J. Trop. Agric. and Fd. Sc. 51 (2)(2023): 51 – 60

Extraction of protein and antioxidant and antihypertensive properties of protein hydrolysates derived from black tilapia (*Oreochromis placidus)*

Kasran, M.[1]*, Mohamed, N.[2] and Ishak, Z.[1]

[1] *Food Science and Technology Research Centre, MARDI Headquarters, Persiaran MARDI-UPM, 43400 Serdang, Selangor*
[2] *Food Science and Technology Research Centre, MARDI Kuala Terengganu, 21200 Kuala Terengganu, Terengganu*

Abstract

Protein hydrolysates were produced from black tilapia protein isolate. The degree of hydrolysis was determined and antioxidant and antihypertensive properties of protein hydrolysates were evaluated. Protein solubility was tested within pH range between 3 to 8 before protein extraction. Black tilapia proteins were most insoluble at pH 5.2 and hence proteins were extracted at pH 5.2. SDS-PAGE electrophoretogram showed black tilapia having molecular weight ranging from 10 to 150 kDa. Amino acids analysis revealed that there were no significant difference of the individual amino acids in protein isolate and protein hydrolysates/100 g protein where glutamic acid was the most abundant. The degree of hydrolysis of black tilapia reached an optimum value after 4 h of hydrolysis. The ferric reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays were used to determine the antioxidant properties of protein hydrolysates. Antihypertensive activity of protein hydrolysates was performed via angiotensin converting enzyme (ACE) inhibitory activities. In vitro, the bioactivity of these hydrolysates showed high antioxidant activity (DPPH inhibition, 90% with IC_{50} value of 0.47 mg/mL and FRAP value, 166 mM Fe(II)/g dry wt. sample) while anti-hypertension activity (ACE inhibitory activity) was 81%. The results showed black tilapia protein hydrolysates exhibited antioxidant and antihypertensive activities.

Keywords: protein extraction, protein hydrolysates, DPPH, FRAP, ACE inhibitory

Introduction

Food proteins in addition to supplying essential nutrients could confer additional health benefits beyond nutrition. Food proteins and food-derived peptides are now recognised to elicit beneficial physiological effects; therefore have the potential to influence health and may help reduce the risk of chronic disease (Rutherfurd-Markwick 2012). Bioactive peptides are specific protein fragments with peptides ranging in sizes from 2 to 20 amino acid residues (Zaky et al. 2022). In addition to acting as sources of nitrogen and amino acids, these peptides have numerous potential physiological functions within the body which includes opioid, immunomodulatory, antibacterial, antithrombotic and antihypertensive activity (Murray and Fitzgerald 2007). Moreover, some of these peptides may exhibit multifunctional properties (Miesel et al. 2006). In general, these peptides are encrypted within the sequence of parent protein and may be released during gastrointestinal digestion and/or during food processing (Harnedy and FitzGerald 2012).

Antihypertensive and antioxidative peptides derived from food proteins are the most comprehensively studied bioactive peptides. Many studies have shown that peptides with enhanced bioactivity have been produced through enzymatic digestion of fish. They are considered to be safe and healthy compounds with low molecular weight, low cost, high activity and easy absorption (Sánchez and Vázquez 2017; Bhat et al. 2019). In recent years, numerous protein hydrolysates from aquatic products and by-products such as mackerel protein, jumbo squid skin, yellowfin sole frame protein, hoki frame protein, Alaska pollack frame protein and whey protein hydrolysates have been reported to possess antioxidant activity (Wu et al. 2003; Jun et al. 2004; Je et al. 2005a; Mendis et al. 2005; Kim et al. 2007; Peng et al. 2009).

A large numbers of bioactive peptides with antihypertensive activities derived from various marine organisms such as tuna (Hwang 2010), sardine by-product protein hydrolysates (Bougatef et al. 2008), Alaska pollack (Byun and Kim 2002), shark (Wu et al. 2008), bullfrog (Qian et al. 2007), blue mussel (Je et al. 2005b), oyster

Article history
Received: 24.3.23
Accepted: 18.5.23

Authors'full name: Madzlan Kasran, Nor Salasiah Mohamed and Zuwariah Ishak
Corresponding author: madzlan@mardi.gov.my

(Je et al. 2005c), sea cucumber (Zhao et al. 2007) and algae (Sheih et al. 2009) were studied. Moreover, Phadke et al. (2014) reported angiotensin-I converting enzyme (ACE) inhibitory activity and antioxidant activity of ngari, a fermented fish product. In addition, Neves et al. (2017) studied bioactive peptides from Atlantic salmon (*Salmo salar*) with angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory and antioxidant activities.

In Malaysia, black tilapia is an underutilised fish compared to red tilapia. Red tilapia (*Oreochromis niloticus*) accounts for 44.7% of the total freshwater aquaculture production, followed by catfish (36.7 %) and carps (10.08%). In terms of value of production, tilapia contributes 49.37%, followed by catfish (37 %) and carps (10 %). In terms of value, red tilapia yields the highest value at USD 27 million. The black tilapia, which was introduced in the 1950's, did not augur well, due to its colour compared to the red hybrid tilapia (FAO Fisheries and Aquaculture - National Aquaculture Sector Overview - Malaysia). Since black tilapia is an underutilised fish, transforming black tilapia proteins into corresponding protein hydrolysates with antioxidant and antihypertensive properties would add to its value.

The present study is to extract protein from black tilapia **(***Oreochromis placidus) flesh* and to investigate the antioxidant and antihypertensive activities of their corresponding *protein hydrolysates*. The extracted protein or protein isolate was hydrolysed into protein hydrolysates. Antioxidant and antihypertensive activities of the *protein hydrolysates were* then investigated. The ferric reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays were used to determine antioxidant properties of protein hydrolysates and antihypertensive activity of protein hydrolysates were performed via angiotensin converting enzyme (ACE) inhibitory activities.

Materials and methods

Materials

Black tilapia about 5 to 6 months of age and an average weight of 450 to 500 g was supplied by the retailer. All chemicals used in this study were of analytical grade unless otherwise specified. Sodium tetraborate decahydrate, sodium chloride, papain, ethyl acetate, DPPH (1,1-diphenyl-2-picrylhydrazyl), angiotensin converting enzyme (ACE) from rabbit lung and hippuryl histidine leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium acetate trihydrate, Anhydrous Ferric (III) chloride, Ferrous heptahydrate were purchased from Nacalai Tesque, Japan, 2,4,6-Tri(2-pyridyl)-s-triazine was purchased from Acros Organics, Belgium. Ascorbic acid, hydrochloric acid (HCl), glacial acetic acid, sodium acetate, methanol, sodium chloride, carvedilol and tris-HCl were purchased from Sigma (USA).

