



Antioxidant Activity of Hydrolysate Protein in Skipjack Fish (*Katsuwonus pelamis*) Using the ABTS Method

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Abstract

Skipjack tuna (Katsuwonus pelamis) bones are a byproduct of the fishing industry that are rich in protein and have potential as a source of bioactive peptides. This study aimed to determine the antioxidant activity of protein hydrolysates from raw and cooked skipjack tuna bones using the ABTS method. Hydrolysis was performed using bromelain at concentrations of 4%, 6%, and 8% at 55 °C for 4 hours pH 7.0), followed by enzyme inactivation at 85 °C for 20 minutes. Antioxidant activity was measured by IC₅₀ determination using a UV-Vis spectrometer at 734 nm. The results showed that the antioxidant activity of skipjack tuna bone samples with the highest total protein content was observed at an enzyme concentration of 8%, namely 35.55% (raw fish bones) and 36.24% (cooked fish bones). The highest degree of hydrolysis in the raw fish bone sample reached 82.15 % and in the cooked fish bone sample reached 86.81 % with 8 % enzyme. Qualitative amino acid tests (ninhydrin, Biuret, xanthoprotein, and Millon) yielded positive results, indicating the presence of aromatic amino acids (tyrosine, tryptophan, phenylalanine) that contribute to antioxidant activity. The lowest IC₅₀ value was obtained in the raw fish bone sample at an 8 % concentration, namely 10.03 mg/ml, and in the cooked fish bone sample at 6.19 mg/ml at an 8 % enzyme concentration. The higher the enzyme concentration, the higher the antioxidant activity.

Keywords: ABTS; antioxidants; bromelain; protein hydrolysate; skipjack fish bones

Introduction

Gorontalo Province is known for its substantial marine fisheries potential. One of its main resources is skipjack (*Katsuwonus pelamis*). According to the BPS (2024), skipjack fish production in Indonesia is 411.753 tons, and in Gorontalo Province, it is 6.224 tons. The part of the fish that is used is the meat part, while the fish bones that cannot be used will become waste. Fish bones account for 10 % to 20 % of the total weight of fish that have not been utilized optimally. Fishery industrial waste still contains protein that can be used as a raw material for the manufacture of peptides, collagen, and other materials (Meilani et al., 2022).

Protein hydrolysate is the result of the process of breaking down proteins into peptides and amino acids (Hermaya et al., 2021). Protein hydrolysate is generally produced using enzymatic hydrolysis, which is considered more efficient because it can prevent damage to certain amino acids. Enzymatic hydrolysis is the most commonly used method for producing bioactive peptides, due to its gentle processing conditions, ease of reaction control, and minimal by-product formation. One

protease that can hydrolyze proteins is bromelain (Sholahuddin & Prayoga, 2023).

Bromelain is a proteolytic enzyme in the hydrolase group. This enzyme belongs to the group of cysteine proteinases, which catalyze protein hydrolysis by cleaving peptide bonds in proteins or polypeptides into smaller molecules, namely amino acids (Nugraha, 2022). Bromelain enzymes show good thermal stability at an optimal pH of 7.0. Its primary source is agricultural waste, making it an economical natural alternative and a potential therapeutic agent (Ilyas et al., 2020). The bromelain enzyme has an optimal temperature range of 50-60 °C (Varilla et al., 2021).

Protein hydrolysate can act as an antioxidant, a substance that blocks or neutralizes free radicals to prevent oxidative damage to cells (Warda et al., 2025). Fish protein hydrolysate exhibits antioxidant activity due to its hydrolysis. This process breaks down complex proteins into small-sized peptides rich in bioactive amino acids, such as tyrosine, methionine, cysteine, histidine, lysine, and tryptophan. These amino acids then act as electron or proton donors to capture free radicals, thereby preventing oxidative damage (Hadjji et al., 2025).

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To date, various studies have successfully revealed the antioxidant potential of fish protein hydrolysates. However, a significant research gap remains regarding two important aspects. First, nearly all existing studies have focused on meat, skin, or internal organs, while the bones of skipjack tuna (*Katsuwonus pelamis*), which are also rich in protein, have not yet been studied in depth. In fact, the volume of bone waste from this flagship Indonesian commodity is abundant, particularly in eastern regions such as Gorontalo. Second, no studies have specifically compared protein hydrolysates from raw and cooked skipjack tuna bones, particularly using the ABTS method. In practice, bone waste often originates from cooked fish, whether from household consumption, markets, or the food service industry. The cooking process causes changes in protein structure due to heat, which can certainly affect hydrolysis results and biological activity. Without a deep understanding of these differences, efforts to utilize skipjack tuna bone waste as a source of bioactive peptides will not be fully effective.

