Characteristics of *Pindang* Boiling Water and Its Use to Produce Antioxidative Peptides through Trypsin Hydrolysis

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Abstract

Pemindangan is a traditional fish processing method in Indonesia, where fish are preserved by boiling in saltwater. This process results in a liquid as a by-product, which is often discarded without any further processing. The liquid contains a substantial amount of protein, reaching 1.04±0.03 mg/mL, which make it a potential source of bioactive peptides. This study aimed to produce bioactive peptides from *pindang* liquid which was hydrolysed by enzymes isolated from skipjack viscera. The hydrolysates were prepared by using extracted trypsin, which was also from a fish by-product. The trypsin-assisted hydrolysis was carried out at a temperature 60 °C and pH 8.0, while for the semi-purified trypsin, the process was run at 50 °C and pH 8.0. The hydrolysis time was operated at different periods of 15, 30, and 60 min. The results showed that the *pindang* liquid dominantly contained hydrophobic amino acids and the hydrolysate was still rich in protein content ranging from 0.56 to 0.65 mg/mL. Furthermore, hydrolysis time significantly influenced antioxidant activity of the peptide. The best antioxidant activity was shown at hydrolysis time of 15 min by crude extract enzymes, resulting in antioxidant activity of 0.90±0.01 mg ascorbic acid equivalent antioxidant capacity (AEAC)/mg protein and reducing power of 4.49±0.60 mg trolox equivalent antioxidant capacity (TEAC)/mg protein. Hydrolysis by using semi-purified trypsin showed antioxidant activity equivalent to 0.73±0.05 mg AEAC/mg protein and reducing power 6.54±0.34 mg TEAC/mg protein. Overall, the results revealed the conversion of *pindang* liquid into bioactive peptides showing antioxidant activity, and therefore, they might be potentially developed as functional foods.

Keywords: Amino acids, antioxidant, bioactive peptide, fishery processing byproduct, trypsin protease

Introduction

Pindang is a popular traditional fish product in Indonesia, made by combining salting and boiling processes without drying. The process produces a vast quantity of boiled waters which end up as a by-product, and this might cause serious environmental problems when they are released into rivers, causing water pollution and bad smell. On the other hand, the liquid contains protein reaching >1 mg/mL, which is a viable source of peptides. Increasing added value of the *pindang* by-product by converting it into bioactive peptides will pave the way for production of industrial functional food ingredients.

Protein hydrolysis with enzymes is the most preferred method for the production of bioactive peptides, because pH and temperature-controlled enzymatic hydrolysis have advantages related to bioactivity and quality of the final product over chemical hydrolysis (Madende & Hayes 2020; Cabral, 2021). Enzymatic hydrolysis can produce bioactive peptides by converting proteins into short-chain compounds that exert functional properties such as antidiabetic, antioxidant, antimicrobial, antiadipogenic (anti-obesity), anticancer, antihypertensive, and immunomodulatory peptides (Pavlicevic et al. 2020; Daroit & Brandelli 2021; Ortizo et al. 2023). Fish bioactive peptides may have a variety of uses,

including in food, agriculture, cosmetics, pharmaceuticals, and nutraceuticals industries (Alzaydi et al., 2023; Behera et al., 2022). In this case, antioxidant activity can be further considered.

Antioxidants prevent oxidation reactions by binding to free radicals, and thus inhibiting cell damage. Antioxidant peptides from fish produced by commercial protease enzymes has been reported by previous researchers, for example, peptide hydrolysate produced from carp fish with alkalase (González-Serrano et al., 2022), skipjack with flavourzyme (Wang et al., 2022), round scad with neutrase and trypsin (Hu et al., 2020), salmon with alcalase (Vázquez et al., 2019), yellowfin tuna with protamex (Pezeshk et al., 2019), *Pangasius* sp. with bromelain (Nurdiani et al., 2024), oyster with pepsin and trypsin (Qian et al., 2020), skipjack tuna with trypsin and alcalase (Phetchthumrongchai et al., 2022).

The commercial protease enzymes are relatively expensive, and for this reason the enzymes derived from fish digestive organs has been applied to produce antioxidant peptides, including enzymatic extract of visceral organs from several species (Borges et al., 2023). Trypsin (40-60% ammonium sulfate fraction) from the pyloric caeca of snapper and freshwater fish (*Lutjanus vitta*, *Priacanthus tayenus*, and *Nemipterus marginatus*) was reported able to hydrolyse the bark *of L. vitta* (Khantaphant & Benjakul 2008). Additionally, trypsin obtained from pyloric caeca *Aluterus monoceros* hydrolysed meat protein of Indian mackerel (Zamani & Benjakul 2016) while crude enzyme extracts from the offal of zebra blenny (*Salaria basilisca*) hydrolysed muscles of the *Balistes capriscus* (Siala et al., 2016). The study reported by Sripokar et al. (2019) showed that a crude extract of the trypsin enzyme from albacore tuna liver could hydrolyse *Abalistes stellaris* muscle protein and the hydrolysate becomes a functional food ingredient and natural antioxidant.

