Research Article

Bach Trong Nguyen*, Ngan Hong Nguyen, Cuc Thi Kim Nguyen, Huy Minh Do, Bao Nguyen, Witoon Prinyawiwatkul, and Bao Nguyen Duy Huynh

Antioxidant potential of peptide fractions from tuna dark muscle protein isolate: A green enzymatic approach

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Abstract: Tuna dark muscle protein isolate (TDMPI) derived from yellowfin tuna (*Thunnus albacares*) represents a sustainable by-product for bioactive peptide production. In this study, TDMPI was hydrolysed using alcalase, an eco-friendly enzymatic approach, to generate antioxidant-rich hydrolysates. These hydrolysates contained a diverse profile of hydrophobic and negatively charged amino acids, which contributed to their antioxidant properties. Ultrafiltration was employed to fractionate the hydrolysates into three peptide fractions, which were tested for 2,2-diphenyl-1-picrylhydrazyl free radical scavenging (DPPH-RSA), lipid peroxidation inhibition (LPIA), and total reducing power (TRPA). Results showed that peptides <10 kDa exhibited higher antioxidant activity than the unfractionated hydrolysates, with peptides <3 kDa exhibiting the highest DPPH-RSA and TRPA. However, LPIA was higher in peptides >10 kDa. Hydrolysis time significantly affected antioxidant activity: DPPH-RSA increased up to 9 h, while LPIA peaked at 6 h and TRPA at 3 h before declining. This study demonstrated that TDMPI hydrolysates and their peptide fractions exhibited significant antioxidant activities, making them promising natural alternatives to synthetic preservatives. Their utilisation potentially supports functional food applications and aligns with green processing strategies for valorising fishery by-products.

Keywords: antioxidant activities, bioactive peptides, by-product, enzymatic treatment, tuna dark muscle

1 Introduction

Tuna dark muscle represents a significant fraction of the tuna processing industry. Although tuna dark muscle is nutritionally rich, it is often considered a low-value product due to its poor sensory characteristics, limiting its direct application in food products. The tuna dark muscle contains a significant amount of myoglobin (Mb) and unsaturated fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid, which make it highly susceptible to oxidation, especially during hydrolysis. Direct utilisation of tuna dark muscle in food products is limited due to its high susceptibility to oxidation. The tuna dark muscle can be used as sausage ingredients, hydrolysed products, fish meal, etc. A promising approach to valorising tuna dark muscle is enzymatic hydrolysis to generate bioactive peptides with antioxidant properties, contributing to sustainable food processing. These antioxidants can replace synthetic antioxidants, especially in the field of food processing and preservation.

Food oxidation reduces consumer acceptability and may be harmful to human health by deteriorating nutritional and sensory qualities, especially color and flavor. This degradation is accelerated by lipid peroxidation, which is a major concern for the food industry. Concerns over the potential health risks of synthetic antioxidants, such as their possible carcinogenic effects, have driven interest in natural, eco-friendly alternatives derived from plants and marine sources. Natural antioxidants derived from plants [1–4] and aquatic sources [1,5] have shown promise, especially peptides extracted from seafood by-products [6–13]. Among these, peptides with antioxidant

* **Corresponding author: Bach Trong Nguyen,** Faculty of Food Technology, Nha Trang University, Khanh Hoa, 57000, Vietnam, e-mail: ntbachnt@ntu.edu.vn
Ngan Hong Nguyen, Bao Nguyen, Bao Nguyen Duy Huynh: Faculty of Food Technology, Nha Trang University, Khanh Hoa, 57000, Vietnam
Cuc Thi Kim Nguyen: Institute of Biotechnology and Environment, Nha Trang University, Khanh Hoa, 57000 Vietnam
Huy Minh Do: Department of Analytical Chemistry, Faculty of Chemistry, University of Science, 227 Nguyen Van Cu, District 5, Ho Chi Minh City, 70000, Vietnam; Viet Nam National University, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, 70000, Vietnam
Witoon Prinyawiwatkul: School of Nutrition and Food Sciences, Louisiana State University, Agricultural Center, Baton Rouge, LA, 70803, United States of America

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properties have been successfully prepared from various fish processing by-products, such as yellowfin tuna viscera [9], head of tuna (*Thunnus albacares*), parrotfish (*Chlorurus sordidus*), and Tra catfish [7,13,14], tuna eye (*Thunnus* sp.) [8], skin of common carp fish [11], dried squid head [10], backbone blend of Tra catfish [13], dark muscle of Tra fish, tuna dark meat [13,15], abalone viscera [12], and fish milt and roe [16].

Enzymatic hydrolysis is an environmentally friendly and efficient approach to protein valorisation, reducing chemical usage while enhancing the functionality of derived peptides. Among protease enzymes, alcalase is commonly used to hydrolyse fish protein to produce peptides with high antioxidant activity due to its strong hydrolytic ability and its capability to generate short-chain peptides rich in hydrophobic amino acids such as leucine, valine, and proline, which are known for their free radical scavenging and lipid peroxidation inhibition properties [17]. As a serine endopeptidase, alcalase works effectively at pH 7–9 and temperatures of 50–60°C, which are suitable for hydrolysing seafood proteins and optimising the recovery of functional peptides [18]. Furthermore, studies have demonstrated that peptides produced using alcalase exhibit strong metal ion chelating properties, further enhancing their antioxidant activity [19]. Antioxidant activity in these peptides depends on hydrolysis parameters such as degree of hydrolysis (DH) [20], hydrolysis duration [10,13], and the molecular weight of the peptide fragment formed [12,15,21]. According to Nguyen et al. [13], increasing the hydrolysis time increased TRPA and 2,2-diphenyl-1-picrylhydrazyl free radical scavenging (DPPH-RSA) until a certain point, after which they decreased. Likewise, Saidi et al. [21] and Unnikrishnan et al. [15] demonstrated that peptide fractions with a low molecular weight (<3 kDa) had greater antioxidant potential.

