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Recovery of Proteolysate From Salmon By-Product: Investigation of Antioxidant Activity, Optimization of Hydrolysis, Determination of Iron-Binding Activity And Identification of Bioactive Peptides

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ABSTRACT : *In this study, salmon by-product was used as protein source to obtain bioactive peptides. Firstly, the effect of by-product:water on protein recovery yield and effects of hydrolysis condition including enzyme type, pH, temperature, enzyme to substrate (E:S) ratio and hydrolysis time on the antioxidant activity of the proteolysate were investigated. Then, response surface methodology (RSM) was applied to maximize the antioxidant capacity through E:S ratio and hydrolysis time. Next, the proteolysate was fractionated and four collected peptide fractions of 10–30 kDa, 3–10 kDa, 1–3 kDa and <1 kDa were examined for their antioxidant and iron-binding capacity. Next, the fraction with the highest bioactivity was used for peptide identification (molecular weight and amino acid sequence) using mass spectrometer / mass spectrometer (MS/MS) method. The result showed that with the by-product:water ratio of 1:10 (w/v), the protein recovery yield achieved the highest value of 28.8 ± 1.4%. The proteolysate with the highest DPPH scavenging activity of 35.4% was obtained when hydrolyzing salmon by-product with the optimal hydrolysis condition including Flavourzyme, pH 7, 50^{o}C, 7.97 hours and E:S ratio of 49.83 U/g protein. The <1 kDa fraction exhibited the highest antioxidant potential with the 50% DPPH• inhibition concentration (IC_{50}) of 1.86 ± 0.01mg/mL and FRAP value of 253.8 ± 8.1 µM Trolox equivalent (µM TE) and iron-binding capacity of 2882.1 ± 499.2 µg Fe^{2+}/g protein. Two peptides, GAAEKGVPLF and GVDNPGHPF, were detected from <1 kDa fraction with their molecular weights of 987.5 Da and 938.4 Da, respectively. These small peptides have been supposed to be easily absorbed in the human body. In addition, these bioactive peptides could be applied as a pharmaceutical or a natural food additive which can substitute for synthetic compounds. This finding suggested a new utilization trend of salmon by-product which is a waste from salmon processing industry.*

KEYWORDS *: antioxidant activity, iron-binding activity, salmon by–product, hydrolysate.*

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I. INTRODUCTION

Antioxidant is a substance that significantly inhibits oxidation of another substances when present at low concentrations compared to that of an oxidizable substrate [1]. Many synthetic antioxidants such as Butylated HydroxyAnisole (BHA), Butylated HydroxyToluene (BHT) have been utilized as food additives to prevent rancidity in food [2]. Even though these synthetic agents possess strong antioxidant capacity, there has been a risk to human health and they have begun to be prohibited in many countries [3]. Antioxidant peptides have been proven that they could protect the body against reactive oxygen species (ROS), which exert oxidative damage to membrane lipids, protein and DNA, causing numerous diseases such as cardiovascular disease, diabetes, cancer and Alzheimer's disease as well as aging [4].

Iron is one of the most essential elements that plays an important role in immune function, muscle metabolism and neuronal function as well as taking part in structure of cytochrome, several enzymes, hemoglobin and myoglobin [5, 6]. Anaemia, poor cognitive development and increased maternal mortality related to iron deficiency which could be prevented by fortifying iron into food in forms of iron salt, elemental iron and iron-binding peptides [7, 8]. Among these methods for supplying iron, iron salt was low bioavailability and poor taste while elemental iron was merely used in solid dehydrated food because of its insolubility [6, 9]. In addition, at physiological pH in the presence of peptic digestion, ferrous ions are rapidly oxidized to the insoluble ferric form and must be first reduced by the enzyme ferric reductase present on the brush border of the enteric cells before being absorbed [10]. Moreover, experimental evidence also indicated that ferrous sulfate

may promote the formation of hydroxyl radicals, which can start the peroxidation of lipids from biologic membranes, enzyme inactivation and DNA damage [11]. In contrast, iron-binding peptides were able to form a stable soluble complex with ferrous ion, resulting in improving iron absorption, stability and bioavailability [12, 13]. Furthermore, iron-binding peptides were capable of remaining complex with iron under acid condition in the stomach but released at higher pH of duodenum where absorbs iron the most in human body [14]. Recently, there are several publications on finding iron-binding peptides from various sources such as shrimp by-product [15], Alaska pollock skin [7], Pacific cod skin gelatin [16], sea cucumber [17] and anchovy muscle protein [18].

By–product from salmon (*Salmo salar*) processing industry accounted for approximately 45% of the total fish [19]; the viscera and trimmings made up more than 50% of the by–product, while heads and backbones, respectively, accounted for about 7 and 8% [20]. The large quantities of waste can cause serious pollution all over the world. However, the discard is rich in protein, bioactive compounds and essential nutrients that could benefit for human health [21]. Recently, there are many researches on bioactive peptides from salmon by-product including pectoral fin [22, 23], trimmings [24], head [25]. However, there is no published study on investigating antioxidant and iron-binding activity of proteolysate from salmon frame.

The aims of this study were to (i) investigate the effect of by-product:water ratio on protein recovery yield and the effects of hydrolysis condition on antioxidant activity of salmon by-product proteolysate; (ii) optimize hydrolysis condition for maximizing the antioxidant activity of the proteolysate; (iii) fractionate the proteolysate and examine the antioxidant and iron-binding activity of obtained peptide fractions; (iv) identify molecular weight and amino acid sequence of antioxidant and iron-binding peptides.