Methods

Determination of protein solubility

Protein solubility of black tilapia protein as a function of pH was determined by the method of Babiker et al. (1998). Samples solution (1 mg/mL protein) at various pH values (pH 3 – 5, 0.05 M acetate buffer; pH 6 – 8, 0.05 M phosphate buffer and pH 9, 0.05 M tris buffer) were centrifuged at 1,118 x g for 20 min. After centrifugation, each supernatant was decanted and filtered (Whatman No. 1 filter paper) and its protein content was determined by BIO-RAD DC protein assay, Lowry method (Lowry et al. 1951). Protein solubility at various pH were indicated by the ratio of the protein concentration in the supernatant to that in the original protein solution at pH 9.

Extraction of protein from black tilapia fish meat

The proteins were extracted by grinding the black tilapia flesh in a mixer (IKA, A10 Basic, Willmington, NC). Protein was extracted by suspending the minced muscle in water at 1:10 ratio (w/w, minced muscle : water) and homogenised with Ultra Turrax homogeniser, IKA, Germany at high speed for 1 min at 25 ^{o}C. The solution was gradually added with 0.5 M NaOH until the pH constant reached pH 9. Soluble proteins present in the solution were separated by centrifugation at 7,800 x g for 30 min at 4 ^{o}C in a Sorvall RC-5B Superspeed Refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co. Newtown, CT). The supernatant was adjusted to pH 5.2 with 2 N HCl enabling the precipitation of protein. The precipitate was separated by centrifuging at 7800xg for 30 min in a Sorvall RC-5B Superspeed refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co. Newtown, CT) at 4 ^{o}C and the supernatant was discarded. The precipitate was washed with water and re-solubilized in deionised water and the final pH was adjusted to pH 7.0 with 2 N NaOH, freeze dried and stored at -20 ^{o}C for further use. The extracted protein is called black tilapia protein isolate.

Chemical analysis

The moisture, ash, crude protein and crude fat contents of fish proteins were determined by standard methods outlined by AOAC (2005).

Preparation of protein hydrolysate

The hydrolysis was performed according to Madzlan et al. (2006). A 10 g of freeze-dried fish protein was dispersed in 500 ml distilled water to make 2% substrate. The solution was heated at 95 °C for 5 min and then cooled immediately to room temperature. Enzyme was added (enzyme: substrate ratio of 2:100 on a w/w basis) to hydrolyze the extracted protein at a temperature of 60 ^{o}C and pH 6. L-cysteine (free base) was added at concentration of 0.01 M as papain activator. The

hydrolysate solution was incubated for 4 h at 200 rpm. After 4 h, the solution was heated at 95 °C for 10 min to inactivate the enzyme. The hydrolysates were then centrifuged at 7,800 x g in a Sorvall RC-5B Superspeed Refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co. Newtown, CT) at 4 ºC for 30 min followed by filtering through filter paper (Whatman No. 1) to remove free lipids. The filtrates were then freeze-dried and stored at -20°C until further use.

Determination of degree of hydrolysis

The method was based on the ratio of trichloroacetic acid (TCA) soluble nitrogen as discussed by Hoyle and Merritt (1994) and Madzlan et al. (2006). During the course of hydrolysis, 20 mL aliquot was removed after 0, 1, 2, 4, 8, 16 and 24 h to determine the optimum hydrolysis time. At each time period, triplicate samples were taken and 20 ml 20% TCA was added to create a 10% TCA soluble and insoluble fractions. The solutions were centrifuged at 1,118 x g for 15 min and the supernatants were assayed for nitrogen by the Kjeldhal method (AOAC, 2005). Samples taken directly from the hydrolysates were diluted and assayed for nitrogen. The approximate degree of hydrolysis is expressed as:

$$\% \text{ Degree of hydrolysis} = \frac{\text{Soluble N in 10\% TCA x 100}}{\text{Total N in the sample}}$$

SDS-PAGE analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 12%, w/v, acrylamide separating gel and a 5%, w/v, stacking gel both containing 0.1%, w/v, SDS. Fish proteins (3%, w/v) were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2%, w/w SDS, 5%, w/w, mercaptoethanol and 0.012%, w/w, bromophenol blue and heated at 95 ºC for 4 min prior to loading into gel slots. Electrophoresis was carried out at a constant voltage (200 V) for 45 min using a tris glycine buffer (pH 8.3) containing 0.125%, w/w, SDS. The gel was removed from the electrophoresis unit, stained with Coomassie brilliant blue R-250 and destained with deionised water.

Amino acids analysis

Individual amino acids were determined after digestion of the samples in 6 N HCl at 110 ºC as described by Blackburn (1968). The sample was hydrolysed in triplicates by using a sealed hydrolysis tube (Davies and Thomas, 1973). About 0.05 – 0.1 g of the homogenised sample was weighed into a medium wall borosilicate test tube. It was then added with 10 ml of 6 N HCl and the tube was flushed with nitrogen to remove soluble oxygen before being hydrolysed in the oven at 110 ºC for 24 h. After hydrolysis, the sealed end of the tube was opened and the tube was allowed to cool. The hydrolysate was transferred to a 100 ml volumetric flask and 400 ml of 50 mmole/ml of α-amino butyric acid (AABA) was then added before being made up to volume with deionised water. The hydrolysates were then filtered through filter paper (Whatman No. 541) and re-filtered with syringe filter (0.45µm).

Derivatisation was done according to Cohen and De Antonis (1994) using borate buffer and 6-aminoquinolyl-Nhydroxysuccinimidyl carbamate (ACCQ-tag reagent). Borate buffer (70 µl) and 10 µl of sample were mixed and vortex to increase the pH. Then, 20 µl of ACCQ-tag reagent was added and vortex immediately. The sample was kept at room temperature for 1 min for the derivatisation to complete.

The amino acid content of the samples was analysed and quantitatively determined using Waters 510 HPLC system (Waters, USA) and detected by Waters 470 fluorescence detector at Eλ and Em of 250 nm and 395 nm respectively. A volume of 10 µl of derivatised sample was injected in gradient mode into the column and the amino acids were eluted automatically. The column used was Waters AccQ-Tag Amino Acid Analysis Column (3.9 mm id x 150 mm) at 36 °C. The quantity of each amino acid was determined from the chromatogram.