In this study, Alcalase was selected as the proteolytic enzyme for hydrolysing tuna dark muscle protein isolate. Alcalase is a serine endopeptidase from *Bacillus licheniformis* that is well known for its broad substrate specificity and high efficiency in hydrolysing fish proteins. Compared to other enzymes such as papain or trypsin, Alcalase has been widely used to produce peptides with strong antioxidant properties due to its ability to generate low-molecular-weight peptides [22]. Furthermore, several studies have demonstrated that Alcalase-treated fish protein hydrolysates exhibited enhanced bioactivity, making it a suitable choice for the valorisation of fish by-products [23].

Lipid oxidation, primarily driven by the conversion of Mb to metmyoglobin (metMb), is a major factor contributing to quality deterioration in tuna dark muscle [24]. Tuna dark muscle is often considered a low-value product due to its high lipid and Mb contents, which make it more susceptible to oxidation and off-flavor development. This degradation is further intensified during enzymatic hydrolysis, which generates undesirable by-products and diminishes the antioxidant capacity of the resulting hydrolysates. To mitigate these challenges, the pH shift method has emerged as a promising pre-treatment strategy. By adjusting the pH to solubilise and then precipitate proteins, this technique facilitates the removal of lipids and Mb prior to hydrolysis, thereby improving the purity and oxidative stability of the protein isolate. The resulting protein is more suitable for the production of antioxidant peptides with enhanced functional properties. However, the pH shift process may alter protein charge and conformation, which can influence hydrolysis efficiency and conditions compared to untreated fish muscle proteins.

The purpose of this study was to evaluate the effect of enzymatic hydrolysis time on the antioxidant activity of peptides prepared from yellowfin tuna dark muscle using an innovative approach that included protein isolation via the pH shift method, hydrolysis of isolated protein by the alcalase enzyme, and fractionation of protein hydrolysate using molecular weight cut-off (MWCO) membranes. The study emphasised the potential of underutilised tuna by-products used to produce bioactive peptides as a sustainable alternative to synthetic antioxidants.

2 Materials and methods

2.1 Materials

The yellowfin tuna *(Thunnus albacares)* dark muscle, provided by Hai Vuong Group (Khanh Hoa province, Vietnam), was used to prepare TDMPI using the pH-shift method as described by Nguyen et al. [25]. The fresh TDMPI contained 77.30 ± 0.87% moisture, 20.10 ± 0.30% protein, 0.66 ± 0.06% lipid, and 1.40 ± 0.04% ash. Fresh TDMPI was vacuum-packed before freezing at −45°C and storage at −20 ± 2°C.

2.2 Chemicals

Alcalase 2.4L FG (activity: 2.4 AU-A/g) was purchased from Novozymes (Bagsværd, Denmark) and stored at 4 ± 1°C until use. metMb, EPA, thiobarbituric acid (TBA), DPPH, and amino acid standards (HPLC grade) were purchased from Sigma-Aldrich Company (Missouri, USA). Other chemicals and reagents used in this study were of analytical grade.

2.3 Preparation of protein hydrolysate and peptide fractions

Forty grams of TDMPI was homogenised with 60 mL of distilled water (pH = 8) and pre-incubated at 55°C for 30 min prior to enzymatic hydrolysis. Enzyme (Alcalase 2.4 L FG) was added with an enzyme-to-substrate ratio (E/S) of 1% (wt%/wt%). The hydrolysis reaction was performed at different hydrolysis times (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 h) in a water bath (Memmert, Germany) at 55 ± 0.1°C. During hydrolysis, the pH of the mixture was maintained constantly at the value of 8 by the addition of 1 N NaOH. Then, the hydrolysate mixture was placed in a water bath at 90°C for 20 min to inactivate the enzyme. The hydrolysate was centrifuged at 10,000 rpm for 30 min at 4°C using a refrigerated centrifuge (Hettich Mikro 22R, Germany) to remove the unhydrolysed residue. A portion of the supernatant obtained was used for direct analysis or peptide fraction separation.

The peptide fractions were separated based on the MWCO technique using Amicon® Ultra-15 membrane 3 and 10 kDa (PLBC Ultracel-PL membrane, Merck Millipore Corporation, Darmstadt, Germany). The hydrolysate was added to a pre-equilibrated 10 kDa MWCO membrane and centrifuged at 13,000 rpm for 30 min at 4°C to obtain the filtrate (fractions of Mw < 10 kDa) and the fractions with a molecular weight of higher 10 kDa. Then, the filtrate was added to a pre-equilibrated 3 kDa MWCO membrane and refrigerated centrifuged at 13,000 rpm for 30 min at 4°C to separate the filtrate (fractions of Mw < 3 kDa) and fractions with 10 kDa > Mw > 3 kDa. The obtained peptide fractions were determined for their antioxidant activities.

Protein hydrolysate and peptide fractions were standardised to a protein content of 1 mg/mL prior to use in all antioxidant assays, including DPPH-RSA, TRPA, and LPIA.

2.4 Determination of protein recovery (PR)

The PR from protein hydrolysates at different hydrolysis times was calculated using Eq. 1 and reported as a percentage:

$$\mathrm{PR}(\%) = \frac{\text{Protein content of hydrolysate}}{\text{Total protein content of TDMPI}} \times 100 \quad (1)$$

The protein content of the hydrolysates was determined according to the Lowry method described by Nalinanon et al. [26]. The protein content was quantified from a standard curve made with bovine serum albumin with concentrations ranging from 0.1 to 1.5 g/L. The protein content of the TDMPI was measured using the Lowry method according to the method described by Maehre et al. [27]. One gram TDMPI sample was homogenised with 60 mL of 0.1 M sodium hydroxide in a 3.5% sodium chloride solution. The homogenate was incubated in a water bath at 60°C for 90 min, followed by centrifugation at 4°C for 30 min at 5,000 rpm (Hettich Mikro 22R, Germany).