II. MATERIALS AND METHODS

2.1. Materials

2.1.1. Salmon by-product

The salmon frames including bones, fins, tails and some remaining flesh attached to the frames were purchased from Annaseafresh company in Ho Chi Minh city. The by–products were transported on ice to the Biochemical laboratory of Bach Khoa University within 4 hours, individually packed in polyethylene bags, labelled and stored at –20°C until used. The chemical composition of salmon by–product which contained 61.9 ± 0.2% moisture, 44.3 ± 0.7% crude protein, 45.4 ± 1.1% crude lipid and 10.2 ± 0.2% ash (on dry weight basis) was determined using the method of AOAC [26].

2.1.2. Chemicals and enzyme preparation

Alcalase, Neutrase, Protamex, Flavourzyme and Corolase were obtained from Novozymes (Denmark) and AB enzymes (Germany). Chemicals were purchased from Sigma-Aldrich and Merck. All reagents were of analytical grade. Double-distilled water was used in experiments.

2.2. Methods

2.2.1. Preparation of hydrolysate

The preparation of hydrolysates was performed according to the procedure of Bhaskar and Mahendrakar [27] with slight modification. Water was added with the selected ratio and the mixture was heated at 90ºC for 10 minutes to deactivate endogenous enzymes. After the required hydrolysis time, the reaction was terminated by heating the hydrolysates for 10 min at 90ºC in order to deactivate the enzymes. The hydrolysates were then centrifuged at 5000g for 15 min to collect the supernatant. A portion of collected supernatant was lyophilized and stored at 4 °C for further use.

2.2.2. Effect of by-product:water ratio on protein recovery yield

Protein recovery yield was determined by the percentage of protein content in the proteolysate comparing to the crude protein content of the material. For this experiment, Alcalase was used for hydrolysed at its recommended pH and temperature, E:S ratio of 50 U/g protein, hydrolysis time of 8 hours and salmon by-product: water ratio in range from 1:1 to 1:12 (w/v).

2.2.3. Effect of hydrolysis condition on the antioxidant activity of *Acetes* proteolysate

The effect of five factors including enzyme type, pH, temperature, E:S ratio and hydrolysis time on the antioxidant capacity of the proteolysate were examined using single factor test method which was performed by one factor varied with different levels while other factors fixed.

2.2.4. Determination of antioxidant activity

DPPH radical–scavenging capacity. The DPPH radical scavenging potential was assayed employing the method of Sharma and Bhat [28] with slight modification. The mixture of sample and DPPH was incubated in the dark at room temperature for 30 minutes. The absorbance at 517 nm was determined by a spectrophotometer. The scavenging activity was calculated with the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{A_0-(A_1-A_2)}{A_0} * 100\% \quad (2)$$

Where A_0 denotes the absorbance of the blank (distilled water instead of sample), A_1 is the absorbance of the mixture containing sample, and A_2 is the absorbance of the mixture without DPPH.

FRAP assay. The ferric reducing capacity of hydrolysates was determined using a modified method of Benzie and Strain [29]. This method is based on the reduction of a colorless ferric complex (Fe^{3+}–tripyridyltriazine) at low pH to a blue–colored ferrous complex (Fe^{2+}–tripyridyltriazine) by the action of electron–donating antioxidants. The reduction is monitored by measuring the change of absorbance at 593 nm.

2.2.5. Optimization of E:S ratio and hydrolysis time for maximizing the antioxidant activity of the proteolysate

A randomised, quadratic central composite circumscribe response surface design was used to optimize the hydrolysis via E:S ratio and hydrolysis time. The dependant variable was antioxidant activity of the hydrolysate. The Modde software (version 5.0) was used to generate experimental planning and to process data. Each factor in the design was investigated at five different levels ($-\sqrt{2}$, -1, 0, +1, $+\sqrt{2}$). The total number of experiments was 13 and the number of central experiments was 5.

2.2.6. Fractionation of proteolysate

The proteolysate was further fractionated using ultrafiltration centrifugal devices of 30 kDa, 10 kDa, 3 kDa and 1 kDa (Thermo–Fisher Scientific, Pall, USA). Four peptide fractions of 10–30 kDa, 3–10 kDa, 1–3 kDa, and < 1 kDa were collected and tested for their antioxidant capacity.

2.2.7. Determination of iron-binding capacity

Iron-binding assay was performed using the method of Carter [30] with a slight modification. After demineralization by macroporous resin (Amberlite IRC-748I sodium form, Acros), 1 ml of each peptide fraction was mixed with 2.5 ml of acetate buffer (0.1M, pH 5) and 0.6 ml of $FeSO_4$ solution (0.2 mM), in order. After 30 minutes, 0.3 ml of Ferrozine (5 mM) was added and the absorbance of Fe^{2+}-Ferrozine complex was recorded at 562 nm. The iron-binding capacity was calculated using the following formula:

$$Iron - binding\ activity = \frac{A_c - A_s}{A_c} * \frac{m_{Fe^{2+}}}{m_{protein}} (\mu g\ Fe^{2+} / g\ protein)\ (1)$$

Where:

A_c denotes the absorbance of the blank (distilled water instead of sample)

A_s is the absorbance of the sample.