Determination of antioxidant activity

DPPH radical scavenging activity

Radical scavenging activities by black tilapia protein hydrolysates were evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals according to a slightly modified method of Blois (1958). DPPH solution was prepared at a concentration of 0.2 mM in ethanol. The free radical scavenging activity was measured by measuring the decrease in absorbance of ethanolic DPPH solution at 517 nm in the presence of peptides (Ismail and Hong, 2002). About 1.0 mL diluted sample (at different concentration) was mixed with 1.0 mL DPPH solution in test tube, stopper with glass ball and mix well. The solution was stored in the dark for 30 min and the absorbance was determined at 517 nm against diluted blank. Diluted blank was prepared by adding 1.0 mL water with 1.0 mL DPPH solution and the absorbance was determined using a UV-VIS spectrophotometer (Lambda 35; Perkin Elmer, Inc. Waltham, Massachusetts, USA). The antioxidant activity was expressed as;

$$\text{DPPH radical scavenging ability (\%)} = [(A_{control} - A_{sample})/(A_{control}] \times 100\%$$

IC_{50} value was determined from the plotted graph of scavenging activity versus the concentration of protein hydrolysates, which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%. Triplicate measurements were carried out and their activity was calculated by the percentage of DPPH scavenged.

Ferric reducing activity (FRAP Assay)

The ferric reducing activity of the black tilapia protein hydrolysates was estimated based on the Ferric reducing ability of plasma (FRAP) assay developed by Benzie and Strain (1996). The solutions for this assay consisted of 300 mmol/L acetate buffer, 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L of HCl and 20 mmol/L $FeCl_3.6H_2O$. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) with 16 ml glacial acetic acid and brought to 1 l with distilled water. Reagent for this assay was prepared fresh by mixing 25 mL acetate buffer with 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$. The FRAP assay was performed by warming 1 mL of dH_2O at 37 °C before adding 25 µL of sample and 1 mL of reagent and incubating at 37 °C for 4 min. A reagent blank (without adding samples) was incubated at 37 °C for 4 min.The absorbance at 593 nm was determined using a UV-VIS spectrophotometer (Lambda 35; Perkin Elmer, Inc. Waltham, Massachusetts, USA). A standard curve was prepared using various concentrations (200 -1000 µM) of ferrous sulphate ($FeSO_4$. $7H_2O$). The total antioxidant capacity of samples was determined against a standard of known FRAP value. The difference between sample absorbance and blank absorbance was calculated and used to calculate the FRAP value. In this assay, the reducing capacity of the protein hydrolysates tested was calculated with reference to the reaction signal given by a Fe2+ solution. FRAP values were expressed as mmol Fe2+/g of sample. All measurements were done in triplicate.

Angiotensin converting enzyme (ACE) inhibition assay of protein hydrolysate

The ability of the peptides produced enzymatically from black tilapia protein isolate to inhibit the activity of ACE *in vitro* was measured according to the spectrophotometric method (Cushman and Cheung 1971; Sarmadi et al. 2011) with slight modification. Briefly, 100 µL of peptides and 100 µL of hippuryl-L-histidyl-L-leucine (HHL, 12.5 mM in 0.05 M sodium Borate buffer containing NaCl 0.4 M, pH 8.3) were incubated at 37 ºC for 5 min. Then, 150 µL of ACE was added and the mixture was incubated for 1 h. The enzymatic reaction was stopped by adding 250 µL of 0.5 N HCl. The hippuric acid formed by the action of ACE on HHL was extracted from the acidified solution into 1.5 mL ethyl acetate by vortexing for 15 s. The mixture was centrifuged at 3,290 x g for 10 min at 4 ºC, and a 0.5 mL aliquot of each ethyl acetate layer was transferred to clean tubes and evaporated by heating at 85 ºC until dry on a heating plate. The hippuric acid was redissolved in 3 mL of 1 M NaCl, and the absorbancewas measured at 228 nm using an UV-VIS spectrophotometer (Lambda 35; Perkin Elmer, Inc. Waltham, Massachusetts, USA). Assay mixture without peptides was referred as control. The extent of inhibition was calculated as:

$$\%ACE\ inhibition = (A_{control}-A_{sample} / A_{control}) \times 100$$

Statistical analysis

All experiments were performed in triplicates. Statistical analysis was performed by using SPSS ver. 20. The results were first checked for normality before proceeding to one-way ANOVA, two-way ANOVA and paired Sample T-test analysis. The data is considered statistically significant at $p \leq 0.05$ or $p \leq 0.001$.

Results and discussion

Protein solubility

Protein solubility was carried out before the extraction of protein. Protein solubility was carried out as a function of pH from 3 – 8 as shown in *Figure 1*. The solubility of protein in solution is influenced by pH. As the pH of solution is adjusted to the isoelectric point of protein, the net charge of proteins is reduced, which results in the precipitation of protein. It was found that the isoelectric point of black tilapia is at pH 5.2 where proteins are most insoluble due to zero net charge. Therefore proteins were extracted from black tilapia at pH 5.2. The results were similar to those of Rodriguez et al. (1994) where the precipitate of solid content obtained was significantly higher at around pH 4.5. Precipitation of protein has usually been performed by adjusting the pH to isoelectric point of protein for fish protein (Del Valle and Aguilera 1990).

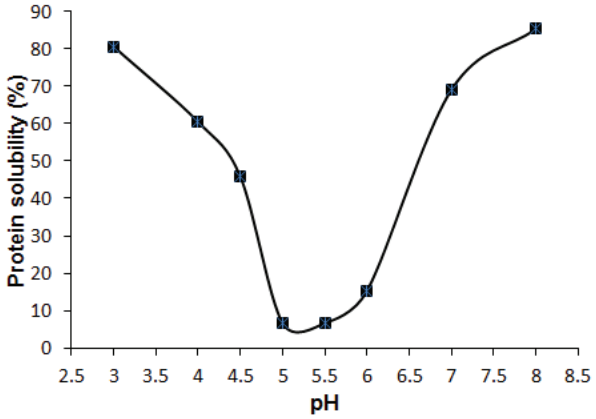

Figure 1. Protein solubility of black tilapia fish meat protein as a function of pH from 3 to 8. Results represent means ± SD of three independent experiments

Chemical composition of fresh black tilapia fish meat and their protein isolate

Aqua-cultured fish and shellfish waste contain significant amount of high quality protein which can be a good source of bio-peptide mining (Harnedy and FitzGerald, 2012). Chemical composition analysis was carried out on black tilapia fish meat and its protein isolate as shown in *Table 1*. In fresh fish meat, black tilapia contains small amount of fat and ash (0.58% ± 0.05 and

1.23% ± 0.09 respectively) while the protein content was 20.28% ± 0.65. Dried powder of black tilapia protein isolate (extracted at pH 5.2) contained high amount of protein (92.53% ± 1.52) and minute amount of fat (0.08% ± 0.05). The ash content was slightly higher than fresh fish meat. This might be due to the formation of sodium chloride during neutralisation of protein solution with sodium hydroxide.

