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Research Article

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# Potential Antioxidant Activities from Milkfish Bone with Different Retorting Time

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## Abstract

Bioactive peptides derived from fish-processing by-products have attracted considerable attention as natural antioxidants. This study investigated the production and antioxidant properties of hydrolysates obtained from milkfish (*Chanos chanos*) bone through high-temperature extraction at 121 °C for 30, 60, 90, and 120 min. The hydrolysates were centrifuged, neutralized with phosphoric acid, freeze-dried, and evaluated for molecular weight distribution, degree of hydrolysis (DH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, and ferrous ion-chelating ability. The dried hydrolysates contained 85.10 - 89.27% protein with DH values ranging from 67.88% - 72.10%. The hydrolysate extracted for 30 min was mainly distributed within the 30 - 70 kDa molecular weight range, whereas longer extraction times predominantly produced peptides with molecular weights below 14.4 kDa. The hydrolysate obtained after 90 min exhibited the highest DPPH radical-scavenging activity (98.50 ± 0.89% at 12 mg/mL), whereas the hydrolysate produced after 30 min showed the greatest ferrous ion-chelating ability (80.86 ± 0.57% at the same concentration). Further prolongation of retorting time did not improve antioxidant performance. These findings indicate that controlled high-temperature extraction effectively generates milkfish bone hydrolysates with strong antioxidant potential, supporting their potential application as functional ingredients in food and nutraceutical products.

## Keywords

Antioxidant activity, Hydrolysate, Milkfish bone

## Introduction

Natural antioxidants that are effective, safe, and derived from food sources have attracted considerable research interest in recent years. In addition to widely recognized natural antioxidants such as vitamins, carotenoids, and bioflavonoids, bioactive peptides have also demonstrated promising antioxidant mechanisms [1, 2]. Compared with intact proteins, bioactive peptides possess smaller molecular structures that contribute to greater biological diversity and enhanced antioxidant potential. Furthermore, these peptides are generally considered safe and easily digestible [3-5]. Consequently, fish-processing by-products have increasingly been explored as alternative sources of bioactive peptides during the past decade.

Fish by-products are commonly processed into fish protein hydrolysates (FPH) prior to fractionation into specific bioactive components. FPH exhibits several desirable functional properties, including water-holding capacity, gel formation, emulsifying ability, and texture improvement [6-10]. In addition to their functional characteristics, FPH-derived peptides have been associated with

various biological activities, including antioxidant, anticancer, and angiotensin-converting enzyme (ACE) inhibitory effects [11-16].

Despite the growing interest in fish-derived hydrolysates, studies on the production of hydrolysates from milkfish by-products using retorting technology remain limited. Previous studies have reported that hydrolysates derived from milkfish scales exhibit antioxidant and ACE-inhibitory activities, potentially associated with a high DH, low molecular weight peptides, and elevated levels of hydrophobic amino acids [17]. Nevertheless, milkfish bone remains underutilized despite its considerable potential as a raw material for value-added functional products, including collagen, gelatin, and protein hydrolysates.

Milkfish bone contains collagen characterized by a unique amino acid composition, particularly high levels of glycine, alanine, and proline, which are commonly found in collagenous materials. In addition, elevated concentrations of hydroxylysine and hydroxyproline have also been identified in fish collagen, similar to those observed in collagen from terrestrial animal sources [18]. Previous studies have shown that membrane fractionation can influence peptide molecular weight distribution and bioactivity. Peptide fractions with lower molecular weights and enriched in amino acids such as alanine, histidine, leucine, phenylalanine, proline, and tyrosine tend to exhibit stronger radical-scavenging activity compared with larger peptide fractions [19]. Several amino acids, including tyrosine, methionine, histidine, lysine, proline, and tryptophan, are also recognized for their antioxidant properties, particularly when present within peptide structures [20-24].