Based on these two gaps, this study offers the following innovations. First, to address the first gap, this study focuses primarily on skipjack tuna bones and is the first to test their antioxidant activity using the ABTS method. Second, to address the second gap, this study specifically compares protein hydrolysates from raw and cooked skipjack tuna bones, thereby providing a more realistic picture consistent with actual waste conditions in the community. Third, as a complement, this study integrates the results of antioxidant activity tests (IC_{50} values) and qualitative amino acid tests (ninhydrin, biuret, xantoprotein, and Millon) on the same samples.

Based on this background, this study aims to characterize skipjack tuna bone hydrolysates and their antioxidant activity using the ABTS method.

Methods

Tools

The equipment used for the research includes various laboratory supplies such as test tubes (Pyrex), volumetric flasks (Pyrex), measuring cylinders (Pyrex), Erlenmeyer flasks (Pyrex), an analytical balance (Ohaus), a centrifuge (Hettich), pH paper (Nesco), filter paper (Whatman 93), dropper pipettes (Onemed), a stirrer, a Bunsen burner (Delpi Alkes), a water bath (Mettmert), a Kjeldahl apparatus, and a UV-Vis spectrophotometer (PG Instruments).

Material

The main raw material is skipjack tuna bones obtained from the central market in Gorontalo City, Gorontalo Province. The chemicals used are ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), 1 M NaOH, HNO_3 , 1 M HCl, distilled water, bromelain enzyme, 10 % TCA (trichloroacetic acid), K_2SO_4 (potassium persulfate), 0.1 % $CuSO_4$

(Copper(II) sulfate), Millon's reagent, 0.1 % ninhydrin solution, and vitamin C.

Skipjack bone sample preparation

Skipjack tuna bones were obtained by filleting fresh, dead fish and skipjack tuna that had undergone cooking processes such as traditional grilling or smoking at temperatures ranging from 30 °C to 90 °C (Andhikawati & Pratiwi, 2021). A total of 300 grams of bones were then weighed and ground in a blender for 5–10 minutes. The resulting paste is then analyzed for protein content and stored in a refrigerator for the next stage of analysis (Idowu et al., 2019).

Manufacture of protein hydrolysate

Aquades is added to the bones of the mashed skipjack fish in a ratio of 1: 5. The mixture is then centrifuged at 8.000 rpm for 10 - 15 minutes. Next, the mixture is adjusted to pH 7.0 using 0.1 M HCl or 0.1 M NaOH, then heated to 60 °C for 15 minutes. The bromelain enzyme is then added at concentrations of 4%, 6%, or 8% based on protein weight. Hydrolysis takes place at 55 °C for 4 hours, followed by enzyme inactivation at 85 °C for 20 minutes. The protein hydrolysate was then centrifuged at 5.000 rpm and 10 °C for 30 minutes to separate the supernatant and the residue. The hydrolyzed supernatant was analyzed to determine the degree of hydrolysis, dissolved protein content (Kjedahl method), and antioxidant activity (ABTS method) (Idowu et al., 2019).

Method Kjeldhal

The protein analysis method used followed the procedure from AOAC (2005). The principle of this method is to measure the protein content of an ingredient, and its implementation comprises three main components: digestion, distillation, and titration (Syahfitri et al., 2025). At the destruction stage, 1 gram of the sample is placed in the Kjeldahl flask along with 1 grain of selenium catalyst and 20 mL of sulfuric acid (H_2SO_4), and the mixture is heated to 410 °C until the solution turns clear. After that, the distillation stage is carried out by adding 50 mL of aquades and 20 mL of 40% sodium hydroxide (NaOH), then the mixture is distilled, and the steam is flowed into an Erlenmeyer flask containing 25 mL of boric acid (H_3BO_3) 2 % and the indicators of bromocresol green 0.1 % and methyl red 0.1 %. The last stage is titration, in which the distilled solution is titrated with 0.2 N HCl until it turns blue-green, and the volume of HCl used is recorded to calculate nitrogen levels. Protein levels can be calculated using formulas 1 and 2.