Degree of hydrolysis

Degree of hydrolysis was used as a measure of the extent of hydrolysis. The degree of hydrolysis was measured as a function of hydrolysis time from 0 to 24hr. The degree of hydrolysis (DH) of black tilapia protein isolate hydrolyzed by papain is shown in *Figure 2*. It was found that there was an initial rapid phase of hydrolysis and the degree of hydrolysis reached an optimum value of 84% after 4 h of hydrolysis. Shahidi et al. (1995) found that the enzymatic hydrolysis of fish muscle proteins is characterized by an initial rapid phase, during which a large number of peptide bonds are hydrolyzed, after which the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place. A high concentration of soluble fish peptides was released in the reaction mixture during the initial phase of hydrolysis.

SDS-PAGE electrophoretogram of unhydrolysed and hydolysed black tilapia

SDS-PAGE electrophoretogram of black tilapia protein its hydrolysate is shown in *Figure 3*. Due to high degree of structural diversity, fish proteins provide a large range of substrate material. Black tilapia has molecular weight ranging from 15 kDa to 250 kDa. These may represent various intact myofibrillar proteins. Fish muscle proteins are classified into three main groups, i.e, sarcoplasmic, myofibrillar and stroma proteins. Myofibrillar proteins are structural proteins and account for 65 – 75% of the total protein in fish muscle (Venugopal, 2009). Actin and myosin are the two muscle proteins responsible for muscle contraction. Myosin ranging from 50% to 58% (w/w) is the most abundant protein present in the contractile apparatus while actin accounts for 15 – 20% (w/w) (Vareltzis 2000). Other structural and regulatory proteins associated with the contractile apparatus and myofibrillar proteins include tropomyosin, troponin, actinin, desmin, nebulin and the C and N proteins.

SDS-PAGE was used to characterise the molecular weight of peptides through enzymatic hydrolysis. Figure 3 shows the SDS-PAGE electrophoretogram pattern and molecular weight of unhydrolysed and hydrolysed black tilapia proteins by papain after 4 h of hydrolysis. As shown in *Figure 3*, hydrolysis of protein produces peptides

Table 1. Proximate composition of black tilapia fish meat and its protein isolate

	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Black tilapia fish meat	77.18±0.66	20.28±0.65	0.58±0.05	1.23±0.09
Black tilapia protein isolate powder	5.18±0.66	92.53±1.52	0.08±0.05	2.23±0.09

Results represent means ± SD of three independent experiments (n=3).

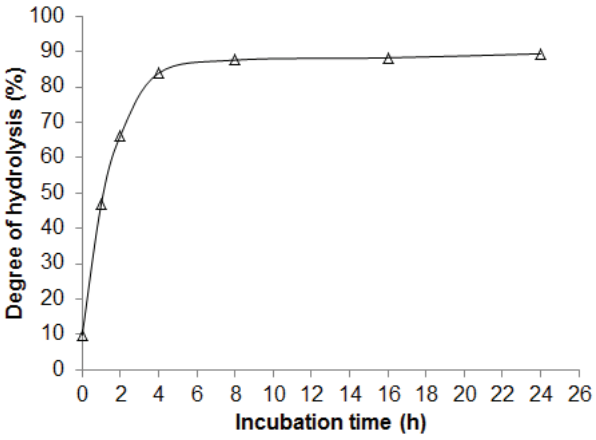

Figure 2. Hydrolysis curves for black tilapia protein isolate by papain as a function of time from 0 to 24 h. Results represent means ± SD of three independent experiments

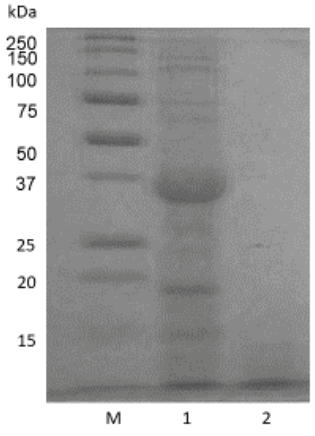

Figure 3. SDS-PAGE electrophoretogram of black tilapia protein isolate and corresponding peptides hydrolysed by papain after 4 hr of hydrolysis; 1 – unhydrolysed protein, 2 – hydrolysed protein (after 4 hr of hydrolysis)

with molecular weight less than 15 KDa indicating the presence of low molecular weight peptides. Previous studies showed that hydrolysis of various species such as catfish, cod and salmon using Protamex, Neutrase and Alcalase yielded low molecular weight peptides at a high percent of degree of hydrolysis (Panyam and Kilara 1996; Liaset et al. 2000; Theodore 2005).

Amino acids composition of black tilapia protein isolate and its protein hydrolysates

Amino acids composition of black tilapia protein isolate and its protein hydrolysates is shown in *Table 2*. Analysis of the amino acid composition revealed that there was no significant difference of the individual amino acid content between black tilapia protein and black tilapia protein hydrolysates (per 100 g protein). Glutamic acid was the most abundant amino acid followed by aspartic acid. Both black tilapia protein and black tilapia protein hydrolysates exhibited high amounts of hydrophobic amino acids. Hydrophobic amino acids amounted 39.73 g and 39.54 g per 100 g protein for black tilapia protein isolate and its protein hydrolysates respectively excluding tryptophan. Many food-derived peptides with hydrophobic amino acids have been observed to exhibit antioxidant activities (Mendis et al. 2005; Sarmadi and Ismail 2010).