Using trypsin enzyme from skipjack tuna to produce protein hydrolysate can reduce the cost of commercial proteases. Overall, this work aimed to utilise a by-product from the skipjack tuna industries, namely pindang industry. The internal organ fish was used as a source of trypsin (Nurhayati et al., 2020; Sripokar et al., 2019). This trypsin was further used to produce bioactive peptides for the second by-product, namely liquid waste of *pindang* processing.

Material and Methods

Material

Pindang boiling water samples were collected from a fish processing industry in Bogor, Province of West Java, Indonesia. Meanwhile, two types of protease (crude extract and semi-purified trypsin) were derived from skipjack tuna viscera. For protein analyses, these chemicals were used, i.e. HCl (Merck), OPA reagent (ortho-phthalaldehyde) and standard amino acids (Merck), Bradford reagent and bovine reagent serum albumin (BSA) (Sigma Aldrich). For antioxidant assay, the chemicals included 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich), methanol 95% (Merck), ascorbic acid, acetic acid, Iron (III) chloride hexahydrate ($FeCl_3.6H_20$) (Merck), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-Tris (2-Pyridil)-S-Triazine (TPTZ) (Sigma-Aldrich). Every other chemical was of analytical quality.

Preparation of pindang *boiling water*

The by-product liquid from the *pindang* processing was obtained by boiling 100 kg of skipjack tuna in 300 L of salt water with a concentration of 4% (w/w) at a temperature of 90-100 °C for 1 h . Once the process completed and the boiling water was cooled, the sample was collected into a bottle. The liquid was stored in a freezer (-20 °C). Prior to use, it was melted and filtered using a straining cloth to remove impurities.

Enzyme preparation

The crude enzyme extract was obtained by preparing skipjack tuna collected from a fish processing industry in Bogor, West Java, Indonesia. The viscera were extracted by cutting and cleaning, then homogenised with buffer solution (10 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$) with a 1:2 ratio. Homogenization was carried out with a homogeniser (Armfield L4R, UK) at a speed of 11,000 rpm (for a duration of one min at 4 °C). The homogenate was centrifuged at 4 °C (Himac CR 21G, Japan), 10,000× g for 15 min. The supernatant was taken and referred to as trypsin crude extract (modification of the method of Bougatef et al., 2007).

The trypsin extract was mixed with ammonium sulfate $(NH_4)_2SO_4$ at a concentration of 40-50% (w/v). Stirring was conducted using magnetic stirrer (Cimarec, USA) for 45 min at a cold (4 °C) temperature. The mixture was precipitated overnight, then the supernatant was obtained by centrifugation (15,000× g, 4 °C, 10 min). The precipitate was dissolved in buffer (0.01 M $CaCl_2$, 0.01 M Tris- HCl pH 8.0, 1:1 ratio). The solution was put into the dialysis membrane (D9277 Sigma, molecular weight cut off 14 kDa,). The dialysis membrane was placed in a buffer pH 8.0 (0.001 M $CaCl_2$ and 0.001 M Tris-HCl), then agitated under cold condition (4 °C) for 30 min. The solution in the membrane was taken and used as a semi-purified trypsin.

The crude extract and semi-purified trypsin from internal organs of skipjack tuna were analysed for enzyme activity using N-*á*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as the substrate (Erlanger et al. 1961), with slight modifications. A total of 43.5 mg of BAPNA was dissolved in 1 mL of dimethyl sulfoxide. The solution was then made up to 100 mL by adding 50 mM Tris HCl (pH 8.0). A 2500 ìL aliquot of the BAPNA solution was then mixed with 50 ìL of enzyme solution and incubated for 10 min at the optimum temperature (37 °C). After the first incubation, the enzymatic reaction was terminated by the addition of 1 mL of 30% glacial acetic acid, and the mixture was then incubated again for the same time and temperature. A spectrophotometer (2450 UV-Vis, Shimadzu Corp., Kyoto, Japan) was used to measure absorbance at 410 nm.

Preparation of peptide/hydrolysate from liquid pindang *byproduct*

The procedure of fish protein hydrolysate processing referred to Sripokar et al. (2019), by altering the type and ratio of enzyme, buffer solution, temperature, pH and time of hydrolysis. Hydrolysed fish protein was made from the by-product liquid of *pindang*, and two types of enzymes (crude and semi-purified trypsin from skipjack offal tuna) were applied to hydrolyse the protein. The pH level of the liquid was adjusted with 100 mM Tris-HCl buffer (pH 8.0). Then, the solution was allowed to equilibrate for 10 min at an optimum temperature. The crude extract trypsin was applied at 60 °C and pH 8.0, while semi-purified trypsin was used at 50 °C and pH 8.0. Enzyme hydrolysis was initiated by adding 1 mL of the enzyme solution (with activities of 0.55 U/mL and 1.01 U/mL, respectively) to 2.5 mL of the *pindang* liquid, and incubated for different times (15, 30, and 60 min) in a shaking water bath (PolyScience, USA) at 150 rpm. The enzyme was inactivated by heating at 90 °C for 15 min to stop hydrolysis, and the mixture was then centrifuged at 3000 rpm and 4 °C for 30 min. The supernatant was collected and stored at -20 °C for analysis.