$$\% \text{ Nitrogen} = \frac{(V_{HCl \text{ sample}} - V_{HCl \text{ blanko}}) N_{HCl} \times 14.00}{\text{Total proteins}} \times 100 \quad (1)$$

$$\% \text{ Nitrogen} = \% N \text{ total} \times \text{conversion factor} \quad (2)$$

Hydrolysis degree test

Each hydrolysate sample is taken in 5 mL portions, and 5 mL of 10% TCA is added after the mixture has sat for approximately 30 minutes. Then

the mixture, left to sit, is centrifuged for 20 minutes at 5.000 rpm. The obtained supernatant was then analyzed using the Kjeldahl method (Hermaya et al., 2021). The degree of hydrolysis (% DH) can be calculated using formula 3.

$$\% DH = \frac{\text{Soluble proteins } 10\%}{\text{Total proteins}} \times 100 \quad (3)$$

Protein hydrolysate quantitative test

Protein qualitative tests on skipjack bone hydrolysates were carried out through four methods. The ninhydrin test is carried out by taking 1-2 mL of the sample, adding 5 mL of ninhydrin solution, heating at 100 °C for 15 minutes, and a positive result is indicated by a blue-purple color change (Taniyo et al., 2021). The biuret test was performed by adding 2 mL of 10 % NaOH and 5 - 10 drops of CuSO₄ 0.1% into 1 - 2 mL of sample, then stirred slowly, and positive results were characterized by the formation of a reddish to purple color (Taniyo et al., 2021). The xantoprotein test is performed by adding a few drops of concentrated HNO₃ to 1 - 2 mL of the sample, stirring, and heating for 3 - 5 minutes, until the mixture turns yellow. A few drops of 10% NaOH are added, and a positive result is indicated by a change in color to orange (Hadji et al., 2025). The Millon test is performed by adding a few drops of Millon reagent to a 1-2 mL sample, stirring, heating for 3-5 minutes, then cooling, and a positive result is indicated by a change in color to red (Dwiningrum et al., 2019).

ABTS method antioxidant activity test

The testing of the antioxidant activity of the ABTS method begins with the preparation of the solution, namely 3.5 mg of potassium persulfate dissolved in 5 mL of aquades, and 7.1015 mg of ABTS dissolved in 10 mL of aquades, then 5 mL of ABTS solution is mixed with 5 mL of potassium persulfate solution and then incubated for 12 - 16 hours in a dark place at room temperature, after which it is diluted with aquades to a volume of 25 mL. A total of 100 mg of hydrolysate samples was dissolved in aquades up to 10 mL to make a 10.000 ppm stock solution, then diluted into a series of concentrations of 500 ppm, 1000 ppm, 2500 ppm, and 5000 ppm.

A total of 0.5 mL of each sample concentration was pipetted. Then, 1.5 mL of ABTS solution was added, incubated for 6 minutes, and the absorbance was measured at 734 nm using a spectrophotometer. As a control, a 100 ppm vitamin C stock solution was made from 2.5 mg of vitamin C in 25 mL aquades, then diluted to concentrations of 20 ppm, 40 ppm, 60 ppm, and 80 ppm, then 0.5 mL of vitamin C solution was mixed with 1.5 mL of ABTS solution, incubated for 6 minutes, and measured in absorbance at wavelength 734 nm (Sari et al., 2024).

IC₅₀ calculation

An IC₅₀ value usually expresses the antioxidant capacity of a compound. This value indicates a concentration of compounds capable of suppressing free radical activity by up to 50%. To calculate it, the linear regression equation $y = ax + b$ is used. By entering the value $y = 50$ into the equation, the resulting value of x is the value of IC₅₀ (Tumanggor et al., 2025).

Results and Discussion

Protein up to hydrolysate protein

The analysis showed that the initial protein content in raw skipjack bones was 21.2%, while in cooked fish bones it was 22.59%. After hydrolysis, dissolved protein levels increased significantly across all treatments, as shown in **Table 1**. The increase in bromelain enzyme concentration from 4% to 8% was positively correlated with higher hydrolysate protein levels in both raw and cooked samples.