2.2.8. Identification of antioxidant peptides

Dried samples were firstly cleaned using C_{18} columns as manufacturer's instruction before being submitted to a mass spectrometer. In brief, 50 µg of each dried sample was re–suspended in 100 µL of loading buffer C containing 0.1% trifluoroacetic acid (TFA) in 3% of acetonitrile (ACN), and then loaded onto a C_{18} MicroSpin™ column (The Nest Group, USA). The column was washed twice with buffer A consisting of 0.1% formic acid (FA) in 3% ACN, peptides were eluted from the column with 100 µL of buffer B (0.1% FA in 97% ACN), and then dried in a vacuum concentrator (Eppendorf, USA). Cleaned peptides were re–dissolved in 20 µl of buffer A before 4 µl of sample was withdrawn and submitted onto an amazon ETD ion trap mass spectrometer (Bruker Daltonics, Germany) coupled with a nano uHPLC 3000 system (Dinonex, UK) operated at a flow rate of 0.3 µl/min. Peptides were separated using a C18 column with a 70 min gradient of buffer B as follow: 3% for 5 min, then ramped up to 10% for 5 min, 50% for 45 min, 90% for 1 min, then kept at 90% for 4 min before ramped back to 3% buffer B for 1 min then maintained at 3% for 9 min. The MS was operated in positive mode with a m/z scan from 300 – 1500 m/z, ICC target of 1.5 x 10^5 and accumulating time of 50 ms. A full MS auto scan mode was used with 3 precursors applied. Four independent biological samples were applied for identification of active peptides.

All raw MS data files were submitted to PEAKS Studio for peptide identification against *Salmo salar* databases downloaded in December 2018 from Uniprot (http://www.uniprot.org). No specific enzyme mode was used for searching with max missed cleaves of 2, minimum peptide length of 4 and maximum peptide mass of

1,100 Da were set; tolerances of 1.2 and 0.8 Da were applied for MS and MS/MS respectively. A False Discovery Rate (FDR) of 0.05 was used for identification of both peptides and proteins. Only peptides observed from at least 3 independent biological replicates were used and their MS/MS annotations were manually examined to ensure that good coverage of b– and y– series ions observed.

2.2.9. Statistical Analysis

Data were presented as means ± standard deviations of triplicate determinations. Analysis of variance (one-way ANOVA) was performed on the data, and the significance was determined using Tukey method ($P < 0.05$) [31]. These analyses were performed using the Statgraphics Centurion 18 software.

III. RESULTS AND DISCUSSION

3.1. Effect of by-product:water ratio on protein recovery yield

The figure 3.1 showed profile of effect of by-product:water ratio on protein recovery yield. The yield reached the peak of 28.8 ± 1.39% at the ratio of 1:10 (w/v). It can be explained that the solubility of protein in water was proportional to the amount of water. However, the presence of large amount of the solvent in the mixture could limit the contact between by-product and hydrolysis enzyme, decreasing in releasing of protein from the by-product during hydrolysis. In the study of Halldorsdottir et al. [32], cod protein was hydrolyzed using Protease P "Amano" 6 with minced cod:water ratio of 1:5 to obtain antioxidant proteolysate. Halim et al. [33] found antioxidant peptides from proteolysate obtained by hydrolyzing eel protein using Alcalase with the material:solvent ratio of approximately 1:1. These differences of material:solvent ratio was attributed to the variance of protein source and hydrolysis enzyme. In this study, the by-product:water ratio of 1:10 was used for further analysis.

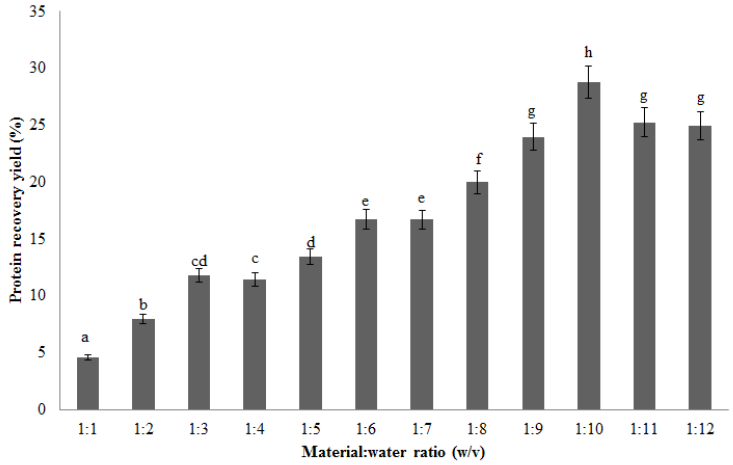

Figure 1. Effect of by-product:water ratio on protein recovery yield. Bars with different letters indicate significant differences ($P<0.05$).

3.2. Effect of hydrolysis enzyme type on antioxidant activity of proteolysate

Huang et al. [15] reported that the biological activities of protein hydrolysates depend on the protein substrate, the specificity of the enzyme used for the proteolysis. The proteolytic enzymes cleave the peptide bond between amino acids, releasing a mixture of peptides with various molecular weights and free amino acids. The selection of enzyme types to produce compounds with defined physiochemical and nutritional properties is essential due to the variable in mechanism of enzymes to hydrolyze protein substrates [34]. The result showed that Flavouryzme hydrolysate expressed the highest antioxidant activity with DPPH scavenging capacity of 35.2 ± 0.2% and FRAP value of 112.3 ± 0.2 µM TE (Fig. 2). Zamora-Sillero et al. [35] reported that protease, its nature and specificity, has a vigorous effect on antioxidant activity of the proteolysate via generating different proteolysates in terms of amino acid composition and sequence, size of peptides. Flavourzyme preparation is a fungus-originated enzyme containing both exopeptidases and endoproteases which has a broad substrate specificity, releasing more antioxidant peptides [36]. This finding was in agreement with those of Thiansilakul et al. [37] who reported that Flavourzyme was the best candidate to obtain the round scad muscle proteolysate with the highest DPPH scavenging activity and reducing power. Moreover, the Flavourzyme proteolysate of salmon by-product and *Mytilus coruscus* mussel showed the highest antioxidant activity [38, 39]. Flavourzyme was used as hydrolysis enzyme for further investigation in this research.

