, followed by the addition of 0.3 g selenium catalyst and 20 mL concentrated H₂SO₄, and heated at 410°C until a clear greenish-yellow solution was obtained, then cooled for 15 minutes. The distillation stage was conducted by adding 300 mL of distilled water and 100 mL of 40% NaOH to the digest, after which the distillate was collected in a 125 mL Erlenmeyer flask containing 10 mL of 0.1 N H₂SO₄ with mixed indicators (0.1% methylene blue and 0.1% methylene red in a 2:1 ratio), resulting in a bluish-green solution. Finally, in the titration stage, the distillate was titrated with 0.1 N HCl until the solution changed to pink, and the volume of titrant used was recorded for protein content determination. The protein percentage was then calculated using the appropriate formulas.

$$\text{Nitrogen content (\%)}: \frac{\text{volume HCl (mL)} - \text{volume blank (mL)} \times N \text{ HCl} \times 14}{\text{Sample weight (mg)}} \times 100\% \quad (4)$$

$$\text{Protein content (\%wb)} = \%N \times 6.25 \quad (5)$$

$$\text{Protein content (\%db)}: \frac{\text{Protein content (\%bb)}}{100 - \text{moisture content (\%bb)}} \times 100\% \quad (6)$$

Description:

wb: Wet basis

db: Dry basis

N: Normality of HCl solution

2.3.5 Degree of hydrolysis

The degree of hydrolysis was measured to determine the ability of protease to hydrolyze protein. A 20 mL sample of FPH was taken, and 20 mL of 20% (b/v) TCA was added. The mixture was allowed to stand for 30 minutes to facilitate precipitation, followed by centrifugation at $7,800 \times g$ for 15 minutes. The obtained supernatant was analyzed for nitrogen content using the Kjeldahl method. The degree of hydrolysis was calculated using the following formula:

$$\text{Degree of Hydrolysis (\%)} = \frac{\text{Nitrogen content in supernatant}}{\text{Total nitrogen content in sample}} \times 100\% \quad (7)$$

2.3.6 Protein concentration by Bradford method

Protein concentration was determined using the Bradford method in a 96-well plate. A series of standard Bovine Serum Albumin (BSA) solutions were prepared at concentrations of 0; 0.1; 0.3; 0.5; 0.7; and 0.9 mg/mL. Protein hydrolysate samples of catfish were also prepared. A volume of 5 μL of either standard or sample solution was added to each well, followed by 250 μL of Bradford reagent. The mixture was gently mixed with a pipette or shaker for 15–30 seconds to ensure homogenization, then incubated at room temperature for 5–45 minutes. Absorbance was measured at 595 nm using a microplate reader. The standard absorbance values were used to construct a calibration curve (Y-axis: absorbance, X-axis: BSA concentration), and protein concentration in the samples was determined by interpolating their absorbance values on this curve.

2.3.7 Molecular weight determination by SDS-PAGE

Samples were dissolved in 5% SDS solution and heated in a water bath at 85°C for one hour followed by centrifugation at $8,500 \times g$ for 5 minutes at room temperature. Sample buffer solution containing 60 mM Tris-HCl (pH 6.8), 2% SDS, and 25% glycerol was added at a ratio of 1:10, with additional 10% β -mercaptoethanol. The mixture was heated in boiling water for 2 minutes. Subsequently, 15 μL of the sample was loaded into polyacrylamide gels consisting of 15% separating gel and 5% stacking gel. Electrophoresis was carried out at a constant current of 50 mA per gel for 2 hours. Gels were stained with 0.05% Coomassie Brilliant Blue R-250 in 15% methanol and 5% acetic acid for 1 hour, then destained with 30% methanol and 10% acetic acid for 1 hour, followed by a de-staining process for 30 minutes.