2.5 Determination of DH

The DH was determined by the dinitrofluorobenzene (DNFB) method, as described by Nguyen et al. [13]. DH was defined as the ratio between the number of broken peptide bonds (h) and the total number of peptide bonds per mass unit (h_{tot}) as shown below:

$$\mathrm{DH}(\%) = \frac{h}{h_{\mathrm{tot}}} \times 100 \quad (2)$$

where h is determined by measuring the amount of free α-amino group formed in the hydrolysed protein products based on the formation of a yellow complex between the amino groups in the amino acids with a DNFB. The absorbance of the solution was measured at 410 nm by a UV–Vis spectrophotometer (Libra S50 Bio, Cambridge, England). As h_{tot} is the number of peptide bonds, with 8.6 mol of peptide equivalent per kg for fish protein [28], Eq. 2 can be rewritten as

$$\mathrm{DH}(\%) = \frac{A \times D_{\mathrm{F}}}{P \times 8.6} \times 100 \quad (3)$$

where D_{F} is a dilution factor, P is the total protein content (g) in 1 g of the hydrolysate solution, and A indicates the amount of amino groups (mol/L) formed based on a standard curve made with glycine, with concentrations ranging from 0.2 to 1 µM.

2.6 Determination of antioxidant properties

2.6.1 Determination of DPPH-RSA

DPPH-RSA of the peptide was determined according to the procedure described by Bersuder et al. [29] with minor modifications. About 2 mL of the peptide was placed into a test tube containing 1 mL of methanol and 1 mL of 0.1 mM DPPH. The reaction occurred in the dark at room temperature for 30 min, and then the mixture was measured at 517 nm using the UV–Vis spectrophotometer. The control

sample was without the 2 mL of the peptide. DPPH-RSA of the peptide was calculated using the following equation:

$$\text{DPPH} - \text{RSA}(\%) = \frac{A_0 - A}{A_0} \times 100 \quad (4)$$

where A and A_0 are the absorbances of the peptide solution and control sample read at 517 nm, respectively.

2.6.2 Determination of total reducing power ability (TRPA)

TRPA of the peptide was determined according to the procedure described by Oyaizu [30] with minor modifications. A mixture of 0.5 mL of 1% potassium ferricyanide, 0.5 mL of peptide solution, and 0.5 mL of 0.2 M phosphate buffer (pH = 6.6) was mixed in the lidded heat-resistant test tube and kept at 50°C for 20 min. A mixture of 0.5 mL of 10% trichloroacetic acid, 2 mL of distilled water, and 0.4 mL of 0.1% ferric chloride was added to the test tube and was shaken well. TRPA was determined based on the absorbance of the reaction mixture after a 10 min reaction measured at 700 nm using a UV–Vis spectrophotometer. A standard curve was made using a standard vitamin C solution spanning a concentration range of 0–25 mg/L. TRPA was expressed as mg equivalent of vitamin C per g protein of hydrolysate or peptide solution (mg equiv. VTM C/g protein).

2.6.3 Determination of lipid peroxidation inhibition activity (LPIA)

LPIA was determined following the procedure described by Binh et al. [31] with minor modifications. In a heat-resistant test tube, 0.1 mL of the peptide was mixed with 0.1 mL of 50 µM metMb and 0.1 mL of 200 µM hydrogen peroxide in a phosphate buffer (50 mM, pH = 7.4). The reaction was allowed to proceed for 3 min at room temperature under continuous shaking conditions, and then 0.2 mL of Tween 20 containing 50 µM EPA was added. The mixture was vortexed and incubated at 37°C for 30 min. Subsequently, 0.2 mL of the incubated mixture was transferred to another heat-resistant test tube containing 0.5 mL of 1.15% potassium chloride, 3 mL of 1% phosphoric acid, 1 mL of 0.6% TBA, and 0.1 mL of 0.1% butylated hydroxytoluene. The mixture was continuously vortexed and incubated at 95°C for 45 min, then quickly cooled down to room temperature under running tap water. After cooling, 4 mL of *n*-butanol was added to the test tube, and the solution was shaken and allowed to stand for 10 min. The resulting red *n*-butanol layer was separated to measure absorbance at 535 nm using a UV–Vis spectrophotometer. The control sample was prepared similarly but replacing 0.1 mL of peptide with 0.1 mL of *n*-butanol. LPIA was calculated using the following equation:

$$\text{LPIA}(\%) = \frac{A_0 - A}{A_0} \times 100 \quad (5)$$

where A and A_0 are the absorbances of the peptide solution and the control sample at 535 nm, respectively.

2.7 Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Tricine–SDS–PAGE)

The molecular size distribution of peptides from the TDMPI hydrolysate at different hydrolysis times was analysed by Tricine–SDS–PAGE according to Berlian et al. [32] with minor modifications. The peptide samples were mixed at a 1:1 (v/v) ratio with the 2X sample buffer (100 mM Tris-Cl [pH 6.8], 1% [w/v] SDS, 4% [v/v] 2-mercaptoethanol, 0.02% [w/v] Coomassive Brilliant Blue G250, and 24% glycerol), heated at 90°C for 5 min, and then separated in the resolving gel. The resolving gel (15%) and the stacking gel (4%) were in 2.5 M Tris–HCl (pH 8.8). Electrophoresis was performed at 150 V per gel in tank buffer (25 mM Tris, 25 mM Tricine, and 0.05 [w/v] SDS) using the Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Following electrophoresis, the gels were stained with 0.03% Coomassie Blue R250 in 10% (v/v) acetic acid for 4 h. The gel was finally destained with 10% (v/v) acetic acid. Molecular weights of peptides were estimated using the molecular weight standards (Invitrogen, USA) in the range from 3.5 to 260 kDa.