Amino acid composition (ICI Instrument Method, 1988)

The protein sample was hydrolysed with HCl 6 N in a closed container filled with nitrogen gas and using an autoclave at 110 °C under a pressure of 4 to 5 psi for 24 h. The hydrolysis results were cooled at a room temperature and dried using a rotary evaporator for 15 min. The dry sample was mixed with 10 mL of HCl 0.01 N, then filtered (millipore paper) and buffer (potassium borate, pH 10.4) was added with a ratio of 1:1. Subsequently, 5 µL of the mixture was mixed with 25 µL of OPA reagent and left for 1 min to complete derivatisation. For separation, a 5 ìL sample was injected into the HPLC system (Shimadzu CBM-20A Corp., Japan), which was connected to a Thermo Scientific ODS-2 Hyersil column at 40 °C, with a fluorescence detector (A Ch1) set to a wavelength range of 350 nm to 450 nm. Mobile phase A was consisted of 0.02% Na-Acetate (pH 6.5), 0.005% Na-EDTA, 9% methanol, and 1.5% tetrahydrofuran (THF), while mobile phase B was composed of 95% methanol and 1 L of high-purity water. Separation was performed at a flow rate of 1 mL/min using a gradient programme, beginning with 5% phase B for 0,01 min, followed by a gradual increase to 70% phase B over 20 min. Subsequently, the column was washed with 100% phase B and equilibrated with phase A (0% phase B), all within a total analysis time of 35 min. The concentration of amino acids in the sample was calculated by making a standard chromatogram using amino acids standard (ready to use which was treated similarly as the protein sample).

Determination of protein concentration

Protein was analysed following Bradford (1976). Bradford reagent was prepared by combining 5 mL ethanol, 10 mg Coomassie brilliant blue, and 10 mL phosphate 85% in a 500 mL solution and the protein standard was BSA. Sample 400 ìL was mixed with 4 mL of Bradford reagent, then vortexed and incubated for 15 min at 30 °C. For the blank, the sample solution was replaced with distilled water. Absorbance at 595 nm was determined using a Shimadzu UV-Vis-2450 spectrophotometer. A 1 mg/mL BSA stock solution was used to prepare dilution series ranging from 0.1 to 1 mg/mL. For each standard, 400 ìL of the diluted solution was pipetted into a test tube containing 4 mL of Bradford reagent, then incubated at 30 °C for 15 min. The absorbance was determined at ë 595 nm. Protein concentrations for both standards and samples were measured in triplicate.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) method

A total of 10-20 ìL of the *pindang* liquid and hydrolysate, with concentrations from 0.56 to 0.65 mg/mL (prepared from 15 min hydrolysis by crude or semi-purified trypsin), were injected separately into the electrophoresis gel following the Laemmli (1970) method, with modifications to both the separating and stacking gel, which were prepared at 12% (v/v) and 4% (v/v) concentrations, respectively. A 5× sample buffer consisting of Tris-HCl (60 mM, pH 6.8), glycerol (25%), SDS (2%), and bromophenol blue (0.1%) was

used to dilute the sample. The samples were then heated to 100 °C for 5 min prior to being injected into the gel. The denatured samples were loaded into the gel wells, and electrophoresis (Hoefer, Inc., San Francisco, CA, USA) was performed for 4 h at 70 volts and 20 A. After the protein separation was completed, the gel was removed, and staining was conducted using 0.25% CBB-R250 on 10% acetic acid and 50% methanol. Destaining was then performed with 7.5% acetic acid and 5% methanol until the protein bands became visible. A protein marker (SMOBIO Technology Inc., Taiwan) with a molecular weight range of 10-180 kDa was applied to estimate the size of the protein bands that were observed.

Determination of antioxidant activity by DPPH method

Analysis of antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used, referring to a method described by Henriques et al. (2021) with a slight modification in DPPH concentration. A total of 1 mL of sample solution were mixed with 1 mL of DPPH (250 mM in methanol). The mixture was shaken and left in the dark for 30 min at a room temperature, and afterwards the absorbance was determined at a wavelength of 517 nm with a UV-2450 UV-vis spectrophotometer (Shimadzu Corp, Japan). The solution mixture in the absence of sample was used as a control. DPPH radical inhibitory activity was calculated based on the equation:

$$Inhibition\ (\%) = \frac{(B - A)}{B} x100$$

where A refers to absorbance of the sample and B is absorbance of the control.