2.3.8 Determination of peptides containing phenolic groups

. A total of 125 μL of the sample was mixed with 125 μL methanol and 625 μL distilled water in a test tube. Then, 62.5 μL of 50% (v/v) Folin–Ciocalteu reagent was added, and the mixture was homogenized using a vortex. After 5 minutes of incubation, 125 μL of 5% Na_2CO_3 solution was added and allowed to react for 60 minutes at room temperature. Absorbance was measured at 725 nm using a UV-Vis spectrophotometer. Total phenolic

content was calculated from a gallic acid standard curve and expressed as mg gallic acid equivalents per gram of fresh sample (mg GAE/g).

2.3.9 DPPH antioxidant activity

The analysis measured the ability of samples to reduce the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). Samples were prepared at concentrations of 2, 5, 10, 20, 30, and 40 ppm. A total of 150 μL of each sample was placed into microplate wells, followed by 50 μL of 0.1 mM DPPH solution. Stock solutions were prepared at 100 ppm, while the blank consisted of 150 μL methanol p.a. and 50 μL DPPH solution. Vitamin C (1–5 ppm) was used as a positive control. The microplate was incubated for 30 minutes at room temperature in the dark, and absorbance was measured at 517 nm using a UV-Vis spectrophotometer.

2.3.10 ABTS antioxidant activity

The ABTS radical cation (ABTS^+) was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) in a 1:1 ratio (v/v), followed by incubation in the dark for 18 hours at room temperature. The resulting solution was diluted with methanol to an absorbance of 0.700 ± 0.02 at 734 nm. Samples were prepared at concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, and 2 ppm. A total of 500 μL of each sample solution (1 mg/mL) was mixed with 500 μL of ABTS^+ solution and incubated for 10 minutes at room temperature. Then, 150 μL of each mixture was transferred into microplate wells, and absorbance was measured at 734 nm using a UV-Vis spectrophotometer. Antioxidant activity was expressed as IC_{50} values obtained from linear regression of percent inhibition versus sample concentration.

3 Result and discussion

3.1 Proximate analysis

This analysis aimed to assess the suitability of catfish for the production of protein hydrolysates. The results of the analysis were then used as the basis for evaluating the nutritional quality and functional potential of the raw material. The chemical composition of catfish flesh (*C. gariepinus*) is presented in Table 1.

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

Parameter (%)	Catfish (<i>C. gariepinus</i>)
Moisture	82.04 ± 0.07
Ash	0.84 ± 0.11
Fat	1.20 ± 0.02
Protein	15.51 ± 0.53

Catfish (*C. gariepinus*) flesh contained 15.51% protein, placing it within the 15–20% range typical of high-protein fish. Catfish can be considered a suitable raw material for producing protein hydrolysates with high nutritional value.

3.2 Characteristics of catfish protein hydrolysate

Catfish (*C. gariepinus*) protein hydrolysate was produced through enzymatic hydrolysis using Alcalase, an enzyme that acts as a biocatalyst to accelerate the hydrolysis reaction and thereby enhance the efficiency of protein breakdown. Hydrolysis is a chemical reaction that

degrades complex protein molecules into simpler forms such as peptides and amino acids. In this study, Alcalase was applied at concentrations of 1%, 2%, and 3% (b/v). Prepared catfish flesh was mixed with Phosphate Buffered Saline (PBS) at a ratio of 1:4 (b/v), and the hydrolysis process was carried out at 55 °C for 2 hours. The resulting supernatant represented the catfish protein hydrolysate, as shown in Figure 1.

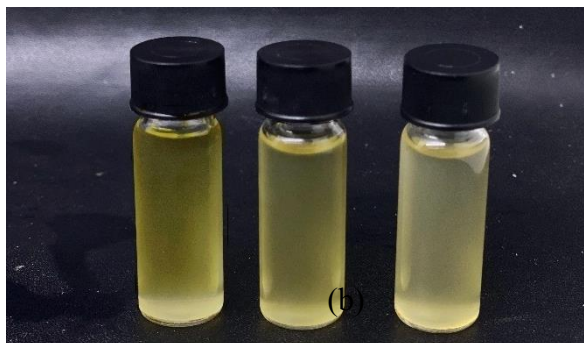


Fig 1. Catfish (*Clarias gariepinus*) protein hydrolysates obtained with Alcalase enzyme concentrations of (a) 1%, (b) 2%, and (c) 3%.