2.8 Determination of amino acid composition

The amino acid composition of the hydrolysates and peptide fractions was determined using an automatic amino acid analyser (Biochrom 30+, Biochrom, Cambridge, UK), according to Wang et al. [33], with minor modifications. One gram of samples was oxidised at 0°C for 16 h with a mixture of performic acid and phenol. The excess oxidation reagent was removed with sodium sulphite. After oxidation, the sample was hydrolysed using 6 mol/L HCl containing 1 g of phenol per L at 110°C for 24 h. The hydrolysate was adjusted to pH 2.2 using 6 mol/L NaOH and citrate buffer, shaken well, and filtered through a 0.22 µm membrane. The

resulting clear solution was subject to ion exchange chromatography using an amino acid analyser. The separated amino acids were reacted with ninhydrin in a high-temperature reaction coil. Each amino acid was quantified at 570 and 440 nm.

The tryptophan analysis was performed as described in the ISO method 13904:2016 [34] with some modifications. The sample was hydrolysed under alkaline conditions with a saturated barium hydroxide solution and heated to 110°C for 20 h. After hydrolysis, an internal standard was added, and the solution was filtered through a 0.22 µm membrane before injection into the HPLC column. The tryptophan and the internal standard in the hydrolysate were determined by a reversed-phase C18 HPLC with fluorescence detection (excitation at 280 nm and emission at 356 nm).

2.9 Statistical analysis

All experiments were conducted in triplicate. Means and standard deviations for all measurements were calculated using Microsoft Excel (Microsoft Inc., USA). Statistical differences among samples were assessed using one-way ANOVA followed by Tukey's HSD test, using the R software version 4.2.2 (http://cran.R-project.org), and a significance threshold was at $P < 0.05$.

3 Results and discussions

3.1 PR and DH

During the enzymatic hydrolysis of TDMPI, the large complex structured protein molecules were broken down into smaller peptides and free amino acids. Figure 1 shows that PR and DH increased with increasing time of hydrolysis. They increased sharply with about 2.5 times increment of DH and PR from 0.5 to 3 h of hydrolysis. Afterward, PR and DH continued to increase slowly and reached approximately 78.6% and 29.7%, respectively, after 9 h of hydrolysis. Although the increase became less pronounced in the final stages, there was no significant decrease observed. This plateau likely indicates that the hydrolysis process had reached its maximum extent, possibly due to enzyme saturation or inhibition by accumulated peptides, as reported in previous studies [10,13,35]. Hsu [35] reported a DH value of 30% for the dark muscle of *Thunnus tonggol* after 6 h of hydrolysis using orientase and protease XXIII.

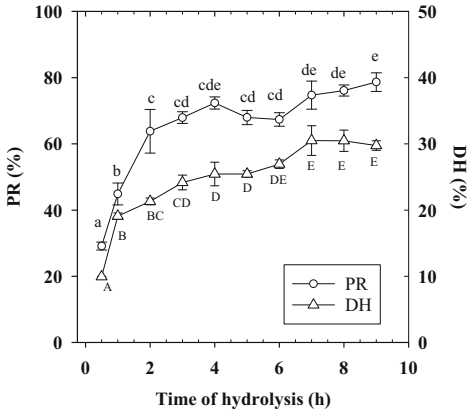

Figure 1: PR and DH of TDMPI hydrolysates at various enzymatic hydrolysis times. Data were mean ± SD (n = 3). Values with different uppercase or lowercase letters were significantly different ($P < 0.05$).

In a study by Nguyen et al. [13] on the production of fish protein hydrolysate from Tra catfish by-products, the DH value ranged from 18.9% to 31.5% after hydrolysis times of 1.5 to 9.0 h.

3.2 Tricine–SDS–PAGE

Hydrolysis reactions with different times cleaved higher molecular weight proteins into shorter peptides chains and free amino acids. Tricine–SDS–PAGE profiles of hydrolysates at different times and SDS–PAGE of TDMPI are shown in Figure 2. The peptide chains were broken down during protein isolate preparation before hydrolysis [25]. The SDS–PAGE patterns of unhydrolysed TDMPI clearly showed some bands with molecular weight higher than 15 kDa that corresponded to myosin heavy chain (223 kDa), tropomyosin

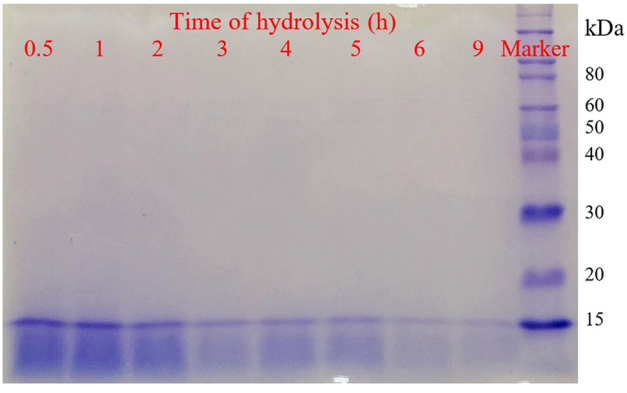

Figure 2: The SDS–PAGE patterns of TDMPI hydrolysates at various enzymatic hydrolysis times. The loaded protein concentration was 5 g/L in each lane.

(37 kDa), and actin (42 kDa) [25]. After hydrolysis, the pattern distribution of peptides in hydrolysates indicates that all observed peptides showed prominent bands below 15 kDa between 0.5 and 3 h of hydrolysis (Figure 2), which could explain the significant increase in DH (Figure 1). After 3 h of hydrolysis, short peptide chains below 15 kDa were continuously broken down into smaller fragments and free amino acids, which were not well visualised on SDS–PAGE. As a result, the bands below 15 kDa became faint or disappeared. Additionally, some low-molecular-weight peptides might have formed dimers or aggregates, slightly increasing their apparent molecular weight and further reducing the intensity of the bands in this region. Our findings were similar to the results reported by Theodore et al. [20] for protein hydrolysates prepared from alkaline-aided channel catfish protein isolates. Although the expected peptide sizes were below 15 kDa, tricine–SDS–PAGE was employed to visualise the hydrolysate profile and assess the hydrolysis efficiency under specific experimental conditions.