Hydrolysates can be produced through retorting at temperatures ranging from 115 to 121 °C. Previous findings demonstrated that retorting temperature significantly affects antioxidant activity [25]. Retorting promotes collagen denaturation and thermal hydrolysis, thereby facilitating hydrolysate formation [26]. Moreover, high-pressure hydrolysis has been reported to accelerate the release of antioxidant compounds and enhance the antioxidant capacity of hydrolysates [27].

Therefore, the present study aimed to produce and characterize hydrolysates derived from milkfish bone. In addition, the antioxidant activities of hydrolysates produced under different retorting durations were evaluated.

## Materials and Methods

Milkfish (*C. chanos*) used in this study were obtained from aquaculture ponds in Sidoarjo, Indonesia. Following harvest, the fish were transported on ice to a processing facility in Surabaya, where the bones were collected as processing by-products. The samples were subsequently transported to the laboratory in insulated containers packed with ice. Upon arrival, the bone by-products were separated, packed in polyethylene bags, and stored at -20 °C until further use. Prior to hydrolysate preparation, the frozen bones were thawed under running tap water, manually cleaned to remove adhering flesh, cut into smaller pieces, and subjected to retorting treatment.

Hydrolysates were prepared according to the method of Yang et al. [26] with slight modifications. Initially, the bones were treated with 0.1 M sodium hydroxide to remove fat and non-collagenous proteins. The samples were then retorted at 121 °C using a water-to-bone ratio of 2:1 (v/w) for 30, 60, 90, and 120 min. After retorting, the softened bones were homogenized and centrifuged at 8,000 rpm for 15 min at 4 °C. The supernatant fraction was collected and treated with calcium hydroxide, followed by a second centrifugation step under the same conditions. The resulting supernatant was subsequently neutralized using phosphoric acid prior to further precipitation and centrifugation.

The study evaluated hydrolysates produced under four different retorting times (30, 60, 90, and 120 min). The hydrolysates obtained from each treatment were subsequently characterized for proximate composition, DH, molecular weight distribution, DPPH radical-scavenging activity, and ferrous ion-chelating ability.

Nitrogen content was determined using the Kjeldahl method according to Association of Official Analytical Chemists. Protein content was calculated using a nitrogen-to-protein conversion factor of 5.95 and expressed on a dry-weight basis. All analyses were performed in triplicate.

DH was determined using the o-phthaldialdehyde (OPA) method described by Church et al. [28] with slight modifications. Freshly prepared OPA reagent was used for analysis. Hydrolysate samples were mixed with OPA reagent and incubated for 2 min before measuring absorbance at 340 nm using a spectrophotometer. A 1.0 mM Leu-Gly solution was used to prepare the standard curve (Figure 1), while gelatin extracted at 50 °C for 1 h served as the control sample (Equation 1).

$$\text{DH (\%)} = (A/B) \times 100 \quad (1)$$

Where, A represents the absorbance of the sample at 340 nm, and B represents the absorbance of the control at 340 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to the discontinuous tris-hydrochloride/glycine buffer system described by Laemmli [29], using a 12.5% resolving gel and a 4% stacking gel. Hydrolysate samples were dissolved in a sample buffer containing glycerol, tris-hydrochloride, SDS, and β-mercaptoethanol, followed by heating for 5 min prior to

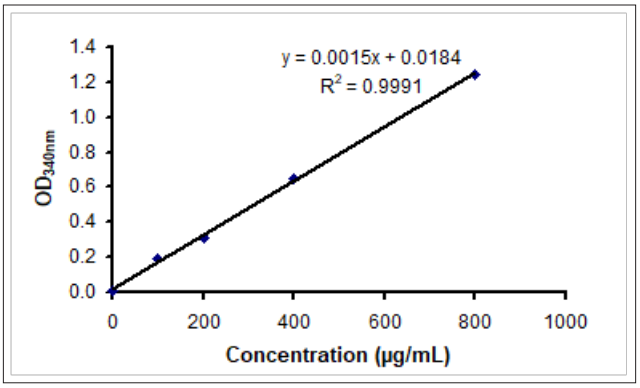


**Figure 1:** Standard curve of Leu-Gly by OPA method.

electrophoresis. Electrophoresis was conducted at a constant current of 20 mA. Protein bands were visualized using Coomassie Brilliant Blue R-250 staining solution, followed by destaining with a methanol–acetic acid solution.