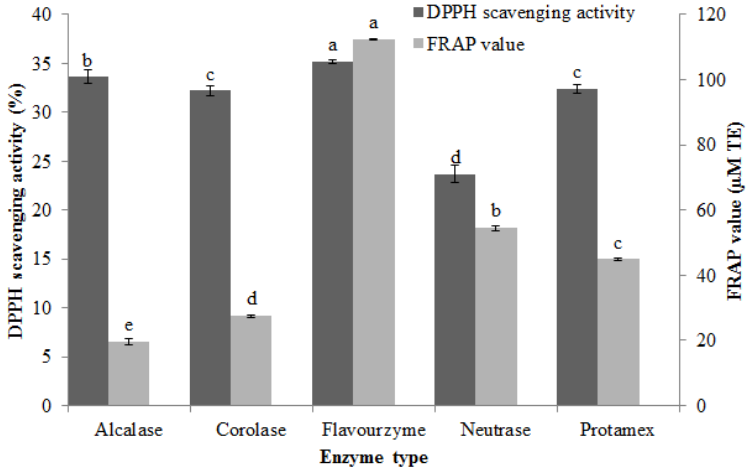

Figure 2. The effect of enzyme type on the antioxidant activity of proteolysate. Bars with different letters indicate significant differences (P<0.05).

3.3. Effect of pH on antioxidant activity of proteolysate

Fig. 3 demonstrated profile of effect of pH on antioxidant activity of proteolysate. Both DPPH scavenging activity and FRAP value achieved the highest value of 34.8 ± 0.2% and 113.9 ± 0.9 µM TE, respectively, at pH 7, optimal pH. It can be explained that the environmental pH had a significant effect on the ionization ability of substrate and enzyme through changing their charge distribution and conformation, affecting on catalytic activity of enzyme and antioxidant activity of proteolysate [40, 41] which depends on its amino acid composition and sequence of peptides present in it [42]. At non-optimal pH (pH= 5-6.5), the amount of antioxidant peptides diminished due to low enzyme catalytic activity [40]. Hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe enhanced antioxidant activity by increasing the solubility of peptides in lipid phase [43]. Tyr, His, and Trp possessed antioxidant activity through their indolic, phenolic, and imidazole groups which serve as hydrogen donors [44-46]. Hence, pH 7 was selected for further experiments.

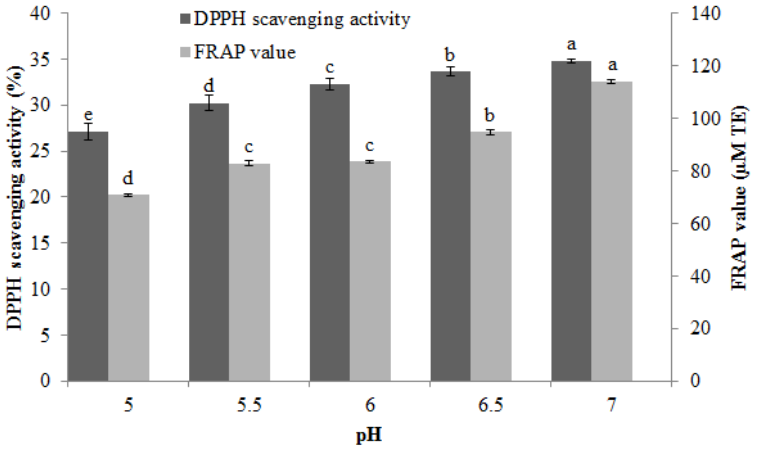

Figure 3. The effect of pH on the antioxidant activity of proteolysate. Bars with different letters indicate significant differences (P<0.05).

3.3. Effect of temperature on antioxidant activity of proteolysate

The temperature – antioxidant activity profile (Fig. 4) showed that both DPPH scavenging activity and FRAP value of the proteolysate reached the peak of 35.6 ± 0.8% and 110.4 ± 0.5 µM TE, respectively, at the temperature of 50^{o}C, optimal temperature. Temperature induced conformational changes of substrate and enzyme, affecting to the thermodynamic parameters of the coordination reaction, changing antioxidant capacity of the proteolysate [18]. The optimal temperature supported the contact between substrate and enzyme beneficially, exposing hydrophobic or hydrogen-donating amino acids which were buried inside the substrate, facilitating the accession of the enzyme on the protein to cleave it [47]. Non-optimal temperature did not facilitate the contact between enzyme and substrate molecules due to the decrease in the movement of these

molecules or change the configuration of enzyme and substrate, reducing the formation of antioxidant peptides. In this experiment, hydrolysis temperature of 50°C was chosen for further investigation.

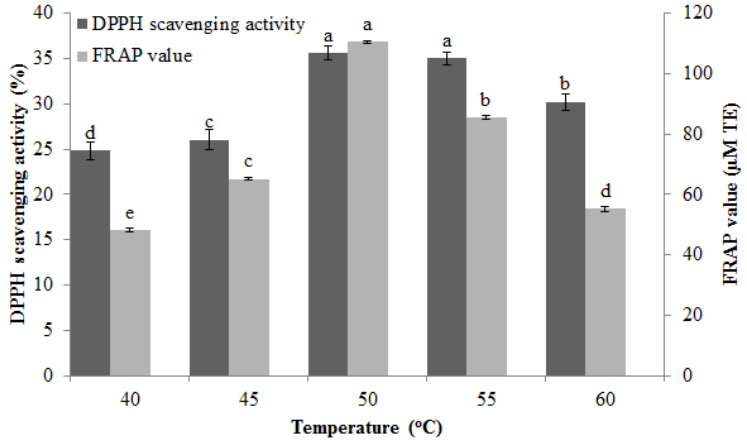

Figure 4. The effect of temperature on the antioxidant activity of proteolysate. Bars with different letters indicate significant differences (P<0.05).