Table 2. Amino acids composition of black tilapia protein isolate and its protein hydrolysates

Amino Acids	Black tilapia protein isolate (g/100 g protein)	Black tilapia protein hydrolysates (g/100 g protein)
Aspartic acid	8.82 ± 0.21	8.77 ± 0.25
Serine	3.63 ± 0.15	4.18 ± 0.05
Glutamic acid	13.32 ± 0.30	14.57 ± 0.30
Glycine*	8.90 ± 0.10	5.40 ± 0.12
Histidine	2.40 ± 0.09	2.56 ± 0.04
Arginine	6.49 ± 0.13	7.30 ± 0.57
Threonine	4.75 ± 0.21	6.36 ± 0.09
Alanine*	6.34 ± 0.23	5.47 ± 0.06
Proline*	4.41 ± 0.30	3.62 ± 0.10
Tyrosine	2.28 ± 0.09	4.05 ± 0.09
Valine*	4.07 ± 0.13	4.43 ± 0.12
Methionine*	2.27 ± 0.08	3.43 ± 0.12
Cysteine	10.46 ± 0.25	8.24 ± 0.30
Lysine	8.08 ± 0.14	5.80 ± 0.28
Isoleucine*	3.43 ± 0.09	4.05 ± 0.09
Leucine*	6.55 ± 0.07	9.51 ± 0.10
Phenylalanine*	3.79 ± 0.04	3.63 ± 0.08

*Hydrophobic amino acids. Results represent means ± SD of three independent experiments (n=3).

Antioxidant activity

DPPH radical scavenging activity

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al. 1997). The DPPH radical model is a quick method and widely-used to evaluate free radical scavenging activity. The effect of antioxidants on DPPH radical scavenging is occurring due to their hydrogen donating ability (Baumann et al. 1979) and this scavenging activity is observed as a change in color from purple to yellow. Therefore, DPPH is often used as a substrate to evaluate antioxidant activity.

Figure 4 shows the DPPH free radical scavenging activity of protein hydrolysates produced from enzymatic hydrolysis of black tilapia protein isolate using papain as a function of hydrolysis time from 1 to 6 h at 1 mg/mL. The results revealed that the optimum hydrolysis time to produce the highest DPPH radical scavenging activity was 4 h. After 1 h of hydrolysis, DPPH radical scavenging activity increased to 76% and reached an optimum DPPH radical scavenging activity to 90% after 4 h of hydrolysis. As the hydrolysis time was increased the DPPH radical scavenging activity decreased as the proteins were hydrolysed into smaller molecular weight. After 6 h of hydrolysis the DPPH radical scavenging activity decreased to 75%. Previous reports revealed that the hydrolysis condition and specificity of enzymes may affect the antioxidant activity of hydrolysates (Tkaczewska et al. 2020). In addition, low molecular weight hydrolysates (below 5000 Da) have higher scavenging activity than high molecular weight hydrolysates (Yu et al. 2018). At 4 h of hydrolysis the hydrolysates effectively quenched DPPH radicals and the IC_{50} value was 0.47 mg/mL. In DPPH assay, the lower the $IC_{50,}$ the better it is able to scavenge the radicals, particularly peroxy radicals which are the propagators of the autoxidation of lipid molecules and thereby break the free radical chain reaction (Frankel 1991).

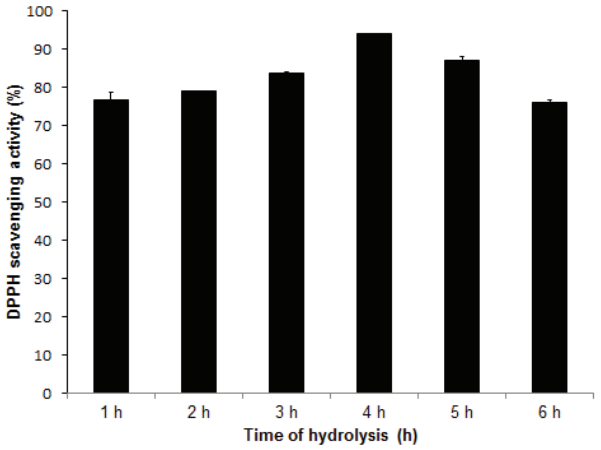

Figure 4. DPPH free radical scavenging activity of black tilapia protein hydrolysates at different hydrolysis time at concentration 1 mg/mL. Results represent means ± SD of three independent experiments. Values with $p < 0.05$ were considered significant

Ferric reducing antioxidant power assay (FRAP)

FRAP assay based on the measurement of the ability of a substance to reduce Fe^{3+} to Fe^{2+} was initially proposed to measure the total antioxidant capacity of plasma (Benzie and Strain 1999). Fe^{2+} is measured spectrophotometrically via determination of its coloured complex with 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) which has a high absorbance at 595 nm. Since the antioxidant activity of a substance is usually correlated directly to its reducing capacity, the FRAP assay provides a reliable method to study the antioxidant activity of various compounds (Benzie and Strain 1999). This method has been frequently used for a rapid evaluation of the total antioxidant capacity of various food and beverages (Benzie and Szeto 1999; Moyer et al. 2002).

In this assay, the antioxidants present in the peptides act as the reductants while the reagent containing excess ferric ions acts as the oxidants. Reduction of the ferric-tripyridyltriazine to the ferrous complex forms an intense blue colour which can be measured at a wavelength of 593 nm. The intensity of the colour is related to the amount of antioxidant reductants in the samples. In this assay, the protein hydrolysates produced after 4 h of hydrolysis was used to determine the ability of protein hydrolysates to reduce Fe^{3+} to Fe^{2+} as 4 h of hydrolysis was the optimum time to reach the optimum degree of hydrolysis. The ability of protein hydrolysates to reduce Fe^{3+} to Fe^{2+} may be attributed to high contents of electron or hydrogen donating peptides by liberating potent peptides. Moure et al. (2006) reported that protein size and concentration clearly influences reducing power. The FRAP value was calculated from standard curve of Fe^{2+} at concentration from 0 to 1000 μM $FeSO_4$. $7H_2O$ and the FRAP value was 166 mM Fe(II)/g dry wt. sample.