3.3 Antioxidant activities of protein hydrolysates and peptide fractions

3.3.1 DPPH-RSA

The DPPH-RSA of TDMPI hydrolysates increased significantly from about 37% to 69% with increasing time of hydrolysis from 0.5 to 9 h (Figure 3a), corresponding to the increase of the DH from 9.9% to 29.7% (Figure 1). This trend was similarly reported by Theodore et al. [20], Prihanto et al. [14], and Nguyen et al. [13]. Theodore et al. [20] showed that the DPPH-RSA of the hydrolysates from alkaline-aided channel catfish protein isolates increased from approximately 68% to 73% to 77% at the DH of 5%, 15%, and 30%, respectively. Prihanto et al. [14] reported that the DPPH-RSA increased with longer hydrolysis times, reaching the highest value of 58.20 ± 0.55% after 24 h at pH 9. Nguyen et al. [13] found that the DPPH-RSA significantly increased from 57.0% to 75.1% with the increasing hydrolysis time from 1.5 to 3.0 h and then decreased to 42% to 59% after 4.5 to 7.5 h of hydrolysis. In our study, the DPPH-RSA somewhat remained unchanged between 6 and 9 h of hydrolysis (Figure 3a); this was consistent with those reported by Wu et al. [36] for the DPPH-RSA of the mackerel protein hydrolysate that remained unchanged after 5 h of hydrolysis. This could be attributed to the protein hydrolysates containing substances acting as electron donors, which reacted with free radicals to form more stable compounds, thereby halting the radical reaction chain [37].

For DPPH-RSA of the peptide fractions, the highest values were found in the <3 kDa peptide fractions (52–90%), followed by the 3–10 kDa peptide fractions (38–70%), and the >10 kDa peptide fractions (32–20%) (Figure 3b). Saidi et al. [21] also found that peptide fractions with molecular weights >4 kDa, 1–4 kDa, and <1 kDa exhibited increasing DPPH radicals scavenging activity in that order. This result could be explained by the fact that low-molecular-weight peptide fractions and free amino acids (<3 kDa) may expose amino acids such as glycine, alanine, or glutamic acid, which then enhance interaction between peptides and lipid substances, thus promoting the stability of free radicals [38].

Moreover, a strong inverse relationship between peptide molecular weight and antioxidant capacity has been observed. As the molecular weight decreases, antioxidant activity tends to increase, likely due to the higher

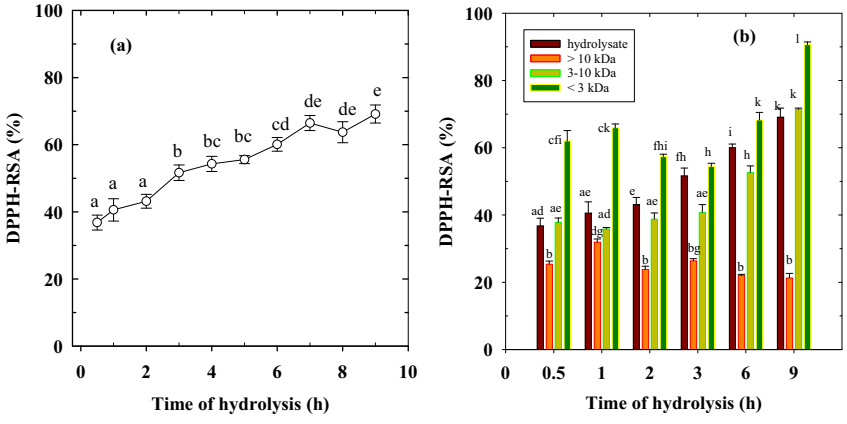

Figure 3: DPPH-RSA (%) of TDMPI hydrolysates (a) and their peptide fractions (>10, 3–10, and <3 kDa) (b) at various enzymatic hydrolysis times. Data were presented as mean ± SD (n = 3). Values (%) with different letters represented significant differences (P < 0.05).

accessibility of functional groups in smaller peptides (<3 kDa). These low-molecular-weight peptides are enriched with specific amino acids such as glycine, alanine, and glutamic acid, which can effectively donate electrons or hydrogen atoms, thereby enhancing their radical scavenging potential [39].

Comparing the DPPH-RSA of the hydrolysate vs the <3 kDa peptides, those of the former one were significantly lower than the latter one across the hydrolysis time (except at 3 and 9 h; higher but not significant) (Figure 3b). This could be attributed to the dilution effect of the mixture of peptide sizes in the hydrolysates, which reduced activity compared to the individual <3 kDa peptide fraction [21]. Furthermore, Hsu [35] reported that the peptide fraction (390–1,400 Da) isolated from tuna dark muscle hydrolysates using enzyme orientase had stronger DPPH-RSA than those in the 1,400–4,500 Da range. Hu et al. [12] found that peptide fractions with molecular weights <1 kDa from abalone viscera exhibited stronger DPPH-RSA than those with larger molecular weights. Other previous studies [9,11,40–42] also reported that peptide fractions with molecular weights below 3 kDa exhibited higher antioxidant activity than those with higher molecular weights.

3.3.2 Total reducing power ability

TRPA is another common criterion for evaluating *in vitro* antioxidant activity. The early stage of hydrolysis (after 0.5 h of hydrolysis) may have increased the availability of reactive groups that contributed to TRPA, although this trend could not be compared with the unhydrolysed material, which was not tested (Figure 4a and b). However, the TRPA of the TDMPI hydrolysates significantly decreased ($P < 0.05$) from 0.55 ± 0.01 to 0.31 ± 0.01 mg vitamin C equivalents per gram protein when the enzymatic hydrolysis time was extended from 0.5 to 3 h (Figure 4a). These observed decreases in TRPA could be attributed to the enzymatic hydrolysis process, which broke down proteins into smaller peptides, free amino acids, and/or other volatile compounds, resulting in a loss of specific chemical groups such as hydroxyl, sulphydryl, and aromatic amino acid residues and consequently leading to a decrease in TRPA [36]. There were no significant differences ($P > 0.05$) in TRPA observed when the hydrolysis time was extended from 3 to 9 h, with the TRPA values ranging from 0.31 ± 0.01 to 0.38 ± 0.01 mg vitamin C equivalents per gram protein (Figure 4a).