3.4. Effect of E:S ratio on antioxidant activity of proteolysate

Fig. 5 presented the effect of E:S ratio on antioxidant activity of the proteolysate. The antioxidant potential based on DPPH scavenging and FRAP methods gained the highest values of 35.4 ± 0.4% and 105.6 ± 2.1 µM TE, respectively, at E:S ratio of 50 U/g protein. The enough amount of enzyme for substrate facilitated the recovery of proteolysate with high amount of antioxidant peptides. Lower or higher enzyme amount may cause the excess or lack of substrate for the hydrolysis reaction, thus, the obtained proteolysate did not possess the best antioxidant activity. This rule was also observed in the research of Somarajan et al. [48] and Gunasekaran et al. [49]. For further analysis, the E:S ratio of 50 U/g protein was selected.

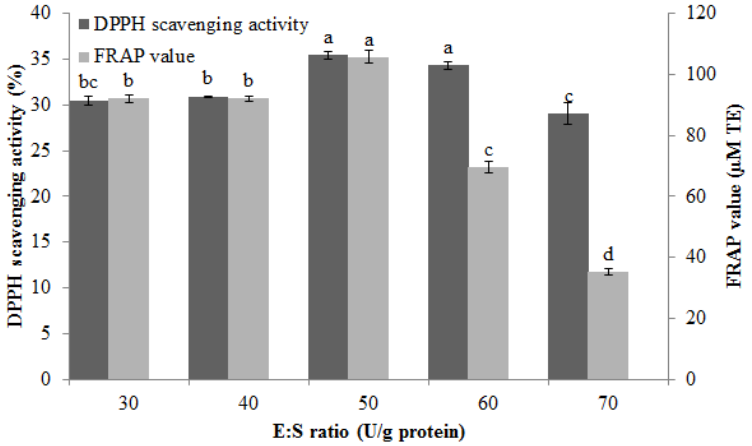

Figure 5. The effect of E:S ratio on the antioxidant activity of proteolysate. Bars with different letters indicate significant differences (P<0.05).

3.5. Effect of hydrolysis time on antioxidant activity of proteolysate

As illustrated in Fig. 6, both DPPH scavenging activity and FRAP value increased and reached the peaks of 35.0 ± 0.5% and 108.6 ± 5.7 µM TE, respectively, at 8 hours of hydrolysis. Longer time of hydrolysis (greater than 8 hours) decreased the antioxidant activity of the proteolysate. Bioactive peptides were released during hydrolysis, however, overlong time would degrade the bioactive peptides formed at early stage [18], lowering the antiradical activity of the proteolysate. The change in the profile of hydrolysis time and antioxidant activity in this research was in consistent with the previous publications on antioxidant activity of proteolysate from stone fish [50], silver carp [51] and salmon skin [52]. In this study, 8 hours of hydrolysis was picked for further experiment.

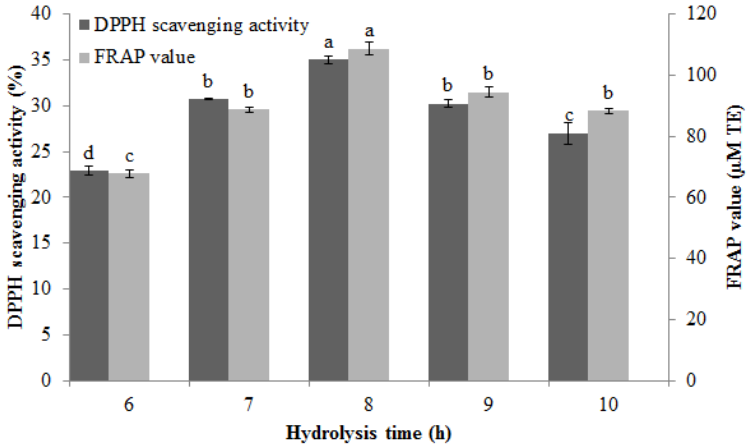

Figure . The effect of hydrolysis time on the antioxidant activity of proteolysate. Bars with different letters indicate significant differences (P<0.05).

3.6. Optimization of E:S ratio and hydrolysis time for DPPH scavenging activity using RSM

To establish the fit model, multiple regression analysis was performed on the experimental data and the final predictive function achieved was as follows:

DPPH scavenging activity (%) = $- 2.56X_1^2 - 1.36X_2^2 - 2.08X_1X_2 + 35.27$ (3)

Where Y, X_1, X_2 were the DPPH scavenging activity (%), hydrolysis time (hour) and E:S ratio (U/g protein), respectively. The E:S ratio was changed from 40 to 60 U/g protein and the hydrolysis time was varied from 7 to 9 hours. The effect of each variable on the response was determined at 95% confidence level. X_1^2, X_2^2, and X_1X_2 were estimated as significant effects while the effect of X_1 and X_2 were insignificant (p>0.05). Similar effect for X_2 variable was also observed in the result of Guerard et al. [53]. In addition, the study of Fang et al. [54] also showed the significant effect of X_1X_2 on DPPH scavenging activity when optimizing the hydrolysis condition for flying squid muscle. Moreover, Sowmya et al. [55] had found a trivial effect of X_1 on antiradical activity of the proteolysate from shrimp head and carapace. The regression model was significant (P < 0.05) with the coefficient of determination (R^2) of 0.98.