In vitro Angiotensin converting enzyme (ACE) inhibitory activity of black tilapia protein hydrolysates

ACE inhibitory activity was reported as percent of ACE inhibition by samples. *Figure 5* shows the ACE inhibitory activity of peptides produced from black tilapia protein hydrolysates using papain as a function of hydrolysis time from 0 to 24 h. The results revealed that the optimum hydrolysis time to produce the highest ACE inhibitory activity was 4 h. Unhydrolysed protein showed very low inhibition activity (7%). After 1 h of hydrolysis, ACE inhibition activity increased to 64% and reached an optimum ACE inhibitory activity to 81% after 4 h of hydrolysis. As the hydrolysis time was increased the ACE inhibitory activity decreases as the proteins were hydrolyzed into smaller molecular weight. After 8 h of hydrolysis, ACE inhibition activity of the hydrolysates reduced to 56% and was further reduced to 45% after 24 h of hydrolysis. The results showed that hydrolysis for 4 h was enough to produce peptides with molecular weight that inhibit ACE activity at optimum condition. The ability of low molecular weight peptides to inhibit ACE activity has also been reported for peptides derived from a variety of fish species such as yellowfin sole (Jung et al. 2006), Alaska pollock (Je et al. 2004) and sea bream (Fahmi et al. 2004).

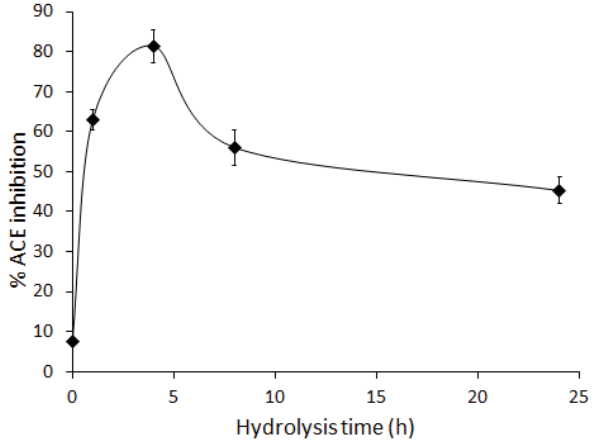

Figure 5. ACE inhibitory activity of black tilapia protein hydrolysates at different hydrolysis time (hydrolysed by papain). Results represent means ± SD of three independent experiments. Values with $p < 0.05$ were considered significant

Peptides rich in hydrophobic amino acids (e.g. Val, Leu, Ile) and cyclic or aromatic amino acids (e.g. Pro, Phe, Tyr) are reported to possess excellent antioxidant activity by donating a proton and scavenging the radicals (Fan et al. 2021; Villamil et al. 2017; You et al. 2010). Hydrophobic amino acids are credited with strong radical scavenging activity in oxidative reactions, especially for enzyme-catalyzed reactions due to the presence of an imidazole ring as an important proton donor (Samaranayaka and Li-Chan 2008). The mechanism of action may be that the antioxidant peptides could smoothly enter into target organs through hydrophobic interactions with membrane lipid bilayer by the aid of their hydrophobicity, enabling them to scavenge free radicals (Pouzo et al. 2016). Several structural features have been identified which appears to influence the biological action of peptides. Murray and FitzGerald (2007) reported binding of the peptide to angiotensin converting enzyme (ACE) in ACE inhibitory activity of peptides being strongly influenced by the presence of tyrosine, phenylalanine, tryptophan, proline, lysine, isoleucine, valine, leucine and arginine.

Recently it has been hypothesised that oxidative stress is a key player in the pathogenesis of hypertension (Montezano and Touyz 2012; Rodrigo et al. 2011). Oxidative stress (OS) is believed to be involved in many types of disease processes. Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense systems. Antioxidants are compounds that are able to trap ROS and thus may be capable of reducing oxidative damage and posibly blood pressure (Baradaran et al. 2014). There might be a correlation between oxidative stress and arterial hypertension (Rodrigo et al. 2011; Montezano and Touyz 2012). A reduction in

superoxide dismutase and glutathione peroxidase activity have been observed in newly diagnosed and untreated hypertensive subjects, which are inversely correlated with blood pressure. Hydrogen peroxide production is also higher in hypertensive subjects (Pedro-Botet et al. 2000). Furthermore, hypertensive patients have higher lipid hydroperoxide production. Oxidative stress is also markedly increased in hypertensive patients with renovascular disease (Lip et al. 2002)

Those findings have led to the use of exogenous antioxidants to reduce blood pressure (Nasri, 2013). Antioxidant inhibits ROS-producing enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) and xanthine oxidase activates and enhances endothelium-derived nitric oxide synthase (eNOS) expression and increases glutathione. These have improved endothelial function, subsequent normalisation of vascular tone and an overall antihypertensive effect (Ward et al. 2005).

Conclusion

Solubility analysis showed that black tilapia proteins are most insoluble at pH 5.2 and hence proteins were extracted at pH 5.2. SDS-PAGE electrophoretogram showed that black tilapia has molecular weight ranging from 10 kDa to 150 kDa and predominantly ranges between 10 kDa to 37 kDa. Amino acid analysis revealed that there was no significant difference of the individual amino acid content in fresh meat, protein isolate and protein hydrolysates/100 g protein where glutamic acid was the most abundant followed by aspartic acid. It was found that the degree of hydrolysis of black tilapia reached an optimum value after 4 h of hydrolysis. Protein hydrolysates were produced from black tilapia protein isolate through enzymatic hydrolysis using papain. In vitro, the bioactivity of these hydrolysates showed high antioxidant activity (DPPH inhibition, 90% with IC_{50} value of 0.47 mg/mL and FRAP value, 166 mM Fe(II)/g dry wt. sample) and anti-hypertension activity (Angiotensin converting enzyme inhibitory activity, 81%).

Acknowledgment

The project was funded by MARDI 10th Malaysian Development Fund (Project Title: P*enyelidikan Produk Ditambah Nilai Tinggi Daripada Rumpai Laut Tempatan, Akuakultur Dan Pertanian* – Project code: 21003001630001). The authors highly appreciate Mohd. Fakhri Hashim, Mazliana Badaruddin and Fadzilah Puteh for their contributions and technical assistance.

References

AOAC (2005). Determination of Moisture, Ash, Protein and Fat. Official method of Analysis, Association of Officiating Analytical Chemists, 18th Edition, AOAC, Washington DC.

Babiker, E. E., Hiroyuki, A., Matsudomi, N., Iwata, H., Ogawa, T., & Bando, N. (1998). Effect of polysaccharide conjugation or transglutaminase treatment on the allergenicity and functional properties of soy protein. *Journal of Agricultural and Food Chemistry*, *46*, 866–871.

Baradaran, A., Nasri, H., & Rafieian-Kopaei, M. (2014). Oxidative stress and hypertension: Possibility of hypertension therapy with antioxidants. *Journal of Research in Medical Sciences, 19*, 358–67.