All analyses were performed in triplicate. Antioxidant activity was represented as ascorbic acid equivalent antioxidant capacity (AEAC, mg/mg protein), determined based on the inhibition of DPPH radicals. This was calculated using the equation obtained from the standard curve of ascorbic acid at concentrations varying from 0.1 to 0.5 mg/mL against DPPH free radicals. The resulting value was then divided by the protein concentration of the hydrolysate sample.

Determination of antioxidant activity using FRAP method

A ferric reducing antioxidant power (FRAP) assay was used as the second antioxidant analysis as prescribed by Mongkonkamthorn et al. (2020) with a modification in the concentration of $FeCl_3.6H_2O$. The FRAP solution contained 25 mL of buffer (0.3 mM acetate, pH 3.6) and 2.5 mL of 10 mM TPTZ in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3.6H_2O$. This reagent mixture was incubated at 37 °C for 30 min. FRAP solutions were always freshly made and were started by mixing 20 mL sample, 1800 mL FRAP reagent, and 180 mL distilled water. The mixture was incubated for 30 min at 37 °C. Absorbance was measured at a wavelength of 595 nm using a UV-vis-2450 spectrophotometer (Shimadzu Corp, Japan). The concentration of trolox used as the standard varied between 0.1 and 0.5 mg/mL. Trolox equivalent antioxidant capacity (TEAC, mg/mg protein) was used to express the antioxidant activity in the samples. The TEAC result in mg/mL was determined using the following equation and was subsequently divided by the protein concentration of the sample.

$$\text{TEAC} = \frac{\text{Abs Sample} - \text{Intercept}}{\text{Slope}}$$

Data Analysis

This study utilised a completely randomised design to investigate the impact of hydrolysis duration on both protein concentration and antioxidant activity. Microsoft Excel 2016 (Microsoft Corp., Washington, USA) was used to calculate the mean ± standard deviation (SD) of the data. A one-way analysis of variance (ANOVA), followed by Duncan's test at a 5% significance level, was used to statistically analyse the data. Statistical Product and Service Solutions version 25 was the software utilised.

Results and Discussion

Amino acid composition of pindang *fish-boiled water*

Pindang liquid contains a variety of amino acids including a hydrophobic group (Ala, Ile, Leu, Met, Pro, Val) and an aromatic group (Phe, Tyr, Trp), as shown in Table 1. These amino acids correspond to 25.82% for hydrophobic and 11.10% for hydrophilic, which mean that the hydrophobic group is 2.33 times higher than the hydrophilic group. The high content of hydrophobic amino acids indicates potential functional properties of the *pindang* liquid.

The high aromatic and hydrophobic amino acid content facilitates antioxidant activity. A well-established antioxidant mechanism has been observed in the antioxidant peptides of the Asian swamp eel: Val and Gly residues (VYGPW) forms two hydrogen bonds with free radicals. The key amino acid residue at the active site of AVLW is Trp-4, which forms a hydrogen bond with the DPPH radical (Wang et al., 2024). Peptides that exhibit vigorous antioxidant

activity have aromatic amino acid residues and hydrophobic amino acid residues (González-Serrano et al., 2022). Amino acid with aromatic residue can donate a proton to an electron-deficient radical (Pezeshk et al., 2019). Ding et al. (2019) found antioxidant peptides from the hydrolysate of skipjack tuna bones with amino acid Gly, Pro, and Met. Peptides containing Met residues showed DPPH radical scavenging activity. The amino acid sequence of Asp-Gly-Pro-Lys-Gly-His contains Asp, Lys, and 2Gly, which may have significant roles in their ability to scavenge radicals (Qiu et al., 2019). Haryati et al. (2020) reported aromatic and hydrophobic amino acids that play significant role in the antioxidant activity of hydrolysate catfish meat. Likewise, positively charged amino acids (Arg, Lys) and negatively charged amino acids (Asp and Glu) can bind and neutralize free radicals (Liu et al., 2021). The composition of amino acids has a more significant impact on the antioxidant properties. Aromatic amino acids exceed 50% of the residues, whereas positive and negative charge amino acids make up 52.46% (Zhao et al., 2022). In our case, the positive charge of amino acid was 34.4%, while the negative charge of amino acid was 17.87%.

Table 1. Amino acid composition of liquid *pindang*

Amino acid type	Amino acid	mg/100g	Percentage (%)
Hydrophobic	Proline (Pro)	273.72	25.82
	Alanine (Ala)	357.63	
	Valine (Val)*	120.82	
	Methionine (Met)*	64.02	
	Ilucine (Ile)*	85.76	
	Leucine (Leu)*	165.74	
Aromatic	Tryptophan (Trp)*	30.00	10.78
	Tyrosine (Tyr)	40.92	
	Phenylalanine (Phe)*	374.63	
Hydrophilic	Glycine (Gly)	209.25	11.10
	Threonine (Thr)*	126.18	
	Serine (Ser)	121.00	
	Cystine (Cys)	2.58	
Positive charge	Histidine (His)*	793.22	34.42
	Lysine (Lys)*	299.38	
	Arginine (Arg)*	330.63	
Negative charge	Aspartic Acid (Asp)	258.92	17.87
	Glutamate (Glu)	480.11	
Total		4134.51	100

*Essential amino acids

Protein concentration

Before hydrolysis, the concentration of dissolved protein reached 1.04±0.03 mg/mL. The enzymatic crude extract (activity 0.55 U/mL) and semi-purified trypsin (activity 1.01 U/mL) were used to hydrolyse the *pindang* liquid at 15, 30, and 60 min. Following hydrolysis, the concentration of protein decreased, leading to the desired outcome presented in Table 2.