DPPH radical-scavenging activity was determined according to the method of Shimada et al. [30] with slight modifications. Sample solution was mixed with 0.1 mM DPPH prepared in 95% ethanol and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a spectrophotometer. Vitamin C (1,000 parts per million (ppm)) was used as a positive control. Radical-scavenging activity was calculated using equation 2.

$$\text{Scavenging effect (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100 \quad (2)$$

Where, $A_{blank}$ represents the absorbance of the blank, and $A_{sample}$ represents the absorbance of the sample, both measured at 517 nm

Ferrous ion-chelating ability was evaluated according to the method of Decker and Welch [21] with slight modifications. The sample solution was mixed with ferrous chloride solution and ferrozine solution, followed by incubation at room temperature for 10 min. Absorbance was subsequently measured at 562 nm. Ethylenediaminetetraacetic acid (EDTA) (1,000 ppm) was used as the positive control. Chelating ability was calculated according to equation 3.

$$\text{Chelating effect (\%)} = [1 - (A_{sample} / A_{blank})] \times 100 \text{ -----} \quad (3)$$

Where, $A_{sample}$ represents the absorbance of the sample, and $A_{blank}$ represents the absorbance of the blank, both measured at 562 nm.

All analytical measurements were performed in triplicate, and the results are expressed as mean ± standard deviation. Statistical analyses were performed using one-way analysis of variance, followed by Duncan's multiple range test to determine significant differences among treatments at the 95% confidence level ($p < 0.05$). Statistical analyses were conducted using IBM Statistical Package for the Social Sciences Statistics version 22.0.

# Results

## Characterization of milkfish bone hydrolysates

The protein contents of the freeze-dried hydrolysates after retorting for 30, 60, 90, and 120 min were 85.67%, 88.47%, 85.10%, and 89.27% (dry basis), respectively (Table 1). The visual appearance of the obtained hydrolysates is shown in figure 2.

The protein content increased significantly after 60 and 120 min of retorting ($p < 0.05$), indicating that prolonged thermal treatment enhanced the recovery of soluble proteinaceous components. This increase may be attributed to enhanced protein solubilization during prolonged retorting. However, a slight reduction was observed at 90 min, although the values were not significantly different from those obtained at 30 min. This decrease may reflect experimental variation or partial thermal degradation during prolonged heating.

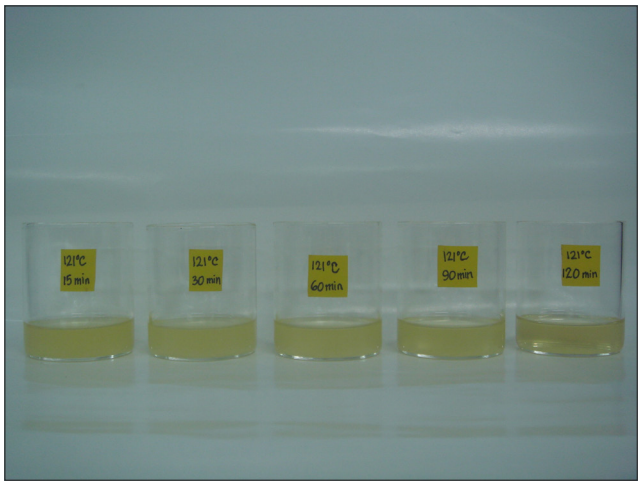


**Figure 2:** Appearance of retorted milkfish bone hydrolysates.

## DH and molecular weight of milkfish bone hydrolysate

The DH increased progressively with increasing retorting time at 121 °C (Figure 3).

The DH values obtained after 30, 60, 90, and 120 min of retorting were 67.88%, 69.26%, 70.58%, and 72.10%, respectively. A significant increase in DH was observed with increasing retorting duration ($p < 0.05$). The highest DH value was achieved after 120 min, suggesting that prolonged retorting promoted greater protein hydrolysis and peptide generation.