In order to determine optimal levels of the variables for the antioxidant activity, a three-dimensional surface plot was constructed according to the quadratic function (3) (Fig. 7). The optimal condition includued the E:S ratio of 49.83 (U/g protein) and hydrolysis time of 7.97 hours with a predictive maximal response of DPPH scavenging activity of 35.4%.

To verify the accuracy of the model, three independent replicates were conducted for measuring antioxidant potential under the optimal condition. The average DPPH scavenging activity was 34.6%, which was nearly the same as the predicted value from quadratic function (3).

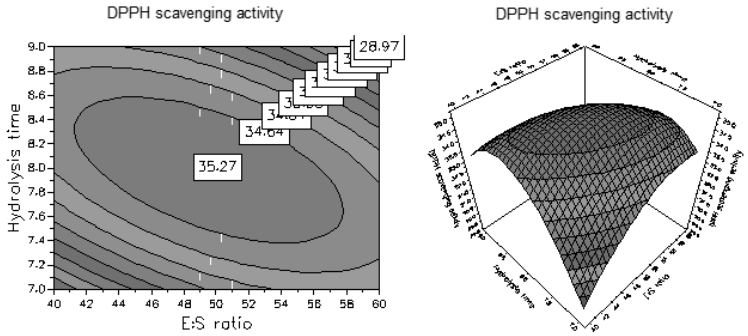

Figure 7. Response surface plot for antioxidant activity of salmon by-product proteolysate using DPPH scavenging method

3.6. Determination of IC_{50}, FRAP value and iron-binding capacity of peptide fractions

It has been published that the fractionation of proteolysate could enhance its specificity towards bioactivity including antioxidant and iron-binding activity [33]. Halim et al. [33] also confirmed that molecular weight of peptide had a significantly strong effect on the bioactivity of proteolysate. In this study, the fractionation result (Fig. 8) revealed that the <1 kDa fraction showed the highest antioxidant potential with IC_{50}

of 1.86 ± 0.01 mg/mL, 250 folds higher than that of vitamin C, FRAP value of 253.8 ± 8.1 µM TE, 270 folds lower than that of vitamin C and iron-binding capacity of 2882.1 ± 499.2 µg Fe^{2+}/g protein, 1.7 times higher than that of disodium ethylenediaminetetraacetate (Na_2EDTA). Peptide size is a key factor for production of proteolysate with desired functional properties to serve as bioactive ingredient [56]. Low molecular weight peptides were proved to have a great contribution to bioactivity of fish proteolysate [57]. Li et al. [58] published that lower molecular weight fraction probably contained more electron-donors which could react with free radicals to transfer these radicals into more stable compounds, ending the radical chain reactions. Besides, these donors contributed to the potential of chelating ferrous ions of the peptide fractions. This finding was in agreement with the report of Wang et al. [59] who indicated that the <1 kDa peptide fraction from croceine croaker scales proteolysate possessed the highest antioxidant activity. In addition, Luo et al. [60], Chi et al. [61] and Chi et al. [62] also revealed that the <1 kDa peptide fraction from proteolysate of *Sphyrna lewini* muscle protein, bluefin leatherjacket skin and bluefin leatherjacket head showed the strongest antioxidant activity. The peptide fraction with the lowest molecular weight from skate cartilage proteolysate also expressed the highest antioxidant potential [63]. Besides, the <1 kDa peptide fraction from sea cucumber ovum hydrolysate also expressed the highest iron-binding capacity [17]. In this study, the <1 kDa fraction was used for identifying sequence and molecular weight of bioactive peptides.

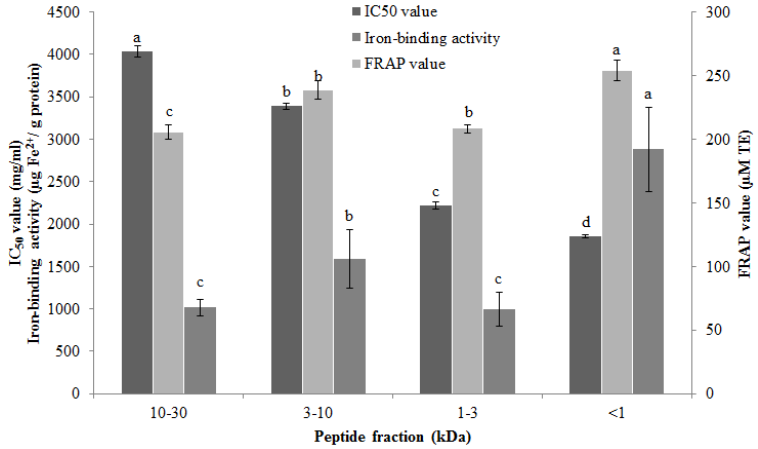

Figure 8. The IC_{50} value, FRAP value and iron-binding activity of peptide fractions from salmon by-product proteolysate. Bars with different letters indicate significant differences (P<0.05).

