Investigation of antioxidant activity of the hydrolysate derived from Tra catfish by-products using Alcalase® 2.4 L FG for application as a natural antioxidant ingredient

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ABSTRACT

In this study, the effects of temperature, pH, enzyme content, hydrolysis time on antioxidant activity of the hydrolysate from Tra catfish (Pangasiushypophthalmus) by-products with Alcalase® 2.4 L FG were investigated using DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method (DPPH• SM) and FRAP (ferric reducing antioxidant potential) method. The chemical composition of the Tra catfish by-products included 58.5% moisture, 33.88% crude protein, 50.14% crude lipid and 15.83% ash (on dry weight basis). The result of antioxidant activity of the hydrolysate showed that the 50% DPPH• inhibition concentration (IC50) of the hydrolysate reached about 6775 μg/mL which was 1645-fold higher than that of vitamin C and 17-fold higher than that of BHT (Butylated Hydroxytoluene) with the degree of hydrolysis (DH) of the hydrolysate of 14.6% when hydrolysis time was 5h, enzyme/substrate (E/S) ratio was 30 U/g protein, hydrolysis temperature was 55^0C, and pH was 7.5. The antioxidant potential of hydrolysate using FRAP method reached about 52.12 μMTrolox equivalent which was 53-fold and 18-fold lower than those of vitamin C and BHT, respectively, when the hydrolysis time was 5h, enzyme/substrate ratio was 30 U/g protein, temperature was 50^0C, and pH level was 8. The result showed that the antioxidant proteolysate derived from Tra catfish by-products has the potential to be used as a natural antioxidant ingredient in nutraceutical and functional food industry.

Keywords: *antioxidant activity; antioxidant peptide; hydrolysate; Tra catfish by-products.*

1. INTRODUCTION

Antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substance when present at low concentrations compared to that of an oxidizable substrate [1]. Many synthetic antioxidants such as Butylated HydroxyAnisole (BHA), Butylated HydroxyToluene (BHT), Tert-Butyl HydroQuinone (TBHQ) and Propyl Gallate (PG) are used as food additives to prevent lipid peroxidation in food [2]. Although these synthetic antioxidants show stronger antioxidant activity than that of natural antioxidants such as α-tocopherol and ascorbic acid, there has been concern about their safety with regard to health. Therefore, the search for natural antioxidants as alternatives to these synthetic compounds has especially attracted the attention of researchers lately.

Recently, protein hydrolysates from different sources of fish processing by-products have been found to possess antioxidant activity. Several researches have described the antioxidant activity of these proteolysates including Alaska Pollack frame [3], tuna backbone [4], cobia skin [5], heads and viscera of Sardinelle [1, 6], Argentine croaker bone [2], salmon pectoral fin [7, 8], tuna dark muscle by-product [9, 10, 11].

Enzymatic hydrolysis of proteins to obtain bioactive compounds has attracted public interest recently. Production of fish protein hydrolysate via enzymatic hydrolysis is one way to add value to proteinaceous fish waste. The main advantage of enzymatic hydrolysis of proteins is that it allows quantification of aspargine and glutamine and other sensitive residues, which are normally destroyed by acid or alkali hydrolysis, and does not cause any racemization during digestion [12].

Alcalase commercial enzyme, a serine bacterial endopeptidase from a strain of Bacillus licheniformis, has been proven as one of the best enzymes by many researchers to be used in the preparation of fish protein hydrolysatewith less bitterness of protein hydrolysate compared with others [13].

In Vietnam, the farming and processing of Tra catfish in the Mekong Delta has been developed very quickly. Fillet is the main product of Tra catfish processing industry with approximately 65-70% of by-products including skin, bone, head, fat and viscera. These by-products have been used as raw materials for production of fish meal for livestock, biodiesel, gelatin, fish oil extraction. Besides, these by-products are also important bio-resources for applications in food, health care products, and pharmaceuticals [9].Until now, no information has been reported on the antioxidant activity of proteolysate obtained from the Tra catfish processing by-products for application of natural antioxidant ingredient.