The molecular weight distribution of the hydrolysates was evaluated using SDS-PAGE (Figure 4). Hydrolysates produced after 30 min of retorting exhibited protein bands predominantly between 30 and 70 kDa.

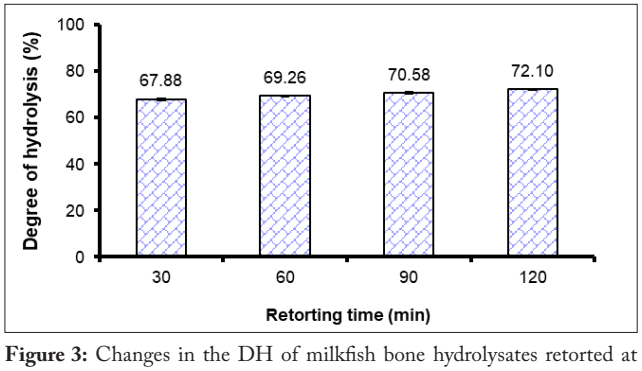


**Figure 3:** Changes in the DH of milkfish bone hydrolysates retorted at 121 °C for different durations. Gelatin extracted at 50 °C for 1 h was used as the control.

**Table 1:** Protein contents of milkfish bone hydrolysates produced at different retorting times.

| Index | Retorting time (min) | | | |
|---|---|---|---|---|
| | 30 | 60 | 90 | 120 |
| Protein content (% dry basis) | 85.67[a] | 88.47[b] | 85.10[a] | 89.27[b] |

**Note:** a, b values within the same row followed by different superscript letters differ significantly ($p < 0.05$) according to Duncan's multiple range test.

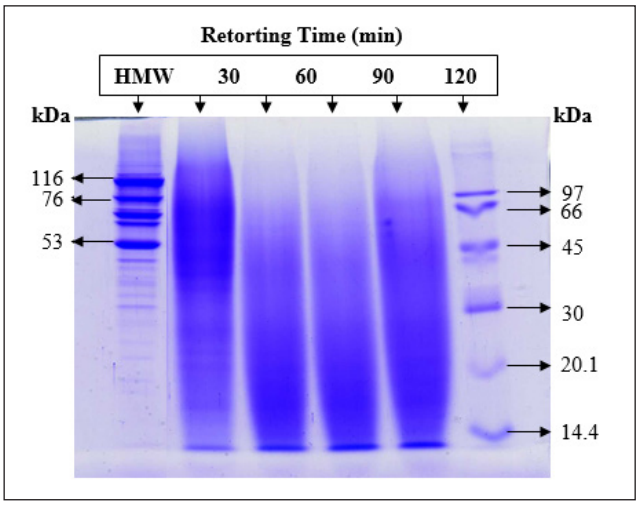


**Figure 4:** SDS-PAGE pattern of milkfish bone hydrolysates with different retorting time, as controls are high molecular weight and low molecular weight in 12.5% gel.

In contrast, hydrolysates obtained after 60, 90, and 120 min were predominantly composed of peptide fractions with molecular weights below 14.4 kDa. These findings indicate that prolonged retorting promoted greater protein hydrolysis, resulting in the generation of smaller peptide fragments.

## Antioxidant activities of hydrolysate

The antioxidant properties of the hydrolysates were evaluated using two complementary antioxidant assays. Hydrolysate samples were dissolved in distilled water at protein concentrations ranging from 2 to 20 mg/mL. Vitamin C and EDTA were used as positive controls for radical-scavenging and metal-chelating activities, respectively.

## DPPH radical-scavenging activity

The DPPH radical-scavenging activities of hydrolysates prepared under different retorting times are shown in figure 5.