Baumann, J., Wurn, G., & Bruchlausen, F. V. (1979). Prostaglandin synthetase inhibiting O_2-radical scavenging properties of some flavonoids and related phenolic compounds, *Naunyn, Schmiedebergs. Archives of Pharmacology, 308*, 27–32.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, *239*, 70–76.

Benzie, I. F., & Szeto, Y. T. (1999). Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay, *Journal of Agricultural and Food Chemistry*, *47*, 633–636.

Bhat, Z. F., Mason, S., Morton, J. D., Bekhit, A. E. D. A., & Bhat, H. F. (2019). Antihypertensive Peptides from Animal Proteins. In Mérillon, J. M., & Ramawat, K. (eds) *Bioactive Molecules in Food*. Reference Series in Phytochemistry. Springer, Cham.

Blackburn, S. (1968). *Amino Acids Determination Methods and Techniques*. New York: Marcel Dekker Inc.

Blois, M. S. (1958). Antioxidant determination by the use of stable free radical. *Nature, 181*, 1533–1535.

Bougatef, A., Nedjar-Arroume, N., Ravallec-Pie, R., Leroy, Y., Guilochon, D., & Baricia, A. (2008). Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (*Sardinelle aurita*) by products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases. *Food Chemistry*, *111*, 350–356.

Byun, H. G., & Kim, S. K. (2002). Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from Alaskan pollack skin. *Journal of Biochemistry and Molecular Biology*, *35*, 239–243.

Cohen, S. A., & De Antonis, K. M. (1994). Application of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Analysis of feed grains, intravenous solutions and glycoproteins. *Journal of Chromatography, 661*, 25–34.

Cushman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin I-converting enzyme of rabbit lung. *Biochemical Pharmacology*, *20*, 1637–1648.

Davies, M. G., & Thomas, A. L. (1973). An investigation of hydrolytic techniques for the amino acid analysis of foodstuffs. *Journal of Science and Food Agriculture*, *24*, 1525–1540.

Del Valle, J. M., & Aguilera, J. M. (1990). Recovery of liquid by-products from fish meal factories: A review. *Process Biochemistry International*, 25(4), 122–131.

Fahmi, A., Morimura, S., Guo, H. C., Shigematsu, T., Kida, K., & Uemura, Y. (2004). Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales. *Process Biochemistry*, 39(10), 1195–1200.

Fan, H., Bhullar, K. S., & Wu, J. (2021). Spent hen muscle protein-derived ras regulating peptides shows antioxidant activity in vascular cells. *Antioxidants, 10*(2), 1–14.

FAO Fisheries & Aquaculture - National Aquaculture Sector Overview - Malaysia

Frankel, E. N. (1991). Recent advances in lipid oxidation. *Journal of the Science of Food and Agriculture*, *54*, 495–511.

Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods*, *4*, 6–24.

Hoyle, N. T., & Merritt, J. H. (1994). Quality of fish protein hydrolysates from herring (*Clupea harengus*). *Journal of Food Sciience*, *59*(1), 76–79.

Hwang, J. S. (2010). Impact of processing on stability of angiotensin I-converting enzyme (ACE) inhibitory peptides obtained from tuna cooking juice. *Food Research International, 43*, 902–906.

Ismail, A., & Hong, T. (2002). Antioxidant activity of selected commercial seaweeds. *Malaysian Journal of Nutrition*, *8*(2), 167–177.

Je, J. Y., Park, P. J., Kwon, J. Y., & Kim, S. K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Journal of Agricultural and Food Chemistry*, *52*(26), 7842–7845.

Je, J. Y., Park, P. J., & Kim, S. K. (2005a). Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Research International*, *38*, 45–50.

Je, J. Y., Park, P. J., Byun, H. G., Jung, W. K., & Kim, S. K. (2005b). Angiotensin I converting enzyme (ACE) inhibitory peptide derived from the sauce of fermented blue mussel (*Mytilus edulis*). *Bioresourse Technology*, *96*, 1624–1629.

Je, Y. J., Park, J. Y., Jung, W. K., & Kim, S. K. (2005c). Isolation of angiotensin I converting enzyme (ACE) inhibitor from fermented oyster sauce (*Crassostrea gigas*). *Food Chemistry*, *90*, 809–814.

Jun, S. Y., Park, P. J., Jung, W. K., & Kim, S. K. (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *European Food Research and Technology*, *219*, 20–26.

Jung, W. K., Mendis, E., Je, J. Y., Park, P. J., Son, B. W., & Kim, H. C. (2006). Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, *94*(1), 26–32.

Kim, S. Y., Je, J. Y., & Kim, S. K. (2007). Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry*, *18*, 31–38.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Liaset, B., Lied, E., & Espe, M. (2000). Enzymatic hydrolysis of by-products from the fish-filleting industry: Chemical characterisation and nutritional evaluation. *Journal of the Science of Food and Agriculture*, *80*(5), 581–589.

Lip, G. Y., Edmunds, E., Nuttall, S. L., Landray, M. J., Blann, A. D., Beevers, D. G. (2002). Oxidative stress in malignant and non-malignant phase hypertension. *Journal of Human Hypertension*, *16*, 333–336.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry, 193*, 265–275.

Madzlan, K., Mamot, S., & Osman, H. (2006). Synthesis of plastein from prawn processing plant wastewater. *Journal of Tropical Agricultural and Food. Science, 34*(2), 277–287.

Meisel, H., Walsh, D. J., Murray, B. A., & Fitzgerald, R. J. (2006). ACE inhibitory peptides. In Mine, Y., & Shahidi, F. (Eds.), *Nutraceutical proteins and peptides in health and disease,* 269–315. Boca Raton: CRC Press.

Mendis, E., Rajapakse, N., Byun, H. G., & Kim, S. K. (2005). Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their in vitro antioxidant effects. *Life Sciences*, *77*, 2166–2178.

Montezano, A. C., & Touyz, R. M. (2012). Oxidative stress, Noxs, and hypertension: Experimental evidence and clinical controversies. *Annals of Medicine*, *44*(1), 2–16.

Moure, A., Dominguez, H., & Parajo, J. C. (2006). Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochemistry*, *41*, 447–456.

Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002). Anthocyanin, phenolics and antioxidant capacity in diverse small fruits: vaccinin, rudbus and ribes, *Journal of Agricultural and Food Chemistry*, *50*, 519–525.