The hydrolysis of catfish (*C. gariepinus*) protein using alcalase produced hydrolysates with distinct visual characteristics at different enzyme concentrations. At 1% concentration, the hydrolysate appeared yellow and highly turbid; at 2%, it became yellow, more transparent, with slight turbidity; and at 3%, it showed a clearer and more transparent yellow solution. The decreasing turbidity with increasing enzyme concentration reflects more efficient hydrolysis, as proteins are broken down into smaller soluble fractions, leading to higher clarity. Turbidity is inversely related to protein solubility and pH, where lower turbidity indicates higher solubility and optimal enzymatic activity [6]. Increasing enzyme concentration provides more active sites for substrate interaction, thus accelerating hydrolysis and increasing product yield, whereas low enzyme concentration slows the reaction due to substrate excess.

3.2.1 Degree of hydrolysis

Enzyme concentration is a critical factor influencing the effectiveness of hydrolysis, as it determines how optimally peptide bonds are cleaved. Higher enzyme concentrations result in greater enzymatic activity, which in turn increases the degree of hydrolysis (DH). DH serves as an indicator of the success of protein hydrolysis, calculated as the percentage of cleaved peptide bonds relative to the total peptide bonds in the substrate. A higher DH value reflects a more efficient hydrolysis process, producing greater amounts of peptides and free amino acids. DH is commonly determined using the SN-TCA (Soluble Nitrogen After Trichloroacetic Acid Precipitation) method, which measures the amount of soluble nitrogen after precipitation of undigested proteins. The degree of hydrolysis of catfish protein hydrolysates is presented in Figure 2.

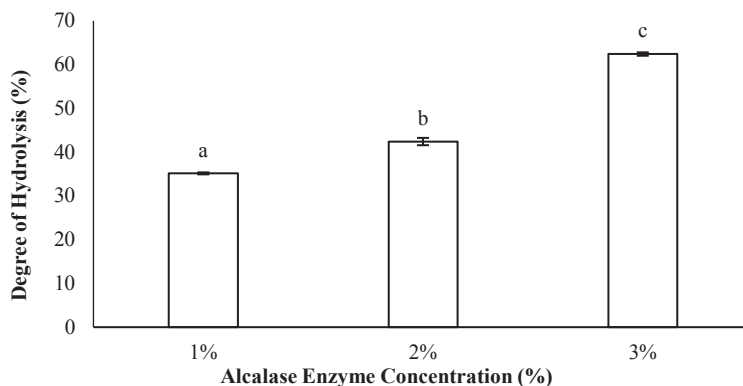


Fig. 2. Degree of hydrolysis of catfish (*C. gariepinus us*) protein hydrolysates.

The degree of hydrolysis (DH) of catfish (*C. gariepinus*) protein hydrolysates increased significantly with higher alcalase concentrations, with the highest DH value of 62.42% at 3% enzyme concentration ($p < 0.05$), as confirmed by ANOVA and Duncan's test. Similar findings were reported by Aprilia *et al* [7], who obtained DH values of 58.19% using 3% calotropin, and Nurhayati *et al* [8], who reported 49% DH with crude papain compared to 25% with commercial papain. A higher DH reflects fewer high-molecular-weight proteins remaining and more small peptides and free amino acids formed, including essential amino acids such as lysine, leucine, and methionine [9].

3.2.2 Soluble protein concentration

The measurement of soluble protein concentration in catfish protein hydrolysates was carried out using the Bradford method, a rapid and practical colorimetric technique for determining total protein concentration in solution. The intensity of the blue color formed is proportional to the protein concentration and can be quantified using a spectrophotometer at a wavelength of 595 nm. The soluble protein concentration of catfish protein hydrolysates can be observed in Figure 3.