The two enzymes were able to hydrolyse the *pindang* liquid by 62.50% (crude extract) and 86.15% (semi-purified) for 15 min of hydrolysis, and extending the hydrolysis time did not affect the protein content considerably. Bahram et al. (2022) reported that hydrolysis for 180 min with papain for *Saurida tumbil* fish protein hydrolysate produced 59.9% protein. Soluble proteins are contained in the hydrolysate product, while insoluble proteins are separated during centrifuge. Insoluble proteins are converted during hydrolysis into soluble nitrogenous compounds, and afterwards they are decomposed into simple compounds (peptides, amino acids).

Table 2 Protein concentration of liquid *pindang* after hydrolysis

Crude extract hydrolysis	Protein concentration (mg/mL)	Semi-purified trypsin hydrolysis	Protein concentration (mg/mL)
P	1.04±0.03^{a}	P	1.04±0.03^{a}
HC15	0.65±0.03^{b}	HD15	0.56±0.04^{b}
HC30	0.61±0.00^{b}	HD30	0.63±0.06^{b}
HC60	0.62±0.02^{b}	HD60	0.64±0.09^{b}

Note: P=control (without hydrolysis); HC15, HC30, HC60=crude trypsin hydrolysate 15, 30, 60 min; HD15, HD30, HD60=semi-purified trypsin hydrolysate 15, 30, 60 min. Different letters in the same column indicate significant differences in values (mean ± SD, n=3) ($p < 0.05$).

In this study, the enzymes used were crude extract and semi-purified trypsin. Trypsin is a serine protease enzyme that is able to break down peptide bonds between the amino acids arginine and lysine into simpler peptides. In another study (Devita et al., 2021), the concentration of dissolved protein after hydrolysis with alcalase and savinase for 6 h was obtained from 6.58-16.37 mg/mL to 1.34-1.99 mg/mL. The variation may result from the variety of enzyme types and hydrolytic activities.

The molecular weight was estimated by using a 12% gel electrophoresis procedure. Both enzymes (crude extract and semi-purified trypsin) could hydrolyse the protein in the *pindang* liquid to smaller peptides. As shown in Figure 1, the protein with a molecular weight of 13, 16, 21, 32, 35, 46, 65, 75 kDa decreased to 10 kDa after hydrolysis. Similarly, the higher molecular weights of protein also showed a considerable drop. After a 15 min hydrolysis by the enzymes, the molecules with <10 kDa were seen, and their presence confirmed enzymatic hydrolysis activity. A similar result was reported by Zamani & Benjakul (2016), resulting in the bands of <6.5 kDa in the protein hydrolysate of muscle mackerel hydrolysed by trypsin from *Aluterus monoceros*. Before hydrolysis, the protein bands were between 25 and 190 kDa. Phetchthumrongchai et al. (2022) found that the hydrolysis of tuna roes using alcalase and trypsin produced protein hydrolysate with a molecular weight of 16-51 kDa. Mohanty et al. (2021) reported that the pattern of *Labeo rohita* hydrolysates (by alcalase) showed bands ranging from 10 to 25 kDa. The profile of molecular weight of the peptides is a crucial characteristic that could impact the antioxidative action of the protein hydrolysate.