Table 1. Increased protein levels

Sample	The concentration of the enzyme	Up to protein hydrolysate (%)
Raw skipjack fish bones	4 %	31.64
	6 %	33.32
Cooked skipjack fish bones	8 %	35.55
	4 %	32.75
	6 %	34.32
	8 %	36.24

The protein content of hydrolysis products is affected by the concentration of proteolytic enzymes used during hydrolysis. The higher the enzyme concentration, the more soluble nitrogen is produced in the protein hydrolysate. The reaction rate increases with the amount of enzyme added. However, as the enzyme concentration increases, the amount of hydrolysate produced remains constant within certain limits. This is because the excess enzyme is inactive. The enzyme-to-substrate ratio must be precise for the reaction to proceed as efficiently as possible. If there is an excess of substrate and a shortage of enzyme, the reaction will proceed slowly, and some of the substrate will remain unreacted (Rahmawati et al., 2021). The protein content before hydrolysis for 21.2 % raw samples increased to 31.64-35.55%, and the cooked sample increased from 22.59% to 32.32-36.24%. The analysis of the two fish bone samples indicates that the protein content of cooked skipjack fish bones is higher than that of raw skipjack bones. The protein content in the bones of skipjack fish that has been cooked has increased because the moisture content in it is reduced, as some of it evaporates during the cooking process. This decrease in moisture content is considered the most important change, as it directly leads to a significant increase in bone protein in cooked fish (Basti'as et al., 2017).

The results of this hydrolysate protein content are in line with previous research that provided information about animal protein levels, including, Warda et al. (2025) it was revealed that the protein content of the head and skin of Venename's shrimp after being hydrolyzed for 4 hours using bromelain enzyme at a concentration of 6 % obtained a protein content of 50.10 %, of which the protein content before hydrolysis was 18.70 %. In research by Susanty & Kusumaningrum (2021), the protein content of toman fish hydrolyzed with bromelain for 4 hours was 40.74%, while the initial protein content before hydrolysis was 21.32%. Based on previous research, the protein content of skipjack bones remains lower, possibly influenced by the type of raw material (skipjack fish), variations in concentration, and the methods used.

Hydrolysis degree analysis

The degree of hydrolysis is a parameter that expresses the percentage of peptide bonds (bonds between amino acids) broken by hydrolysis. The success of the protein hydrolysis process can be monitored by analyzing several hydrolysis parameters, such as dissolved protein content and degree of hydrolysis (% DH) (Ratnayani et al., 2024). The higher the degree of hydrolysis achieved, the greater the effectiveness of proteolytic enzymes in breaking peptide bonds in protein molecules through hydrolysis. This shows that the enzymes work more optimally, resulting in a wider and more efficient breakdown of proteins (Farhan et al., 2025).

Based on the data in **Figure 1**, the results of the measurement of the degree of hydrolysis for hydrolysate with a concentration of bromelain enzyme 4 %, for raw samples of 81.50%, and for mature samples of 83.10 %. Bromelain enzyme concentration is 6% for raw samples, 82.46% for cooked samples, and 85.80% for cooked samples. The concentration of the bromelain enzyme was 8% for raw samples, 82.15% for cooked samples, and 86.81 for cooked samples. Variations in the concentration of enzymes used can affect hydrolysate yield, hydrolysate protein levels, and the degree of hydrolysis (Farhan et al., 2025). Various factors influence the degree of hydrolysis (DH %) during hydrolysis, including pH, temperature, enzyme-to-substrate ratio (E/S), hydrolysis duration, enzyme type, and substrate type. Other factors include the enzyme type and specificity, which affect the degree of hydrolysis and the resulting peptide (Ratnayani et al., 2024). The increase in the degree of hydrolysis is due to an increase in the number of soluble peptides and amino acids in TCA, resulting from the breakdown

of peptide bonds during protein hydrolysis (Susanty & Kusumaningrum, 2021).

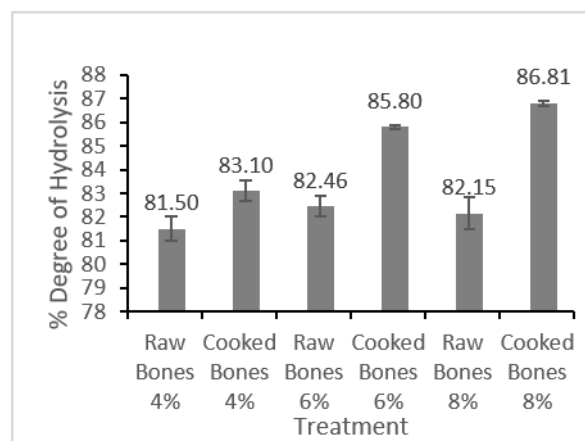


Figure 1. Degree of hydrolysis of skipjack bone protein hydrolysis

Qualitative analysis of protein hydrolysate

Qualitative analysis is a method that aims to determine the presence or absence of specific substances, functional groups, or compounds in a sample. Qualitative analysis of protein content can be performed using various methods, such as the Millon test, the Biuret test, and the ninhydrin test (Dwiningrum et al., 2019). The test results are shown in **Table 2**.