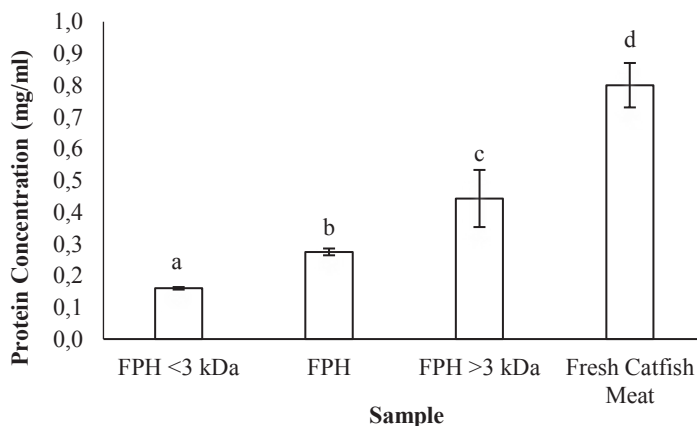


Fig. 3. Soluble protein concentration of catfish (*Clarias gariepinus*) protein hydrolysate.

The measurement of soluble protein concentration using the Bradford method showed that fresh catfish meat had the highest value (0.75 ± 0.072 mg/mL), while the <3 kDa fraction had the lowest (0.16 ± 0.004 mg/mL). The low concentration in the <3 kDa fraction indicates that most proteins were hydrolyzed into small peptides and free amino acids, which are unable to form complexes with Coomassie Brilliant Blue (CBB), making them undetectable. The Bradford method relies on the color shift of CBB G-250 from red to blue upon binding with amino acid residues, with the intensity proportional to protein concentration. The proteolytic activity of alkalase accelerates peptide bond cleavage, promoting further protein degradation and reducing the soluble protein concentration detected, particularly in the <3 kDa fraction.

3.2.3 Catfish protein hydrolysates after ultrafiltration

Ultrafiltration is a pressure-driven membrane separation technique that employs semipermeable membranes with pore sizes ranging from 1 to 200 kDa to separate solutes based on molecular size. Compared to microfiltration, ultrafiltration membranes have smaller pores but remain larger than those used in nanofiltration and reverse osmosis. In this study, ultrafiltration of catfish protein hydrolysates was performed using a 3 kDa membrane (Amicon® Ultra-15 centrifugal filters, 3K MWCO Millipore), producing two main fractions: <3 kDa and >3 kDa. The hydrolysates subjected to ultrafiltration were obtained from treatment with 3% Alkalase, which yielded the highest degree of hydrolysis (62.42%). The results of the ultrafiltration process of catfish protein hydrolysates are shown in Figure 4.

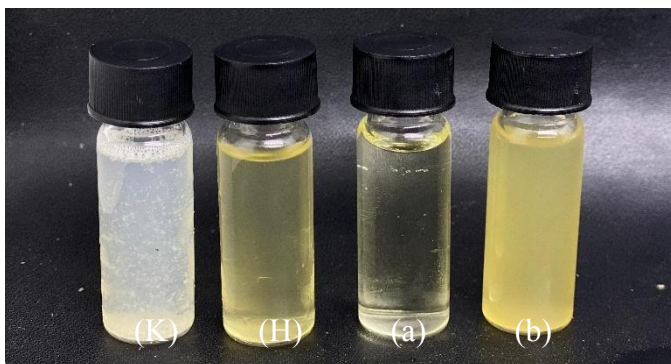


Fig. 4. Fractions of catfish protein hydrolysates: (K) fresh catfish meat, (H) protein hydrolysate, (a) FPH <3 kDa fraction, (b) FPH >3 kDa fraction.