The TRPA values of the <3 kDa peptide fractions were generally highest (except at 9 h of hydrolysis time), being 2–10 times higher than those of the hydrolysates or the peptide fractions (3–10 kDa and >10 kDa) (Figure 4b). Amino acid components play an important role in the TRPA of peptide fractions. For the same hydrolysis time, <3 kDa peptide fractions exhibited the highest TRPA (Figure 4b). This can be explained by the fact that these fractions not only contained amino acids such as glycine, glutamic acid, and aspartic acid [43] but also contained many short-chain peptides, especially the presence of free amino acids [36]. Free amino acids were only present in the peptide fraction with a molecular weight <3 kDa solution (Table 1). The presence of different peptides in higher molecular weight fractions likely diluted the activity, which may explain the lower activity observed in the hydrolysate compared to separated peptide fractions [11,21], especially the <3 kDa peptide fraction in this study.

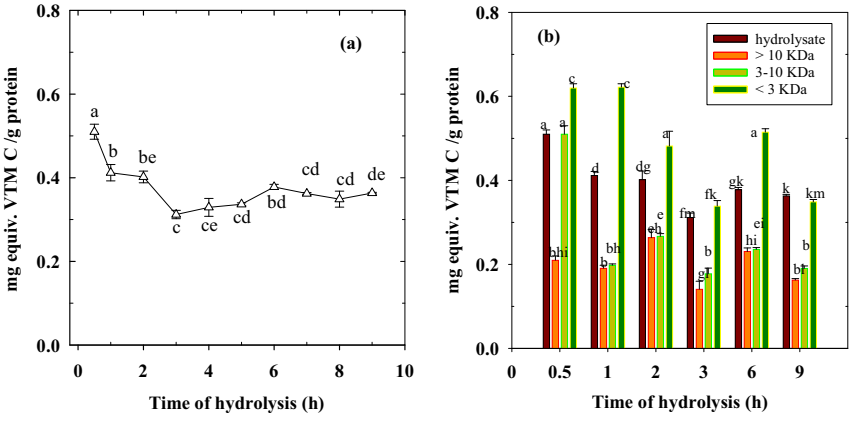

Figure 4: TRPA of TDMPI hydrolysates (a) and their peptide fractions (>10, 3–10, and <3 kDa) (b) at various enzymatic hydrolysis times and expressed as mg equivalent of vitamin C/g protein of hydrolysate or peptides. Data were presented as mean ± SD ($n = 3$). Values with different letters represented significant differences ($P < 0.05$).

Table 1: Free amino acid compositions (μg/mL) of TDMPI hydrolysate and its peptide fractions (>10, 3–10, and <3 kDa)

Amino acid	TDMPI hydrolysate	Peptide fractions		
		>10 kDa	3–10 kDa	<3 kDa
Taurine	7.92	nd	nd	13.22
Glutamine	9.24	nd	nd	27.38
Arginine	7.38	nd	nd	106.00
Aspartic acid	255.68	nd	nd	103.92
Glutamic acid	138.73	nd	nd	133.43
Glycine	87.01	nd	nd	158.79
β-Alanine	22.31	nd	nd	26.00
α-Alanine	253.53	nd	nd	265.64
Proline	31.24	nd	nd	59.49
Methionine	195.19	nd	nd	139.91
Valine	192.00	nd	nd	194.89
Phenylalanine	122.87	nd	nd	111.85
Isoleucine	130.57	nd	nd	124.27
Leucine	244.16	nd	nd	274.72
Ornithine	61.40	nd	nd	93.90
Lysine	5.69	nd	nd	66.40
Histidine	100.64	nd	nd	489.99
Tyrosine	2.58	nd	nd	78.55

nd: not detected.

The TRPA of the hydrolysates was significantly (P < 0.05) higher than those of the 3–10 kDa as well as the >10 kDa fractions during 1 to 9 h of hydrolysis time. Among peptides, it was found that the <3 kDa peptide fractions had the highest TRPA at 1 h of hydrolysis (equivalent to 0.63 mg vitamin C/g protein), but it decreased to 0.37 mg vitamin C/g protein after 9 h of hydrolysis (Figure 4b). This decline may be due to the formation of many lower molecular weight peptides [21,36]. On the contrary, the >10 kDa peptide fractions had lower TRPA values (P < 0.05), regardless of the hydrolysis time.

3.3.3 Lipid peroxidation inhibition activity

The LPIA of the TDMPI hydrolysates decreased significantly (P < 0.05) when the enzymatic hydrolysis time was extended from 1 to 9 h, with the LPIA values dropping from 54.90 ± 1.89% to 22.82 ± 0.54% (Figure 5a). There was no significant difference (P > 0.05) in LPIA observed between 0.5 and 1 h of hydrolysis, with the values ranging from 58.08 ± 3.37% to 54.90 ± 1.89% (Figure 5a). For the peptide fractions, the >10 kDa fraction exhibited significantly higher (P < 0.05) LPIA compared to those of the hydrolysates and peptide fractions with 3–10 kDa and <3 kDa during 0.5 to 6 h of hydrolysis (Figure 5b). Additionally, the 3–10 kDa peptide fractions showed significantly higher (P < 0.05) LPIA than those of the <3 kDa peptide fractions, regardless of the hydrolysis time. From 0.5 to 3 h of hydrolysis, the LPIA values of the >10 kDa peptide fractions were high, ranging from 65.66 ± 0.53% to 70.50 ± 0.79% (Figure 5b); however, they decreased significantly afterward.