At a concentration of 12 mg/mL, the hydrolysate obtained after 90 min of retorting exhibited the highest DPPH radical-scavenging activity (98.50%), which was significantly higher ($p < 0.05$) than those of hydrolysates retorted for 30 min (78.57%), 60 min (84.88%), and 120 min (74.12%). These results suggest that retorting for 90 min produced hydrolysates with the greatest free radical-scavenging capacity among the treatments evaluated.

## Ferrous ion-chelating ability

The ferrous ion-chelating activities of hydrolysates produced at different retorting times are presented in figure 6.

At 12 mg/mL, the hydrolysate retorted for 30 min exhibited the highest ferrous ion-chelating ability (80.86%), which was significantly higher ($p < 0.05$) than those of hydrolysates retorted for 60 min (54.75%), 90 min (59.71%), and 120 min (50.88%). Moreover, the chelating ability of the 30 min hydrolysate was comparable to that of the EDTA positive control (1,000 ppm).

These results indicate that shorter retorting durations were more effective in preserving metal-chelating peptides, whereas prolonged thermal treatment may have reduced the chelating activity of the resulting hydrolysates.

# Discussion

The proximate composition analysis demonstrated that milkfish by-products, including bone, skin, and fins, still contained considerable nutritional value, particularly protein

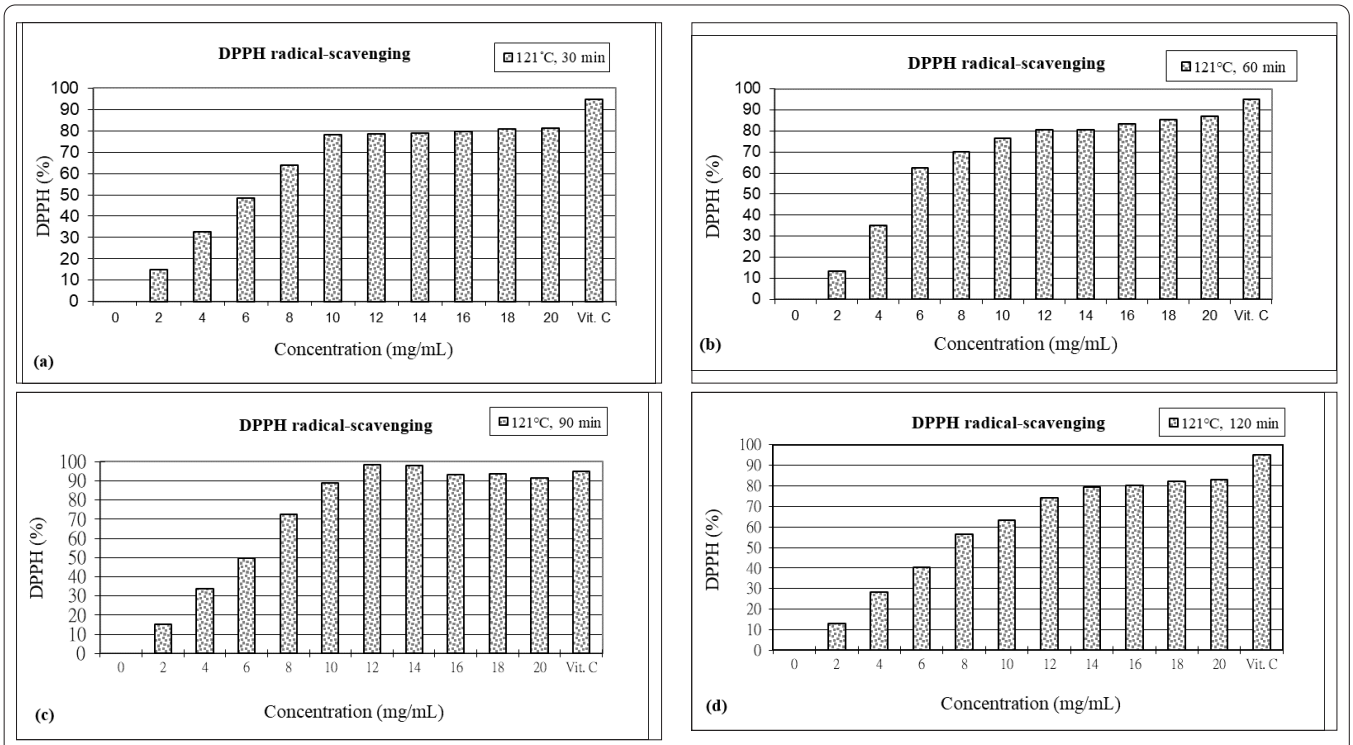


**Figure 5:** DPPH radical-scavenging activity of hydrolysates produced at different retorting times **(a)** 30 min, **(b)** 60 min, **(c)** 90 min, and **(d)** 120 min. Vitamin C (1,000 ppm) was used as the positive control. Values are expressed as the mean ± standard deviation of triplicate analyses.

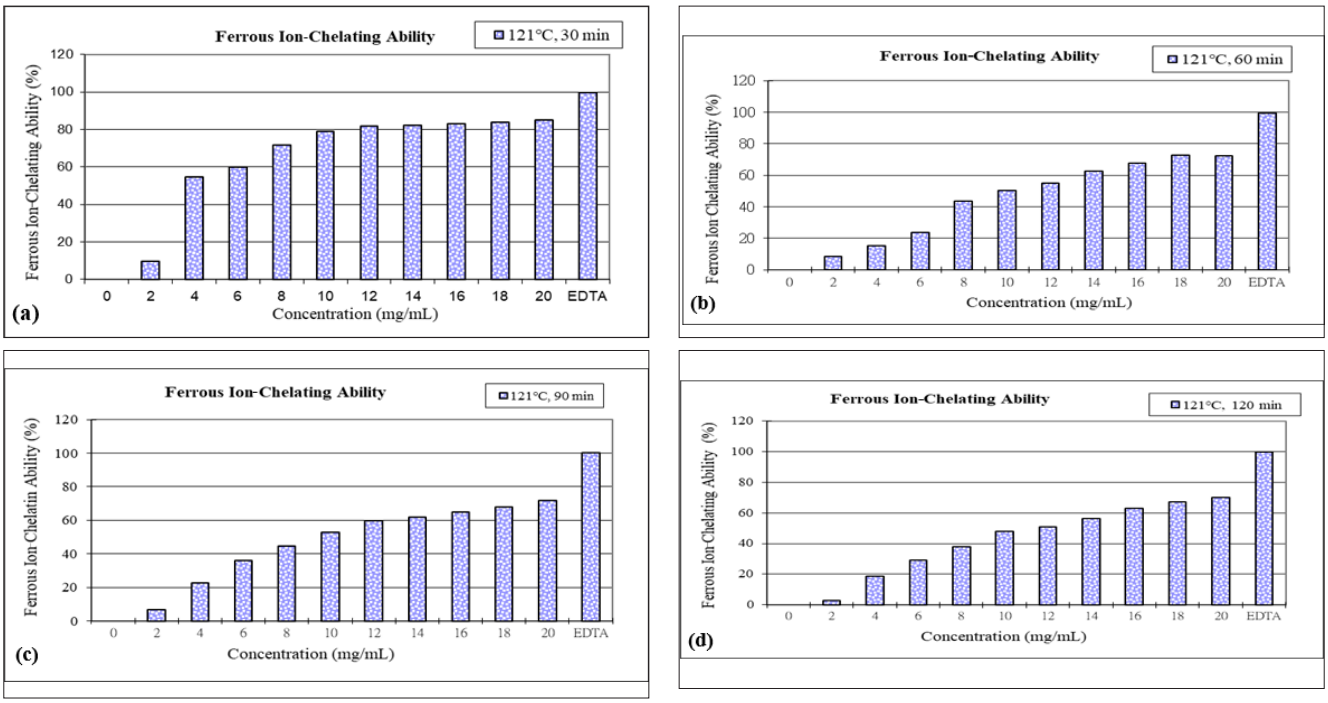


**Figure 6:** Ferrous ion-chelating ability of hydrolysates produced at different retorting times **(a)** 30 min, **(b)** 60 min, **(c)** 90 min, and **(d)** 120 min. EDTA (1,000 ppm) was used as the positive control. Values are expressed as the mean ± standard deviation of triplicate analyses.