The visual characteristics of catfish protein hydrolysates separated using a 3 kDa ultrafiltration membrane showed clear differences between fractions. The >3 kDa fraction appeared more turbid and yellowish compared to the <3 kDa fraction, which was clearer. This difference reflects the principle of ultrafiltration, which separates molecules by size: molecules larger than the membrane's cut-off are retained on the surface, where their accumulation forms a “cake layer” that scatters light and increases turbidity [10]. The efficiency of peptide separation depends on hydrolysate properties such as molecular weight distribution, concentration, hydrophobicity, charge, pH, and ionic strength. They further reported that not only pore size but also membrane arrangement in multilayer systems affects filtrate yield. For example, using a 10 kDa membrane layered above a 5 kDa membrane (10/5) produced more filtrate than two 5 kDa membranes in sequence (5/5), since the upper 10 kDa membrane allowed smoother flow and prevented excessive accumulation on the surface, thereby improving the filtration efficiency of the smaller membrane.

3.2.4 Molecular weight of catfish protein hydrolysates

In this study, SDS-PAGE analysis was performed on fresh catfish meat, catfish protein hydrolysates, and ultrafiltration fractions (>3 kDa and <3 kDa). The SDS-PAGE profiles of catfish meat and hydrolysates are presented in Figure 5. The molecular weight of proteins was determined by comparing the protein bands obtained from SDS-PAGE with standard protein markers (10–250 kDa). The electrophoresis results showed that fresh catfish muscle exhibited thicker protein bands than the hydrolysate, indicating higher protein concentration in intact tissue with a molecular weight range of 10–216 kDa. In contrast, the catfish protein hydrolysate showed degradation into smaller peptides with bands at 6–10 kDa, while the >3 kDa fraction appeared at 7–9 kDa. Similarly, Astiana *et al* [11] reported catfish viscera hydrolysates in the range of 15–16 kDa. These findings indicate that hydrolysis with alcalase effectively degraded proteins into smaller peptides. In the <3 kDa fraction, protein bands were not detected, suggesting that the fragments were either too small (<10 kDa) or present at concentrations too low to be visualized by SDS-PAGE.

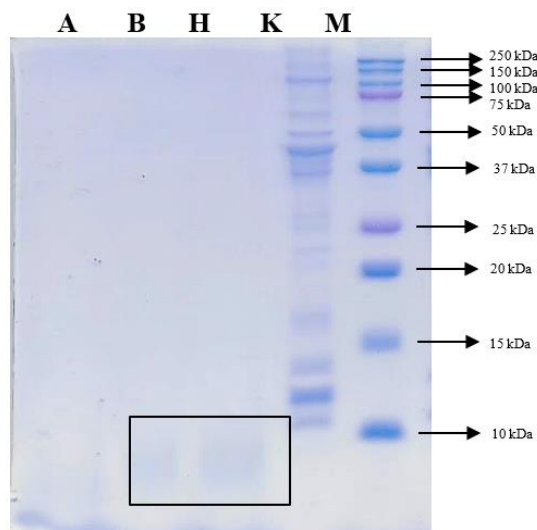


Fig. 5. Molecular weight profile of catfish (*Clarias gariepinus*) protein hydrolysates: (A) <3 kDa fraction, (B) >3 kDa fraction, (H) catfish protein hydrolysate, (K) fresh catfish meat, and (M) protein marker.

3.2.5 Phenolic-containing peptide content

Phenolic compounds characterized by the presence of one or more hydroxyl (-OH) groups attached to an aromatic ring are recognized as one of the most effective groups of natural antioxidants. In this study, the total phenolic content of catfish protein hydrolysates was analyzed to evaluate their contribution to antioxidant activity. The results of the phenolic content analysis of catfish protein hydrolysates are presented in Figure 6.

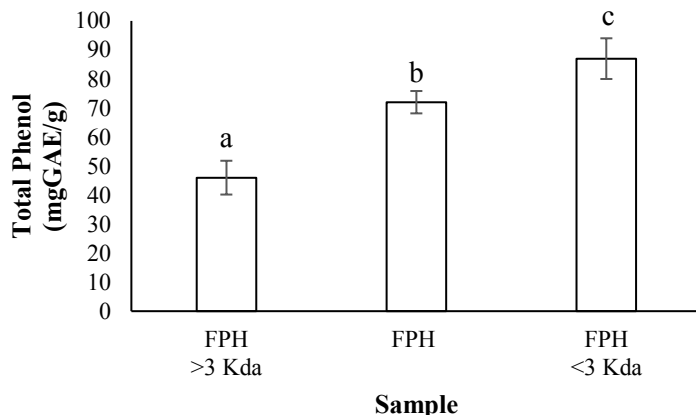


Fig. 6. Phenolic group-containing peptides in Catfish (*Clarias gariepinus*) protein hydrolysates.