3.7. Identification of bioactive peptides

Two peptides (P1: GAAEKGVPLF and P2: GVDNPGHPF) were detected from the <1 kDa fraction presented in Table 1. All (entire) b– and y– series ions were also observed as shown in Fig. 10. The peptide GAAEKGVPLF belongs to protein enolase 3–1, which is a glycolytic enzyme that catalyzes the reversible conversion of 2–phosphoglycerate to phosphoenolpyruvate. This peptide contains 10 amino acids starting from Gly_{172} to Phe_{181} (Fig. 11a) and has a molecular weight of 988 Da with its structure as shown in Fig. 12a. The other peptide GVDNPGHPF belongs to protein Creatine kinase–3, which catalyzes the conversion of creatine and utilizes adenosine triphosphate to create phosphocreatine. The peptide contains 9 amino acids starting from Gly_{110} to Phe_{118} (Fig. 11b). Its molecular weight is 938 Da and its structure was presented in Fig. 12b. The sizes of these peptides were similar to that of antioxidant peptide isolated from tryptic hydrolysate of conger eel muscle protein [56].

The antioxidant activity of these peptides was contributed by their amino acid composition and sequence. The Glu containing peptide (peptide P1) and the Asp containing peptide (peptide P2) were reported that their antioxidant activity related to their excess electrons donated to free radicals [64]. The presence of Pro in these sequences may interrupt the secondary structure of the peptide, improving the availability of antioxidant amino acid residues [65]. Hydrophobic amino acids in the two peptides, Ala, Val and Leu, could increase the presence of peptides at the water-lipid interface, as a result, access to scavenge free radicals from the lipid phase [66, 67]. Moreover, these amino acids can react with polyunsaturated fatty acids, inhibiting the peroxiding chain reaction [68]. Furthermore, the presence of aromatic amino acid, Phe, in both sequences could considerably enhance the antioxidant capacity of these peptides [69]. Besides, Girgiha et al. [69] also reported that Asn partly contributed to antioxidant activity of the P2 peptide via its excess electrons available to be donated during interaction with free radicals. Udenigwe and Aluko [70] and Zou et al. [71] indicated that Lys in P1 peptide supported to its antioxidant activity through superoxide radical scavenging ability. His in the peptide P2 enhanced antioxidant potential though its imidazole ring acting as a hydrogen donor [72]. Sila and Bougatef [3]

explained that imidazole ring of His also had a capability of chelating and trapping lipid radical, improving the antioxidant activity of the peptide.

The iron affinity of the two identified peptides was also contributed by their amino acid composition and sequence. Ying et al. [8] and Storcksdieck et al. [73] revealed that Asp and Glu supported to iron-binding ability of peptides through the coordination between free electrons in carboxyl oxygen atom and empty orbitals of ferrous ion. The same explaination could be applied for the iron affinity of His [16, 73] via its imidazole ring. Besides, Asn was the fourth strongest interaction residue with ferrous ion, followed by Glu, His and Asp [74]. In addition, Lys acted as the iron ligand owing to its Ɛ-amino nitrogen [75]. Also, Phe related to iron-binding potential due to the effects of its aromatic ring and adjacent amide nitrogen [76]. Furthermore, a hydrophobic fence of bulky aliphatic side chains of Val and Ile residues effectively shields one side of the complex plane from the access of water molecules, increasing iron-peptide complex stability [77]. Moreover, the presence of Pro residue increased the bending propensity of peptide chain, stabilizing the Fe(II)-peptide complex through the decrease of the coordination bond length [77]. Likewise, bulky side chains (Phe, Tyr, Trp, Leu and Ile, etc.) are situated over the chelation ring, thus, they may interact with the ferrous ion, enhancing the stability of iron-peptide complex [76].

Table 1. List of detected peptides found in salmon by–product proteolysate

Peptide	Mass (Da)	m/z	Protein name	Amino acid composition
GAAEKGVPLF	987.53	494.89	enolase 3–1	10% acidic 30% neutral 50% hydrophobic
GVDNPGHPF	938.42	470.54	Creatine kinase–3	11.11 % acidic 55.56% neutral 22.22% hydrophobic

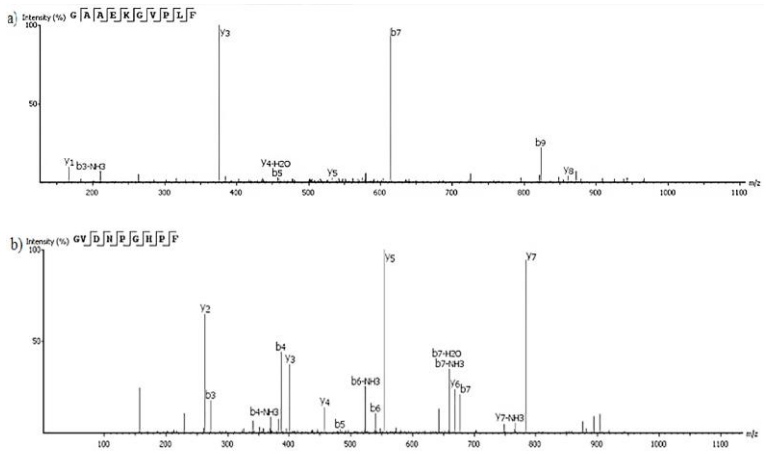

Figure 10. MS/MS annotations of detected peptides of P1 (a) and P2 (b)

a) MSIIKIHAREILDSRGNPTVEVDLYTAKGRFRAAVPSGASTGIHEALELRDGDKSRYLGKGTLK
AVDHVNKDIAAKLIEKKFSVVDQEKIDKFMLELDGTENKSKFGANAILGVSLAVCKA**GAAEK**
GVPLFRHIADLAGHKDVILPCPAFNVINGGSHAGNKLAMQEFMILPIGASNFHEAMRIGAEVY
HNLKNVIKAKYGKDATNVGDEGGFAPNILENNEALELLKSAIEKAGYPDKIIIGMDVAASEFY
KAGKYDLDFKSPDDPARYITGDQLGDLYKSFIKGYPVQSIEDPFDQDDWAAWSKFTAAVDIQ
VVGDDLTVTNPKRIQQAVEKKACNCLLLKVNQIGSVTESIKACKLAQSNGWGVMVSHRSGET
EDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLMRIEEALGDKAKFAGKDYRHPKVN