and mineral contents. Moisture content ranged from 58.27% to 62.60%, while protein content varied between 16.72% and 32.54%. The bone fraction contained 16.72% protein, indicating its potential as a raw material for hydrolysate production. Fish by-products are recognized as valuable sources of proteins, essential amino acids, minerals, and other bioactive compounds that can be utilized in food and nutraceutical applications [32]. Therefore, the valorization of milkfish processing waste into functional hydrolysates may provide both economic and environmental benefits. Similar findings were reported by Fan et al. [33], who suggested that fish by-products possess promising potential for the development of value-added bioactive products when processed appropriately.

Following alkaline pretreatment and retorting extraction, collagen and other structural proteins in the milkfish bone underwent thermal degradation and hydrolysis. Retorting under high-temperature and high-pressure conditions is known to facilitate collagen denaturation and peptide release, thereby enhancing hydrolysate production. Yang et al. [28] reported that thermohydrolysis during retorting effectively converts collagen into smaller peptide fractions. In addition, retorting conditions strongly influence the physicochemical and functional characteristics of FPH, including their antioxidant properties [25].

The DH progressively increased with longer retorting times, reaching the highest value (72.10%) after 120 min at 121 °C. These results indicate that prolonged thermal treatment promoted more extensive cleavage of peptide bonds, resulting in the formation of smaller peptides. Previous studies have demonstrated that the DH directly affects peptide size distribution, hydrophobicity, solubility, and biological functionality [34]. Hydrolysates with high DH generally exhibit improved solubility and enhanced bioactivity due to the increased exposure of functional amino acid residues [4]. Huang et al. [17] further reported that hydrolysates with high DH and low molecular weight fractions showed stronger antioxidant and ACE-inhibitory activities, particularly when enriched with hydrophobic amino acids.

The SDS-PAGE analysis supported the DH results by showing a clear reduction in molecular weight distribution with increasing retorting duration. Hydrolysates produced after 60, 90, and 120 min predominantly consisted of peptides smaller than 14.4 kDa, whereas the 30 min treatment still contained higher-molecular-weight proteins. Similar observations were reported by Yang et al. [26], who found that retorting treatment effectively degraded cobia skin gelatin into low molecular weight antioxidant peptides. Smaller peptides are generally considered more bioactive because they can more easily penetrate cell membranes and interact with free radicals or oxidative targets [35]. Therefore, the formation of low molecular weight peptides during retorting may contribute to the antioxidant potential observed in this study.

Among all treatments, the hydrolysate obtained after 90 min of retorting exhibited the highest DPPH radical-scavenging activity at a concentration of 12 mg/mL. Interestingly, antioxidant activity decreased after 120 min despite the higher DH value. This finding suggests that excessive thermal treatment may negatively affect antioxidant peptides through peptide degradation or modification of amino acid residues responsible for radical-scavenging activity. Similar trends were previously reported in gelatin hydrolysates from Cobia skin and Bullfrog skin, where antioxidant activity peaked at intermediate retorting durations rather than prolonged heating conditions [26]. In addition, Kuda and Yano [36] proposed that antioxidant activity in retorted fish products may also be associated with Maillard reaction

products formed during thermal processing.

The DPPH assay is widely used to evaluate the hydrogen-donating capacity of antioxidant compounds. Peptides capable of donating protons or electrons can neutralize DPPH radicals and reduce oxidative reactions. Fish-derived peptides are considered promising antioxidant agents because of their amino acid composition and peptide sequences. Certain amino acids, particularly methionine and hydrophobic residues, have been associated with strong radical-scavenging activity [37]. Therefore, the enhanced DPPH scavenging activity observed in the 90 min hydrolysate may be related to the formation of optimal peptide structures with suitable molecular size and amino acid composition.