In this study, six types of skipjack bone protein hydrolysates (raw and cooked) will be tested at 4%, 6%, and 8%, using four tests: the ninhydrin test, the biuret test, the xanthoproteic test, and the Millon test. The results showed that all hydrolysate samples with varying enzyme concentrations tested positive across all four test types.

Antioxidant activity of skipjack fish bones

Analysis of the antioxidant activity of skipjack bone protein hydrolysate was carried out to determine the protein hydrolysate's ability to ward off free radicals. Free radicals are molecules that have one or more unpaired electrons on their outer shell. It is very unstable and reactive. Free radicals play an important role in causing tissue damage to the body. If the level of free radicals in the body is abnormal, then it will attack vulnerable compounds, such as proteins. As a result, various diseases can appear. This happens because the amount of oxidants that enter the body cannot be balanced by the antioxidants present in the body (Pratama & Busman, 2020).

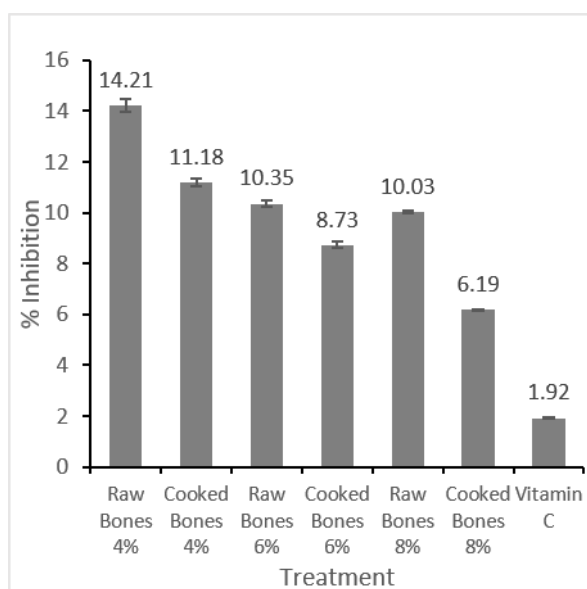
Testing of the antioxidant activity of skipjack bone protein hydrolysate using the ABTS method (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)). The ABTS method works by observing the discoloration of ABTS radical cations, which are greenish-blue and absorb at 734 nm.

Table 2. Qualitative test results of skipjack bone protein hydrolysate

Skipjack fish bone hydrolysate	Types of skipjack fish bone samples	Test ninhidrin	Test burette	Uji xantoprotein	Uji million
Hydrolysate 4 %	Raw	+	+	+	+
	cook	+	+	+	+
Hydrolysate 6 %	Raw	+	+	+	+
	cook	+	+	+	+
Hydrolysate 8 %	Raw	+	+	+	+
	cook	+	+	+	+

This method is widely used to assess the antioxidant activity of complex mixtures and compounds suspected to have antioxidant potential. The ABTS free radical capture by antioxidant compounds can be observed by a decrease in the sample's absorbance at 734 nm. These values will decrease proportionally after the ABTS radical receives electrons or hydrogen from antioxidants (Prastyo et al., 2020).

Based on the data in **Figure 2**, the IC₅₀ values for each enzyme concentration across samples differ. Raw and cooked skipjack bone hydrolysate has a variable IC₅₀ value, where at 4 % enzyme concentration for raw and cooked skipjack fish bones of 14.21 mg/mL and 11.18 mg/mL, at 6 % enzyme concentration of 10.35 mg/mL and 8.73 mg/mL and at 8 % enzyme concentrations of 10.03 mg/mL and 6.19 mg/mL. For Vitamin C, an IC₅₀ of 1.92 mg/mL, this Vitamin C value is lower than the IC₅₀ Values for Raw and cooked skipjack fish bone hydrolysate.