Figure 7 shows that catfish protein hydrolysates phenolic compound varied from 46.49 to 87.24 mg of gallic acid equivalents/100 g of hydrlysate. . Ultrafiltration significantly affected ($p < 0.05$) total phenolic content, with the <3 kDa fraction showing the highest value (87.24 mg GAE/g), followed by whole HPI (71.97 mg GAE/g) and the >3 kDa fraction (46.49 mg GAE/g). This indicates strong antioxidant potential, as low-molecular-weight peptides in the <3 kDa fraction likely contain aromatic amino acids such as tyrosine, tryptophan, and phenylalanine that enhance radical scavenging. Aromatic structure can serve as an active hydrogen donor and gives stability to neighboring amino acid residues. Smaller peptides (<3 kDa) allow free phenolics to interact more effectively, while in the >3 kDa fraction phenolics are bound to larger peptides, reducing their reactivity [12].

3.2.6 Antioxidant activity of catfish protein hydrolysate

a. DPPH antioxidant activity

Antioxidant activity was quantitatively assessed using the DPPH method, based on the reaction between DPPH radicals and antioxidant compounds capable of donating hydrogen atoms. Antioxidant activity was measured using a spectrophotometer at a wavelength of 517 nm. The DPPH antioxidant activity of catfish protein hydrolysates is shown in Figure 7.

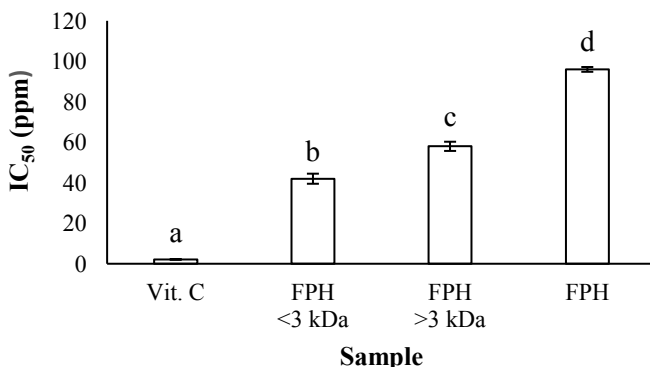


Fig. 7. DPPH antioxidant activity of catfish (*Clarias gariepinus*) protein hydrolysate.

The DPPH antioxidant activity of catfish protein hydrolysates showed that differences in molecular weight fractions obtained after ultrafiltration resulted in significantly different IC_{50} values. Analysis of variance indicated that molecular weight differences significantly affected the antioxidant activity of the hydrolysates ($P < 0.05$), and Duncan's post hoc test confirmed that each fraction had a distinct effect on antioxidant values. Smaller peptides (<3 kDa) exhibited higher solubility, mobility, and proton- or electron-donating capacity, allowing more efficient reduction of DPPH radicals, as evidenced by the color change from purple to yellow. The <3 kDa fraction ($IC_{50} = 41.70$ ppm) was classified as a very strong antioxidant, while the >3 kDa fraction (58.20 ppm) and whole hydrolysate (95.87 ppm) were considered strong antioxidants. Lower IC_{50} values in the >3 kDa fraction compared to whole hydrolysate were attributed to turbidity and larger particles scattering light, which required additional centrifugation for accurate spectrophotometric readings. Similar trends have been reported in silver carp protein hydrolysates [13], where low-molecular-weight peptides exhibited stronger antioxidant activity due to higher concentrations of free amino acids and small peptides.