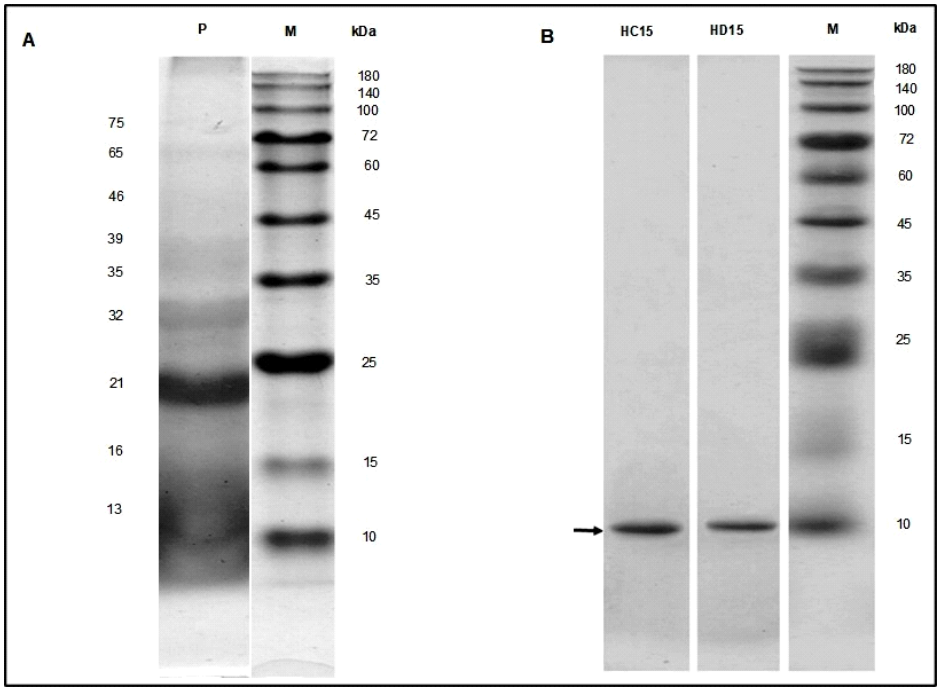

Figure 1. Protein pattern before hydrolysis (A), after 15 min of hydrolysis (B)
Note: P=*pindang* liquid, M=marker, HC15=crude trypsin hydrolysis, HD15=semi-purified trypsin hydrolysis. Protein in the marker: 10 kDa = Lysozyme, 15 kDa = á-Lactalbumin, 25 kDa = Myoglobin, 35 kDa = Phosphorylase B, 45 kDa = Serum albumin, 60 kDa = Glutamate dehydrogenase, 72 kDa = Hsp70, 100 kDa = Myosin, 140 kDa = Vimentin, 180 kDa = Type I collagen

Antioxidant Activity

DPPH free radical scavenging activity

Antioxidant activity is expressed as the antioxidant activity of AEAC in mg/mg protein; the results are illustrated in Figures 2A and 2B. Crude extract and semi-purified trypsin change the protein of *pindang* liquid into peptides, and this increased their antioxidant activity to >5 times higher.

After 15 min of hydrolysis (HC15), the hydrolysate showed the highest antioxidant activity of 0.90±0.01 mg AEAC/mg protein, and this corresponds to 10.96 times higher than the control (996.19%). Meanwhile, at a longer process of 30 min and 60 min, the activity dropped to 0.72±0.04 and 0.60±0.06 mg AEAC/mg protein, respectively.

A similar result appeared in samples treated with semi-purified trypsin. After 15, 30, and 60 min of hydrolysis, the antioxidant activity increased by 8.90,

6.50, and 5.77 times higher than control. *Scomber japonicus* fish protein hydrolysed by trypsin for 3 h showed a higher antioxidant activity over the sample hydrolysed with papain, pepsin, or protease (Ediriweera et al., 2019). Nguyen et al. (2022) found a 1.32-fold rise when the hydrolysis time was increased from 1.5 h to 3 h (maximum time). However, it decreased markedly when the hydrolysis time of catfish hydrolysate with alcalase was extended to 9 h. Alcalase was found able to break down *Lepturacanthus savala* protein with a 5 hour-process, and the antioxidant activity reached the highest (1.80-fold increase) compared to control (Yathisha et al., 2022). Our findings showed a higher increase in antioxidant activity than previous studies.

The higher antioxidant activity occurred in the 15 min-hydrolysis, but it declined as the hydrolysis time increased. In this case, both enzymes (crude extract and semi-purified trypsin) hydrolysed the protein in *pindang* liquid, resulting in peptides with amino acid residues which were able to donate hydrogen, which was then taken by DPPH free radicals. The crude trypsin enzyme had a more significant effect than the semi-purified enzyme. Semi-purified trypsin enzyme was a precipitate fraction of ammonium sulfate dialysed to remove the residual mineral salts. Mineral loss may reduce the rate of enzyme action. Minerals are cofactors required by certain enzymes to support their catalytic activity, although their presence does not consistently enhance enzyme activity and may exert detrimental effects if present in excess. The activity of semi-purified trypsin enzyme was inhibited by ZnCl, at a concentration of 20 mM (Dali et al., 2024). Peredo-Lovillo et al. (2022) reported that enzyme specificity and sample origin affected antioxidant activity of peptides. DPPH assay is widely used to measure the reaction processes of peptide compounds with free radicals because it is the simplest, most stable, and fastest method. Bora (2023) discovered that determination of the reaction rate between antioxidants and DPPH radicals occurs quickly through initial electron transfer. Then, the subsequent transfer of hydrogen atoms occurs slowly (depending on the solvent that accepts neutral hydrogen bonds, namely ethanol and methanol). The rate factor and reaction mechanism is dependent on the ionising characteristics of solvent and intramolecular or intermolecular hydrogen bond interactions, the presence of oxygen, temperature, pH, antioxidants and polarity.