**Figure 2.** IC₅₀ value of skipjack fish bones

The lower IC₅₀ value observed in the hydrolysate of cooked skipjack tuna compared to that of raw skipjack tuna indicates that the increase in ABTS free radical scavenging activity is proportional to the degree of hydrolysis (DH). This is due to the enhanced ability of active peptides to

act as electron and hydrogen donors, reacting with free radicals to convert them into more stable compounds and break the radical chain reaction. Additionally, extensive hydrolysis produces shorter peptides (tripeptides and dipeptides) as well as free amino acids, which are more hydrophilic and therefore more readily interact with water-soluble ABTS radicals (Sbroggio et al., 2016). Consistent with this explanation, differences in IC₅₀ values are also largely determined by the degree of hydrolysis. The higher the degree of hydrolysis, the more low-molecular-weight peptides are formed, thereby increasing the ability to neutralize free radicals. Factors influencing the degree of hydrolysis include hydrolysis duration and enzyme concentration (Mutamimah et al., 2018).

An increase in enzyme concentration increases antioxidant activity; the higher the enzyme concentration, the greater the percentage of protein hydrolysate that inhibits free radicals (Puspawati et al., 2020). Regarding research findings consistent with this statement, a study by Hadji et al. (2025) reported that as enzyme concentration increased from 2%, 4%, and 6%, the highest antioxidant activity was observed at 6% concentration, with the lowest IC₅₀ value. The study by Warda et al. (2025) reported the same finding: among the enzyme concentrations of 4%, 5%, and 6%, the lowest IC₅₀ was observed at 6%, indicating the highest antioxidant activity.

Several studies have reported the antioxidant activity and IC₅₀ values of animal protein hydrolysates. A study by Sholahuddin et al. (2024) found that a tilapia offal hydrolysate produced with the bromelain enzyme had an IC₅₀ of 82.53 µg/mL by the DPPH method. A study by Warda et al. (2025) reported that hydrolysates of shrimp heads and shells produced with the bromelain enzyme yielded IC₅₀ values of 3.14-4.85 mg/mL by the ABTS method. Another study by Noman et al. (2022) reported that a bromelain-hydrolyzed hybrid sturgeon extract yielded IC₅₀ values of 3.14/3.81 mg/mL using the DPPH and ABTS methods, respectively. Meanwhile, a study by Jafar et al. (2025) reported that protein hydrolysate from milkfish meat, produced using the bromelain enzyme, exhibited an IC₅₀ of 1.58 mg/mL in the DPPH assay.

Fish bone hydrolysate has potential as an antioxidant because it contains aromatic amino acids (Tyrosine, Tryptophan, and Phenylalanine), which were identified through qualitative tests. Aromatic amino acids, such as tyrosine and phenylalanine, can donate electrons to stabilize free radicals, thereby increasing biological activity (Botutihe et al., 2024). Aromatic amino acids act as antioxidants because the structure of their aromatic rings can neutralize free radicals. Tyrosine is considered the most effective thanks to its hydroxyl (-OH) group, which can donate a hydrogen atom directly to free radicals, thereby forming stable tyrosyl radicals. Tryptophan also exhibits high radical-capture activity, supported by its electron-rich indole rings, which enable hydrogen transfer and stabilize radicals through resonance. In contrast, phenylalanine with a simple benzene ring has lower antioxidant activity. It tends to play a role via a different mechanism: radical suppression through non-covalent electron transfer. Although their effectiveness varies, the three aromatic amino acids still use their ring structures to neutralize free radicals and protect cells from oxidative damage (Korczyk et al., 2018).

Conclusions

This study demonstrates that protein hydrolysates from skipjack tuna (*Katsuwonus pelamis*) bones, whether raw or cooked, exhibit antioxidant activity as measured by the ABTS method. The highest activity was observed in the cooked bone sample with a maximum concentration. The presence of aromatic amino acids (tyrosine, tryptophan, phenylalanine), detected by qualitative testing, plays a crucial role in the free-radical scavenging mechanism. This study has limitations regarding the variation in enzyme concentration, which was limited to the result, and further characterization of the types of bioactive peptides produced has not yet been conducted. Consequently, skipjack tuna bone waste has potential as a source of natural antioxidants for functional foods or supplements. Further research is recommended using a wider range of enzyme concentrations and hydrolysis times, antioxidant activity assays using alternative methods (DPPH, FRAP), and in vivo studies to confirm safety and efficacy. Broader implication: cooked bones showed higher antioxidant activity than raw bones, proving that thermal processing does not reduce peptide bioactivity and increases the real-world feasibility of utilizing fishery bone waste from households and food industries.

Conflict of Interest

The author has no conflict of interest.

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