Antioxidants can inhibit lipid peroxidation through various mechanisms, with DPPH-RSA and TRPA being the two most common. In the present study, the lipid peroxidation reaction mixture acted as a simulated biological model in which EPA was initiated by H_2O_2 in the presence of metMb. During lipid peroxidation, lipid hydroperoxides are generated and react with the existing metMb in the reaction mixture to form ferrylmyoglobin (ferrylMb) [44], which can capture a hydrogen atom from lipids, generating an alkyl radical. This alkyl radical subsequently forms a peroxyl radical in the presence of oxygen, potentially inducing further oxidation of other lipid molecules [45]. When an antioxidant is introduced into the reaction mixture, it can scavenge the free radicals produced from the interaction between lipid hydroperoxides and metMb. The ferrylMb formed in these reactions accepts electrons

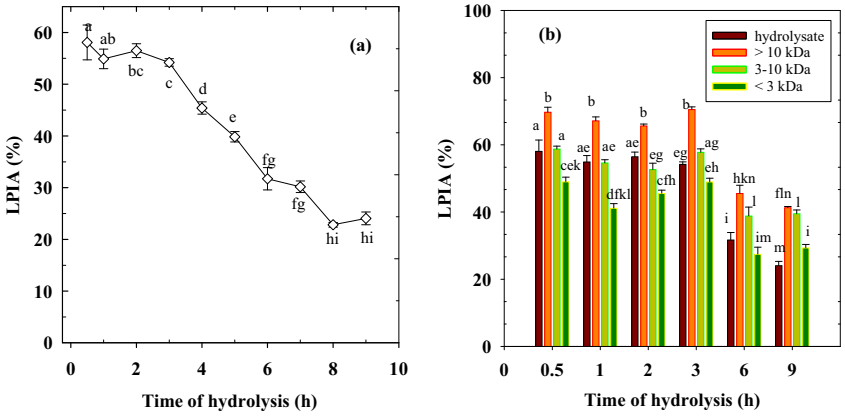

Figure 5: LPIA of TDMPI hydrolysates (a) and their peptide fractions (>10, 3–10, and <3 kDa) (b) at various enzymatic hydrolysis times. Data were presented as mean ± SD (n = 3). Values with different letters represented significant differences (P < 0.05).

from the antioxidant, reverting itself back to metMb. Consequently, lipid peroxidation in the reaction mixture can be suppressed.

These differences in antioxidant trends observed among DPPH-RSA, TRPA, and LPIA across hydrolysis times (Figures 3b, 4b and 5b) may be attributed to distinct chemical mechanisms underlying each assay. DPPH-RSA is mainly associated with hydrogen or electron donation from peptides or amino acids to neutralise free radicals. As hydrolysis progresses, smaller peptides with higher mobility and exposure to antioxidant residues, particularly hydrophobic amino acids such as Leu, Val, Ile, and those with aromatic side chains such as Tyr, Trp, Phe, may increase radical scavenging activity, explaining the continuous rise in DPPH-RSA values [46,47].

In contrast, TRPA reflects the overall reducing power of the hydrolysate, which may be initially enhanced by the release of electron-donating groups such as thiol, amine, and hydroxyl. However, excessive hydrolysis could degrade or fragment peptides with reducing capabilities into smaller units or free amino acids with lower redox potential, leading to a decline in TRPA at later stages [20,48,49]. LPIA, which measures the ability to inhibit lipid peroxidation, often involves complex interactions such as metal chelation and radical trapping in lipid environments. Peptides effective in this assay typically require both amphiphilic properties and specific side-chain functionalities. The reduction in LPIA after extensive hydrolysis may reflect the loss of such structural characteristics or the formation of fragments too small to maintain activity [50–52]. These differences highlight that antioxidant potential is assay-dependent, and no single trend can universally represent the oxidative defense properties of peptides. The peptide size, sequence, and structure all interact with the assay system in unique ways.

In the present study, the hydrolysed protein samples exhibited DPPH-RSA and TRPA (Figures 3a and 4a), which correspondingly demonstrated their LPIA (Figure 5a). At the same enzymatic hydrolysis conditions, peptide fractions with greater molecular weights (i.e., >10 kDa) exhibited significantly higher LPIA (Figure 5b). This may be due to the fact that during enzymatic protein hydrolysis, protein molecules are broken down into peptides and amino acids. In the early stages of enzymatic hydrolysis (0.5–3 h), a higher yield of peptides with >10 kDa was obtained, which corresponded to higher LPIA values. However, with prolonged enzymatic hydrolysis, the concentration of such peptides decreased significantly, resulting in a notable decline in LPIA.

In this study, the antioxidant activity of the original TDMPI was not included due to its very low solubility and limited antioxidant activity, as supported by a previous study [53] reporting that intact or high-molecular-weight proteins typically exhibit minimal antioxidant effects compared to their hydrolysed counterparts. Future work may include a more systematic comparison between native

Table 2: Amino acid composition (g/100 g) of TDMPI hydrolysate and its peptide fractions (>10, 3–10, and <3 kDa)*