Previous studies revealed the antioxidant activity of fish protein hydrolysates hydrolysed with trypsin enzymes (extract, semi-purified, and purified) extracted from the fish digestive tract. Semi-purified trypsin enzymes from the hepatopancreas of Pacific white shrimp were used to hydrolyse seabass (*Lates calcarifer*) skin and they produced more hydrolysates than alcalase under the same enzyme concentration and time. Hydrolysate exerts antioxidative activity by scavenging DPPH radicals (Senphan & Benjakul 2014), and the finding was also found in the protein hydrolysate from zebra blenny (*S. basilisca*) which was hydrolysed by crude extract enzymes from similar fish (Ktari et al., 2020).

The presence of amino acids (sequence, type, and hydrophobicity system) is responsible for the antioxidant activity factor of peptides in *pindang* liquid to neutralise DPPH radicals. Amino acids promote interactions between peptides and radical species via improving peptide solubility in lipids or scavenging lipid-derived radicals via proton donation (Guidea et al., 2020). The hydrophobic/aromatic groups of amino acid residues Trp-Met-Phe-Asp-Trp and Trp-Met-Gly-Pro-Tyr (Zhang et al., 2019), Pro, Tyr, Leu, Val, Ile, Met, Ala, and Phe residues (Qian et al., 2020) was discovered to stabilise free radicals by donating hydrogen. Similarly, Ala, His, Met, and Tyr residues are found in the antioxidant peptide sequences QDHKA, AEHNH, AEM, and YVM. Furthermore, hydrophilic amino acid residues such as Glu, Gln, Asp, Lys, and Asn have a function in radical scavenging (Wang et al., 2022).

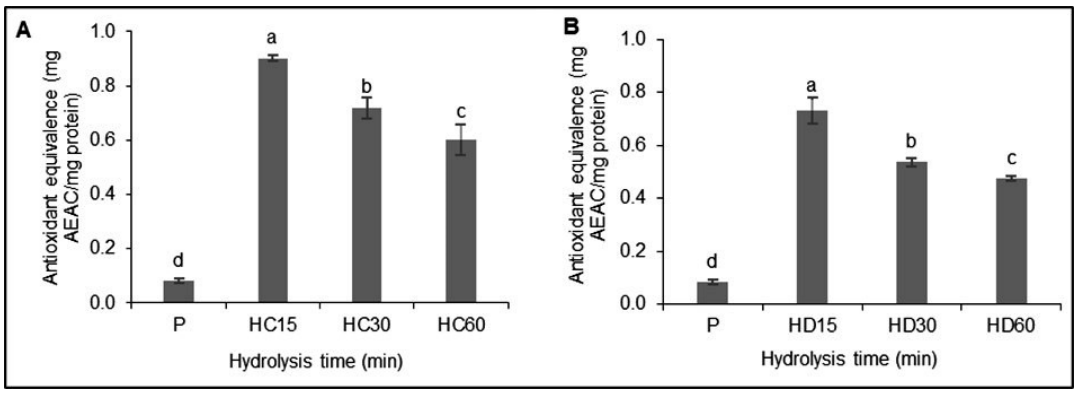

Figure 2. Antioxidant activity of hydrolysate results from scavenging analysis against DPPH radicals, then expressed in mg AEAC/ mg protein, crude extract trypsin (A), semi-purified trypsin (B).

Note: P=control (without hydrolysis); HC15, HC30, HC60=crude trypsin hydrolysate 15, 30, 60 min; HD15, HD30, HD60=semi-purified trypsin hydrolysate 15, 30, 60 min. Different letters in each hydrolysis time indicate significant differences in values (mean ± SD, n=3) ($p < 0.05$).

Ferric reducing antioxidant power

Like a DPPH analysis, the hydrolysis of protein in *pindang* liquid for 15 min significantly increased antioxidant activity. Compared to the DPPH method, the FRAP antioxidant value of peptide is better when a dialysed enzyme (semi-purified trypsin) is used, as depicted in Figure 3. Even though semi-purified trypsin loses minerals as a cofactor during dialysis, this enzyme can still work to hydrolyse proteins in *pindang*. The peptides produced may have more antioxidant capacity in contributing electrons in reduction reactions. Ferri-tripyridyl-triazine (TPTZ) ferric complex (Fe^{3+}) reduction to ferrous complex (Fe^{2+}) was performed to evaluate the antioxidant capability of the peptide compound. This condition provides a blue colour and an elevated absorption value (595 nm). Measuring the strength of iron-reducing antioxidants is straightforward, requires no extra equipment, and immediately determines total antioxidant activity by transferring electrons from antioxidant molecules (Bora, 2023).

The functional qualities of hydrolysate products can be improved over those of the original protein through controlled enzymatic activities (Peredo-Lovillo et al. 2022). In semi-purified trypsin enzyme, the optimum activity as reducing power was demonstrated after 15 min of hydrolysis. The value falls when hydrolysis is extended to 60 min, demonstrating the drop of activity as decreasing power declines. Longer hydrolysis time means producing peptides disassembled into free amino acids. Free amino acids have significantly lower antioxidant activity than peptides (Zhu et al., 2023).