In this study, to recover and utilizeTra catfish by-product protein, enzymatic hydrolysis was performed to obtain bioactive proteolysate. The main objective of the research was to investigate the antioxidant activity of the Alcalase hydrolysate from Tra catfish by-products using $DPPH^{\bullet}$ SM and FRAP methods, with the aim of using these fish by-products as sources of natural antioxidant ingredients.

2. MATERIALS AND METHODS

2.1. Materials

Tra catfish by-products

The Tra catfish frames included heads, bones, fins, tails and some remaining flesh attached to the frames were kindly provided by a local catfish processing plant in Tien Giang province, Vietnam. The by-products were transported on ice to the Biochemical laboratory of Ho Chi Minh City University of Technology within 4 hours, individually packed in polyethylene bags, labeled and stored -80^{o}C until used.

Enzyme source

Alcalase® 2.4 L from *Bacillus licheniformis* was obtained from Novozymes (Bagsvered, Denmark). The optimal working conditions of the enzyme were as follows: temperature between 40 and 65°C, pH between 7 and 9. A declared minimal activity was 2.4 U/g.

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from BDH Chemicals Ltd (Poole, Dorset, UK), acetic acid, $CH_3COONa.3H_2O$, $FeCl_3.6H_2O$, 2,4,6-tripyridyl-s-triazine (TPTZ), Folin, Tyrosin, were purchased from Merck Schuchardt (Hohenbrunn, Germany). Hydrochloric acid 37% and ascorbic acid were purchased fromVWR International (Pennsylvania, USA). Albumin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents were of analytical grade. Double-distilled water was used in experiments.

2.2. Methods

Determination of chemical composition of the by-products

The contents of moisture, crude protein, crude fat and ash were determined according to the methods of AOAC (2000) [14]. The moisture content was evaluated according to oven-drying method at 105ºC until a constant weight. The total crude protein content was determined using Kjeldahl method with Nitrogen conversion factor of 6.25. The crude fat content was evaluated by Soxhlet extraction method. The ash content was determined at 550ºC until white ash was formed.

Preparation of Tra catfish by-product hydrolysates

The preparation of the hydrolysate was performed according to the procedure of Bhaskar et al. (2007) [15] with slight modification. For each batch, by-products were thawed, cut into small pieces, and ground using a 5 mm plate grinder (Vietnam). Then water was added with the ratio of water: by-product of 1:1 (w/v). Next, the mixture was heated at 95ºC for 10 minutes to deactivate endogenous enzymes and the pH value of the mixture was adjusted to the desired value before adding the enzyme for hydrolysis. After that, Alcalase® 2.4 L was added on the basis of standardized activity units which were determined using the method of Anson with slight modification [16]. Hydrolysis temperature was controlled using a water bath (Memmert WB14, Germany) and pH value was monitored every 15 minutes using sodium hydroxide or hydrochloric acid solution of 0.1N.Samples were taken at pre-established time intervals to perform further experiments. After the required hydrolysis time, the reaction was terminated by heating the hydrolysates for 10 min at 90ºC in order to deactivate the alcalase. The hydrolysates were then centrifuged at 6,000 x g for 10 minutes and then cooled down to 4°C to separate the upper fat fraction. Next, the hydrolysates were further centrifuged at 8,000 x g for 10 min to remove insoluble substances and the obtained supernatants were freeze-dried using freeze-dryer (Alpha 1-2/Ldplus, UK). Samples were stored as hydrolyzed protein powder at -80ºC until used.

Evaluation of protein content of hydrolysates

The protein contents of hydrolysates were measured according to the method of Lowry [17] using bovine serum albumin as a standard.

Determination of degree of hydrolysis (DH) of hydrolysate

Nitrogen solubility index was used to determine the DH of hydrolysateusing trichloroacetic (TCA) acid asprecipitating agent[18].Kjeldahl method was used to determine nitrogen content. The formula used is as follows:

% DH = 10% TCA soluble nitrogen in the sample x 100/Total nitrogen in the sample

Effect of proteolysis time on antioxidant activity of proteolysate

In this experiment, the Tra catfish by-products were hydrolyzed at pH 8, 50°C, E/S ratio of 20 U/g protein. The hydrolysis time was controlled from 1 to 6 h. At the time designated, the samples were cooled rapidly in ice water and tested for antioxidant power.