Amino acid	TDMPI hydrolysate	Peptide fractions		
		>10 kDa	3–10 kDa	<3 kDa
Valine	2.76 ± 0.17^{a}	2.36 ± 0.19^{b}	2.25 ± 0.15^{b}	3.74 ± 0.11^{c}
Leucine	5.15 ± 0.08^{a}	4.51 ± 0.04^{b}	4.33 ± 0.05^{b}	4.54 ± 0.12^{b}
Isoleucine	2.51 ± 0.11^{a}	2.22 ± 0.11^{b}	2.17 ± 0.03^{b}	1.94 ± 0.02^{c}
Threonine	3.01 ± 0.14^{a}	2.63 ± 0.09^{b}	2.60 ± 0.13^{b}	2.59 ± 0.03^{b}
Methionine	2.76 ± 0.02^{a}	2.22 ± 0.05^{b}	2.08 ± 0.08^{b}	2.59 ± 0.11^{ac}
Phenylalanine	2.13 ± 0.15^{a}	1.75 ± 0.11^{bc}	1.65 ± 0.10^{b}	1.94 ± 0.02^{ac}
Tryptophan	0.33 ± 0.05^{a}	0.34 ± 0.06^{a}	0.26 ± 0.06^{a}	0.24 ± 0.04^{a}
Lysine	7.77 ± 0.13^{a}	9.97 ± 0.10^{b}	8.15 ± 0.05^{c}	5.18 ± 0.13^{d}
Histidine	1.51 ± 0.08^{a}	1.68 ± 0.02^{b}	1.56 ± 0.02^{ab}	1.30 ± 0.03^{c}
Tyrosine	2.01 ± 0.08^{a}	1.62 ± 0.03^{b}	1.56 ± 0.09^{b}	1.73 ± 0.06^{b}
Arginine	3.89 ± 0.07^{a}	3.64 ± 0.04^{b}	3.12 ± 0.08^{c}	2.81 ± 0.15^{d}
Aspartic acid	7.15 ± 0.15^{a}	7.07 ± 0.12^{a}	6.24 ± 0.12^{b}	5.18 ± 0.12^{c}
Glutamic acid	11.42 ± 0.25^{a}	11.45 ± 0.12^{a}	10.06 ± 0.15^{b}	8.21 ± 0.11^{c}
Proline	2.01 ± 0.06^{a}	3.10 ± 0.03^{b}	2.17 ± 0.05^{a}	1.08 ± 0.08^{c}
Serine	3.01 ± 0.06^{a}	2.63 ± 0.23^{b}	2.51 ± 0.05^{b}	2.59 ± 0.03^{b}
Glycine	2.38 ± 0.13^{a}	2.36 ± 0.06^{a}	1.99 ± 0.13^{b}	1.94 ± 0.05^{b}
Alanine	4.02 ± 0.05^{a}	3.23 ± 0.12^{b}	3.29 ± 0.21^{b}	3.89 ± 0.12^{a}
Cysteine	0.44 ± 0.14^{a}	0.61 ± 0.03^{b}	0.35 ± 0.02^{a}	0.43 ± 0.06^{a}

*Different superscript letters within the same row indicated significant differences among samples ($P < 0.05$).

protein isolates and their hydrolysates to better understand the degree of functional enhancement upon hydrolysis.

3.3.4 Amino acid composition

Table 2 highlights the unique properties of the peptide fractions obtained from TDMPI hydrolysates, which exhibited significant variation in amino acid content ($P < 0.05$). Previous studies showed that the bioactivity of peptides was influenced by their amino acid composition [21,54]. Specifically, antioxidant peptides are often characterised by the presence of amino acids like lysine, histidine, tyrosine, and methionine [21,55,56]. These amino acids contribute to antioxidant activity through various mechanisms: tyrosine and lysine act as hydrogen donors, while histidine scavenges radicals [35,55,56]. Furthermore, short peptides rich in hydrophobic amino acids (alanine, leucine, valine, tyrosine, methionine, and phenylalanine) exhibit strong antioxidant properties. These hydrophobic amino acids enhance free radical scavenging and lipid peroxidation inhibition by donating hydrogen atoms or electrons and interacting efficiently with lipid membranes [21,54–56]. The present study found a significantly higher concentration ($P < 0.05$) of these hydrophobic amino acids in the smaller 3 kDa peptide fractions, which also demonstrated higher antioxidant activity in both DPPH-RSA and TRPA assays (Table 2, Figures 3 and 4). This aligns with previous research highlighting the importance of hydrophobic amino acids in stabilising reactive oxygen species and enhancing lipid membrane interactions for antioxidant activity [21,55].

The results of amino acid composition analysis (Table 2) further support the antioxidant potency of the <3 kDa peptide fractions. These fractions are particularly rich in amino acids such as glycine, alanine, and glutamic acid, which play key roles in radical scavenging and lipid membrane stabilisation. Their presence may help explain the superior antioxidant activities observed in the lower molecular weight peptide fractions [11].

The presence of free amino acids exclusively in the peptide fraction with a molecular weight <3 kDa (Table 1) is a significant finding, as it highlights the potential role of these small molecules in antioxidant activity [21,35]. Free amino acids such as arginine, glutamic acid, glycine, histidine, and tyrosine are particularly abundant in this fraction, with concentrations ranging from 66.40 µg/mL (lysine) to 489.99 µg/mL (histidine). These amino acids are known to exhibit antioxidant properties, primarily through their ability to donate electrons or hydrogen atoms, which is a key mechanism in neutralising free radicals like DPPH [13,21,55].

For instance, histidine, which shows the highest concentration in the <3 kDa fraction, contains an imidazole ring that can act as an electron donor, effectively scavenging free radicals [40,55]. Similarly, aromatic amino acids like tyrosine and phenylalanine possess phenolic and benzene rings, respectively, which can stabilise radicals through resonance, further enhancing their antioxidant capacity [21,55]. The high levels of glycine and proline, which are involved in the synthesis of glutathione (a potent endogenous antioxidant), may also indirectly contribute to antioxidant activity by supporting cellular defense mechanisms [36,43].

The DPPH assay measures the ability of compounds to donate electrons to the stable DPPH radical, thereby reducing it to a non-radical form [13,21]. The abundance of free amino acids in the <3 kDa fraction suggests that these molecules are likely key contributors to the observed antioxidant activity. Their small size allows for efficient interaction with DPPH radicals, facilitating rapid electron donation [15,21]. This aligns with previous studies that have demonstrated the antioxidant potential of free amino acids in low-molecular-weight fractions [13,15,21,36].

4 Conclusion

The present study revealed that enzymatic hydrolysis of tuna dark muscle protein isolate using alcalase effectively produced antioxidant-rich peptide fractions. Hydrolysis for 9 h resulted in optimal antioxidant activity, particularly in peptide fractions below 10 kDa, as evidenced by elevated DPPH-RSA. The presence of hydrophobic and negatively charged amino acids was associated with enhanced antioxidant potential. These findings emphasise the feasibility of valorising tuna dark muscle as a sustainable source of natural antioxidants for functional food applications.

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