b) MPFGNTHNNFKLNFKVEEEYPDLTKHNNHMAKVLTKDMYAKLRDKQTSSGFTLDDVIQT**G**
VDNPGHPFIMTVGCVAGDEESYEVFKDLLDPIISDRHSGYKPTDKHKTDLNFENLKGGDD
LDPNYVLSSRVRTGRSIKGYTLPPHNSRGERRAVERLSVEALDTLDGEFKGKYYPLNKMT
DAEQEQLIADHFLFDKPVSPLLLGAGMARDWPDARGIWHNDAKSFLVWVNEEDHLRVISM
EKGGNMKEVFRRFCVGLKRIEETFKKHNHGFMWNEHLGYVLTCPSNLGTGLRGGVHVKLP
KLSTHAKFEEILGRLRLQKRGTGGVDTASVGGVFDISNADRLGSSEVDQVQMVVDGVKLM
VEMEKKLEKGEAIDGMIPAQK

Figure 11. Position of peptide P1 in the protein enolase 3-1 (a) and position of peptide P2 in the protein Creatine kinase-3 (b).

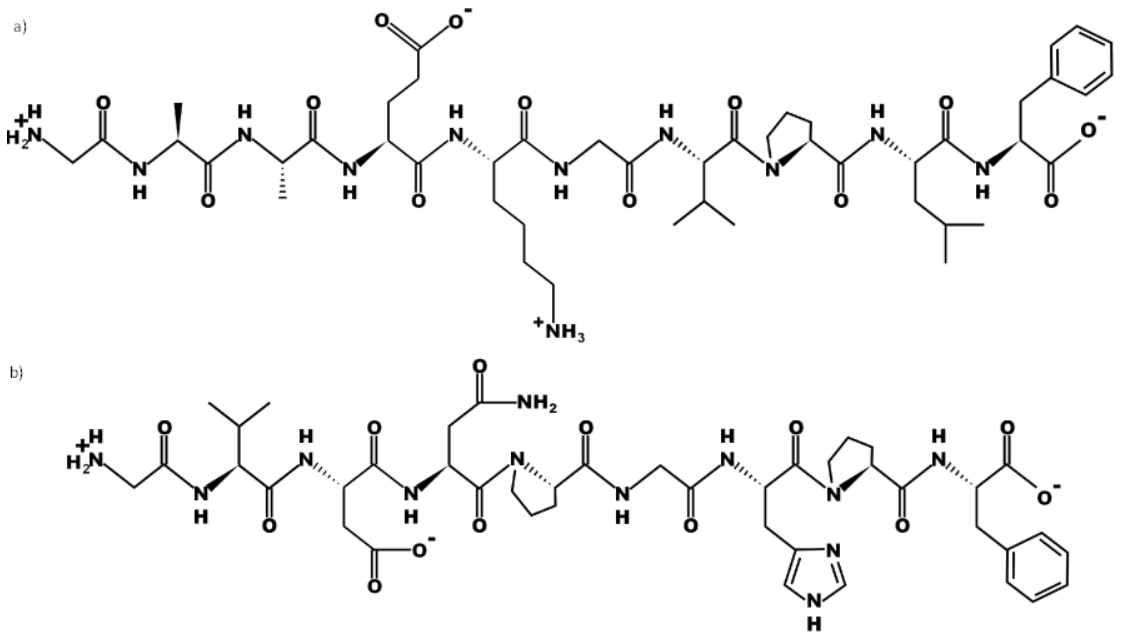

Figure 12. Structure of peptide P1 (a) and P2 (b) drawn from http://pepdraw.com/

IV. CONCLUSION

Fish processing industry discarded a remarkable number of by–product, causing environmental pollution seriously. These by-products were proven to be rich in protein, bioactive compounds and essential nutrients beneficial for human health. The finding new ways of utilization of these by–products has attracted the interest of many researchers. Vietnam is a country who imports salmon and exports its fillet product with a large quantity, discarding a huge amount of by-product every year. The authors tried to utilize these by-products to produce value-added products and contribute to reduce the risk of environmental pollution. In this study, enzymatic hydrolysis was investigated and the peptide fractions from the proteolysate were recovered and tested for their bioactivities. Parameters including E:S ratio and hydrolysis time were optimized for maximizing antioxidant activity of the proteolysate. Two novel peptides, Gly–Ala–Ala–Glu–Lys–Gly–Val–Pro–Leu–Phe (988 Da) and Gly–Val–Asp–Asn–Pro–Gly–His–Pro–Phe (938 Da), were identified and showed the highest antioxidant activity with IC_{50} value of 1.86 ± 0.01 mg/mL, FRAP value of 253.8 ± 8.1 μM TE and iron-binding capacity of 2882.1 ± 499.2 μg Fe^{2+}/g protein. The small peptides were supposed to be easily absorbed in the human body. Besides, these bioactive peptides could be applied as a neutraceutical, supplement or an antioxidant additive which can substitute for synthetic compounds. However, further research on antioxidant and iron-binding activity *in vivo* as well as study on application of these peptides in preservation of some common food products should be done. This study suggested a new trend in using by-product of salmon particularly and by-product of fish generally to both produce value-added products and contributed to solve environmental solution issue.

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