The antioxidant activity of peptides can be attributed to several variables, such as the specific enzyme involved in the hydrolysis process, the duration of hydrolysis, and the specific amino acids present in the peptide structure. The ability of peptides bioactive with semi-purified enzymes to decrease iron ions is greater than that of peptides bioactive with crude extract trypsin enzymes. Similar effects were also noted for the fish protein hydrolysate, which was hydrolysed by the semi-purified trypsin enzyme from the pyloric caeca of the snapper fish (Khantaphant & Benjakul 2008) and the *A. monoceros* (Zamani & Benjakul 2016).

Compared to the control, the reduction power of the 15 min *pindang* liquid peptide hydrolysate with semi-purified trypsin was raised by 3.26 times (225.65%). The obtained result exhibits a reasonably high value compared to the hydrolysis of *Saurida tumbil* protein using alcalase, which resulted in a 1.33-fold increase after 180 min of hydrolysis (Bahram et al., 2022). The iron-reducing power activity correlated with the hydrophobic/aromatic amino acid content, i.e., increasing their solubility at the water-lipid interface, facilitating better interactions with free radicals (He et al., 2019; Hu et al., 2020).

The hydrolysis process increased the antioxidant activity as expressed by the DPPH radical inhibitory activity and reducing power (FRAP). The combination of these assays has the potential to be an excellent method for screening antioxidant activity during the isolation of antioxidant peptides. Different methods of measuring antioxidant activity can provide different results. Conventional methods for measuring antioxidant activity are needed. The FRAP test was offered as a non-radical single electron transfer (SET)-based method with a low connection to other tests of antioxidant activity and a low relation to the hydrogen atom transfer (HAT) mechanism, which is the process of radical extinction that occurs in lipid systems (Munteanu & Apetrei 2021). Therefore, it is advised that this test be performed simultaneously with other techniques to identify the primary pathways for various antioxidants.

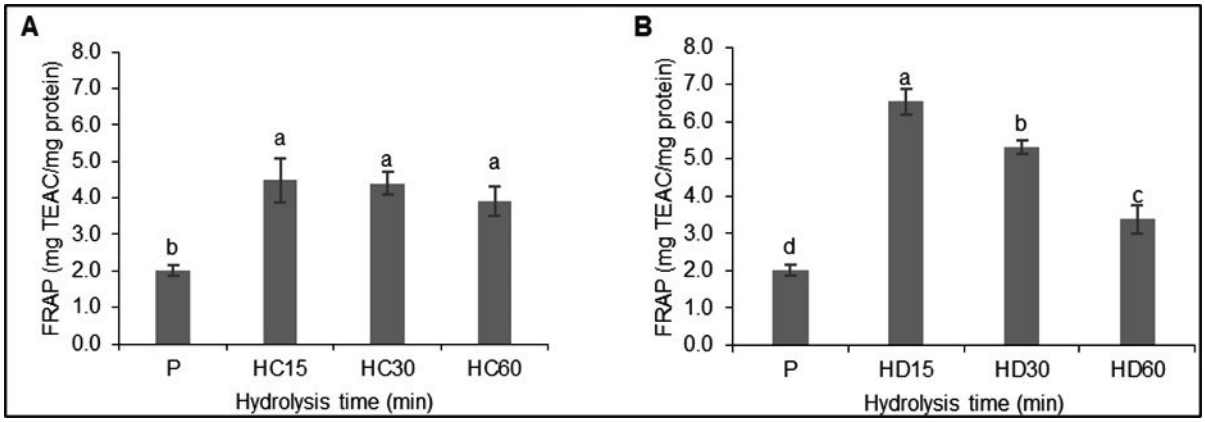

Figure 3. Antioxidant activity with FRAP test by crude extract trypsin (A), semi-purified trypsin (B).
Note: P=control (without hydrolysis); HC15, HC30, HC60=crude trypsin hydrolysate 15, 30, 60 min; HD15, HD30, HD60=semi-purified trypsin hydrolysate 15, 30, 60 min. Different letters in each hydrolysis time indicate significant differences in values (mean ± SD, n=3) ($p < 0.05$).

Conclusion

Two types of enzymes, crude extract and semi-purified trypsin from skipjack tuna viscera, were successfully used to hydrolyse protein from the *pindang* by-product. The peptide hydrolysates showed a high antioxidant activity, and this is a main reason for their further uses as an ingredient of functional foods. Overall, there was a higher antioxidant activity of the hydrolysates over those without hydrolysis, and the 15 min hydrolysis became the best condition. The inhibition was recorded to reach >5 times higher against radicals and >2 times higher against reducing iron. Compared to previous research, the antioxidant activity was 2 times higher. Moreover, the hydrolysates prapared from semi-purified trypsin showed a better activity against reducing iron while those from crude extract trypsin were most appropriate for scavenging DPPH radicals. Additionally, our research might enhance the commercial uses of the *pindang* by-products as an antioxidant source. The fractionation and sequencing of antioxidant peptides need to be further investigated.

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Supplementary Materials

Supplementary materials is not available for this article.

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