Murray, B. A., & FitzGerald, R. J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Current Pharmaceutical Design*, *13*, 773–791.

Nasri H. (2013). Antiphospholipid syndrome-associated nephropathy: Current concepts. *Journal of Renal Injury Prevention*, *2*, 1–2.

Neves, A. C., Harnedy, P. A., O'Keeffe, M. B., & FitzGerald, R. J. (2017): Bioactive peptides from Atlantic salmon (*Salmo salar*) with angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory and antioxidant activities. *Food Chemistry*, *218*, 396–405.

Panyam, D., & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Science & Technology*, *7*(4), 120–125.

Phadke, G. G., Elavarasan, K., & Shamasundar, B.A. (2014): Angiotensin-I converting enzyme (ACE) inhibitory activity and antioxidant activity of fermented fish product ngari as influenced by fermentation period. *International Journal of Pharma and Bio Sciences*, *5*, 134–142.

Pedro-Botet, J., Covas, M. I., Martin, S., & Rubies-Prat, J. (2000). Decreased endogenous anti-oxidant enzymatic status in essential hypertension. *Journal of Human Hypertension*, *14*, 343–3 45.

Peng, X., Xiong, Y. L., & Kong, B. (2009). Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. *Food Chemistry*, *113*, 196–201.

Pouzo, L.B., Descalzo, A.M., Zaritky, N.E., Rossetti, L., & Pavan, E. (2016). Antioxidant status, lipid and colour staility of aged beef from grazing steers supplemented with corn grain and increasing levels of flaxseed. *Meat Science, 111*, 1–8 .

Qian, Z. J., Jung, W. K., Lee, S. H., Byun, H. G., & Kim, S. K. (2007). Antihypertensive effect of an angiotensin I-converting enzyme inhibitory peptide from bullfrog (*Rana catesbeiana Shaw*) muscle protein in spontaneously hypertensive rats. *Process Biochemistry*, *42*, 1443–1448.

Rodrigo, R., Gonzalez, J., & Paoletto, F. (2011) The role of oxidative stress in the pathophysiology of hypertension. *Hypertension Research*, *34*, 431–440.

Rodriguez-Estrada, M. T., Chung, S., & Chinachoti, P. (1994). Solids extraction of cod frame and effects on ultrafiltration of the aqueous extract. *Journal of Food Science, 59*(4), 799–803.

Rutherfurd-Markwick, K. J. (2012). Food proteins as a source of bioactive peptides with diverse functions. *British Journal of Nutrition*, *108*, 149–157.

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2008). Food-derived peptidic antioxidant: A review of their production, assessment and potential applications. *Journal of Functional Foods, 3*, 229–254.

Sanchez, A., & Vazquez, A. (2017). Bioactive peptides: A review. *Food Quality and Safety*, *1*, 29–46.

Sarmadi, B., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides*, *31*, 1949–1956.

Sarmadi, B., Ismail, A., & Hamid, M. (2011). Antioxidant and angiotensin converting enzyme (ACE) inhibitory activities of cocoa (*Theobroma cacao L.*) autolysates. *Food Research International*, *44*(1), 290–296.

Shahidi, F., Han, X-Q., & Synowiecki, J. (1995).Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*), *Food Chemistry, 53*, 285–293.

Sheih, I. C., Fang, T. J., & Wu, T. K. (2009). Isolation and characterization of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. *Food Chemistry*, *115*, 279–284.

Soares, J. R., Dins, T. C. P., Cunha, A. P., & Ameida, L. M. (1997). Antioxidant activity of some extracts of *Thymus zygis*. *Free Radical Research*, *26*, 469–478.

Theodore, A. E. (2005). Bioactive and functional properties of catfish protein hydrolysates and catfish protein isolates. University of Florida, MS Thesis.119.

Tkaczewskaa, J., Dziadkiewiczb, J. B., Kulawika, P., Dudaa, I., Morawskac, M., & Mickowskad, B. (2020). The effects of hydrolysis condition on the antioxidant activity of protein hydrolysate from Cyprinus carpio skin gelatin. *LWT - Food Science and Technology, 117*, 108616.

Vareltzis, K. (2000). Fish proteins from unexploited and underdeveloped sources. In Doxastakis, g., & Kiosseoglou, V. (Eds.), Novel macromolecules in food systems, 133–159. Amsterdam: Elsevier.

Venugopal, V. (2009). Seafood proteins: Functional properties and protein supplements. Marine products for healthcare: Functional and bioactive nutraceutical compounds from the ocean, 51–102. Boca Raton: CRC Press.

Villamil, O., V´aquiro, H., & Solanilla, J. F. (2017). Fish viscera protein hydrolysates: Production, potential applications and functional and bioactive properties. *Food Chemistry, 224*, 160–171.

Ward, N. C., Hodgson, J. M., Croft, K. D., Burke, V., Beilin, L. J., & Puddey, I. B. (2005). The combination of vitamin C and grape-seed polyphenols increases blood pressure: A randomized, double-blind, placebo controlled trial. *Journal of Hypertensio*n, *23*, 427–434.

Wu, H. C., Chen, H. M., & Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, *36*, 949–957.

Wu, H., He, H. L., Chen, X. L., Sun, C. Y., Zhang, Y. Z., & Zhou, B. C. (2008). Purification and identification of novel angiotensin-I-converting enzyme inhibitory peptides from shark meat hydrolysate. *Process Biochemistry*, *43*, 457–461.

You, L., Zhao, M., Regenstein, J. M., & Ren, J. (2010). Purification and identification of antioxidative peptides from loach (*Misgurnus anguillicaudatus*) protein hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Research International, 43*(4), 1167–1173.

Yu, Y., Fan, F., Wu, D., Yu, C., Wang, Z., & Du, M. (2018) Antioxidant and ACE Inhibitory Activity of Enzymatic Hydrolysates from *Ruditapes philippinarum*. *Molecules, 23*(5), 1189.

Zaky, A. A., Simal-Gandara, J., Eun, J. B., Shim, J. H., & Abd El-Aty, A. M. (2022). Bioactivities, Applications, Safety, and Health Benefits of Bioactive Peptides From Food and By-Products: A Review. *Frontier Nutrition*, *8*, 815640.

Zhao, Y., Li, B., Liu, Z., Dong, S., Zhao, X., & Zeng, M. (2007). Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate. *Process Biochemistry*, *42*, 1586–1591.