Peptide fractions with molecular weight <3 kDa consistently demonstrated higher DPPH radical scavenging activity and reduction potential than larger fractions because smaller peptides are more soluble, diffuse more readily, and interact efficiently with free radicals. Antioxidant activity is also strongly influenced by amino acid composition; the presence of hydrophobic residues such as leucine, valine, and phenylalanine, as well as aromatic and sulfur-containing amino acids like tyrosine, tryptophan, and cysteine, enhances electron transfer and radical stabilization. While small peptides excel in radical scavenging, larger fractions may perform better in metal-chelating activity due to a higher number of functional groups [13].

b. ABTS antioxidant activity

The ABTS (2,2'-Azinobis(3-ethylbenzothiazoline)-6-sulfonic acid) method was used to evaluate the antioxidant capacity of a compound by measuring its ability to scavenge free radicals. The ABTS antioxidant activity of catfish protein hydrolysates is presented in Figure 8.

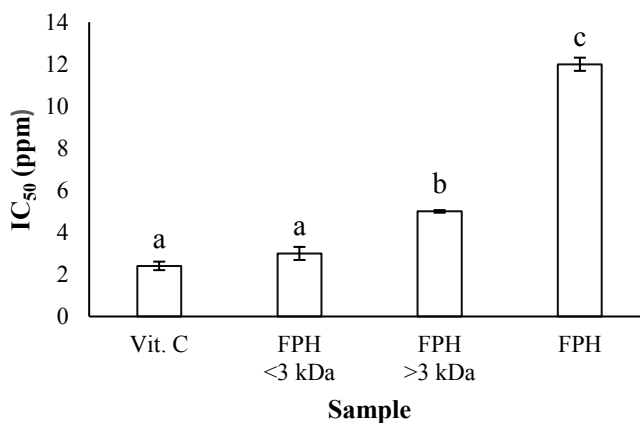


Fig. 8. ABTS antioxidant activity of catfish (*C. gariepinus*) protein hydrolysate.

The antioxidant activity of catfish protein hydrolysates (*C. gariepinus*) measured using the ABTS method showed that molecular weight differences after ultrafiltration resulted in varying IC_{50} values. Analysis of variance indicated that molecular weight significantly affected ABTS antioxidant activity ($P < 0.05$), and Duncan's multiple range test confirmed significant differences among fractions. Lower IC_{50} values indicate higher antioxidant

activity, as smaller peptides can more efficiently donate electrons to reduce ABTS⁺ radicals, causing a decrease in absorbance [14]. The IC₅₀ of unfiltered hydrolysates was higher than the <3 kDa fraction (2.80 ± 0.366 ppm), consistent with studies showing that ultrafiltration produces small peptides and bioactive amino acids that enhance radical-scavenging efficiency [13].

Peptide fractions with lower molecular weight (<3 kDa) exhibited higher antioxidant activity than larger fractions (>3 kDa), as smaller peptides diffuse faster and interact more readily with ABTS radicals. The high activity of the <3 kDa fraction is attributed to the abundance of small peptides containing functional amino acids such as tyrosine, tryptophan, cysteine, leucine, and valine, which donate electrons or hydrogen atoms to neutralize free radicals [12]. ABTS assay results also highlight the high hydrophilic antioxidant content in catfish protein hydrolysates. The ABTS method is considered superior to DPPH in detecting both hydrophilic and lipophilic antioxidants, providing faster reaction times and stable absorbance. While both DPPH and ABTS involve hydrogen atom transfer (HAT) and electron transfer (ET) mechanisms, ABTS is more sensitive to hydrophilic antioxidants and predominantly operates via HAT. In contrast, DPPH is better suited for lipophilic antioxidants and works mainly through ET, sometimes combined with HAT [15].

4 Conclusion

The protein hydrolysate produced using 3% alcalase represents the optimum condition in this current research, yielding the highest degree of hydrolysis. The ultrafiltration process was proven effective in separating peptides smaller than 3 kDa and had a significant impact on enhancing the antioxidant activity of the carp protein hydrolysate. The <3 kDa fraction exhibited higher antioxidant activity compared to the hydrolysate prior to ultrafiltration and the >3 kDa fraction.

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