Metal-chelating ability represents another important antioxidant mechanism because transition metals such as ferrous ion can catalyze lipid oxidation and promote the formation of reactive oxygen species. In this study, all hydrolysates demonstrated ferrous ion-chelating ability, although the ability decreased as retorting time increased. The hydrolysate produced after 30 min exhibited the strongest chelating ability, which was comparable to the EDTA positive control. These findings indicate that shorter retorting durations may preserve peptide structures or functional groups involved in metal binding more effectively than prolonged heating treatments.

The reduction in chelating ability with longer retorting times may be associated with excessive peptide fragmentation. Smaller peptides generated during prolonged hydrolysis may possess lower affinity for metal ions compared to larger peptide structures containing more accessible amino, carboxyl, and imidazole groups. Xing et al. [38] similarly reported that higher molecular weight sulfated chitosan exhibited stronger ferrous ion-chelating ability than lower molecular weight fractions. Amino acids such as glutamic acid, aspartic acid, histidine, lysine, and arginine are known to contribute significantly to metal ion binding through their side-chain functional groups [39]. Comparable antioxidant and metal chelating activities have also been reported in hydrolysates derived from bighead carp bones [16].

Overall, the present findings demonstrate that retorting time plays a crucial role in determining the physicochemical characteristics and antioxidant activities of milkfish bone hydrolysates. A moderate retorting duration enhanced radical-scavenging activity, whereas a shorter retorting time was more favorable for metal-chelation ability. These differences may be attributed to variations in peptide size distribution and structural characteristics generated during thermal hydrolysis.

From a practical perspective, the present findings demonstrate that milkfish bone by-products can serve as an inexpensive and sustainable raw material for producing antioxidant-rich protein hydrolysates. The use of high-temperature retorting without enzymatic hydrolysis offers a simple and economically feasible processing approach that may be readily adopted by the fish-processing industry. The hydrolysates obtained under the optimal retorting conditions have potential applications as natural antioxidant ingredients in functional foods, nutraceuticals, and other value-added products, thereby contributing to the sustainable utilization of fish-processing by-products.

This study has several limitations. The antioxidant activity was evaluated using *in vitro* assays only, and the specific amino acid composition and peptide sequences responsible for the observed bioactivity were not characterized. Consequently, the relationship between peptide structure and antioxidant mechanism could not be fully elucidated. Future studies involving amino acid profiling, peptide identification using liquid chromatography-tandem mass spectrometry, and evaluation of antioxidant stability in food systems are recommended to further characterize the bioactive components and support their industrial application.

## Conclusion

Milkfish bone hydrolysates were successfully produced using high-temperature retorting at different extraction durations. Retorting time significantly influenced the physicochemical characteristics and antioxidant properties of the resulting hydrolysates. Among all treatments, the hydrolysate obtained after 90 min of retorting exhibited the highest DPPH radical-scavenging activity, indicating its potential as a natural antioxidant ingredient for functional food applications.

The present study also demonstrated that high-temperature retorting can effectively generate bioactive peptides from milkfish bone through a relatively simple extraction process. Prolonged retorting increased the DH and promoted the formation of low-molecular-weight peptides, which may have contributed to the enhanced antioxidant activity. However, different antioxidant mechanisms responded differently to retorting duration, with shorter retorting times exhibiting greater ferrous ion-chelating ability.

Overall, these findings highlight the potential of milkfish bone, an underutilized fish-processing by-product, as a sustainable raw material for producing antioxidant-rich protein hydrolysates for functional food and nutraceutical applications. The proposed high-temperature retorting process provides a simple and economically feasible approach for converting fish-processing waste into value-added functional ingredients. Further studies involving enzymatic hydrolysis, peptide purification, amino acid profiling, peptide sequencing, and *in vivo* antioxidant evaluation are recommended to further elucidate and optimize the bioactive properties of these hydrolysates.

## Acknowledgements

None.

## Conflict of Interest

None.

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