Effect of the E/S ratio on antioxidant poteintial of proteolysate

The Tra catfish by-products were hydrolyzed for 5h, pH 8, 50°C. The E/S ratio was controlled from 10 to 60 U/g protein. At the time designated, the samples were cooled rapidly in ice water and tested for antioxidant activity.

Effect of temperature on antioxidant activity of proteolysate

The Tra catfish by-products were hydrolyzed for 5 h, pH 8, E/S ratio of 30 U/g protein. The temperature was controlled using water bath at 40, 45, 50, 55, 60, 65°C. At the time designated, the samples were cooled rapidly in ice water and tested for antioxidant activity.

Effect of pH on antioxidant activity of proteolysate

The Tra catfish by-products werehydrolyzed for 5 h, E/S ratio of 30 U/g protein. pH of the samples were adjusted to 7, 7.5, 8, 8.5 and 9 using sodium hydroxide or hydrochloric acid solution of 0.1N. At the time designated, the samples were cooled rapidly in ice water and tested for antioxidant activity.

Determination of antioxidant activity

DPPH radical-scavenging capacity

The DPPH radical scavenging activity was assayed employing the method of [19] with slight modification. The mixture of sample and DPPH was incubated in the dark at room temperature for 30 min. The absorbance at 517 nm was determined by a spectrophotometer. The scavenging activity was calculated with the following formula:

DPPH Scavenging activity (%)

$$\textit{Scavenging activity}\ (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\% \quad (1)$$

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

Ferric Reducing Antioxidant Potential (FRAP) assay

The ferric reducing capacity of the hydrolysate was determined using a modified method of Benzie and Strain (1996) [20]. 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.

Statistical analysis

Data were presented as means ±standard deviations of triplicate determinations. Mean differences among the measurements were statistically significant at the 95% confidence level. Analysis of variance (ANOVA) was performed using the Statgraphics Plussoftware (version 7.0).

3. RESULTS AND DISCUSSION

3.1. Composition analysis of Tra catfish by-products

Proximate composition analyses of Tra catfish frame in this study revealed that it contained 58.5% moisture, 33.88% crude protein, 50.14% crude lipid and 15.83% ash (on dry weight basis). The protein content was higher than that of silver catfish (Pangasius sp.) frame (without head) which was 25.02 % crude protein reported in the research of Amiza et al. [21]. This supposed that Tra catfish by-product can be used as a protein source for isolation of proteolysate or peptides.

3.2. Effect of proteolysis time on antioxidant activityof protein hydrolysate

The results of the effect of hydrolysis time on antioxidant activity of protein hydrolysate derived from Tra catfish by-products using $DPPH^{\bullet}$ SM and FRAP method were shown in Fig. 1. All treatments produced proteolysates with significantly ($P<0.05$) higher DPPH· radical scavenging activities and FRAP values compared to the non-hydrolyzed samples.

$DPPH^{\bullet}$ scavenging activities and FRAP values of the protein hydrolysates generally increased as the hydrolysis time increased ($P < 0.05$). The increase in proteolysis time led to the decrease in size of peptides; thus, the obtained proteolysates with smaller peptides may possess higher antioxidant activities [22]. This was in agreement with previous reports suggesting the increase of DPPH· radical scavenging capacity and FRAP value due to the extension of hydrolysis time [5, 10].

The antioxidant activity of protein hydrolysate depends on its amino acid composition and sequence [19]. Hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe enhance their antioxidant activity by increasing the solubility of peptides in lipid phase [23].Tyr, Met, His and Lys were also known to possess antioxidant activity [24].Tryp, Tyr and His contains the indolic, phenolic, and imidazole groups, respectively, which serve as hydrogen donors [25]. In addition, His and Tyr can make reactive oxygen species stable through electron/proton transfer [26]. Besides acting as a radical scavenger and reducing power, peptide could serve as a protecting membrane surrounding lipid droplet against oxidation initiators [27].

DPPH• scavenging activity and FRAP value of the protein hydrolysates decreased as the hydrolysis time was greater than 5 hours (P<0.05) (Fig. 1). Another report also showed a decrease in DPPH· radical scavenging capacity of proteolysates with increasing proteolysis time [10].The decrease in radical scavenging activity might be due to the generation of oxidant compounds, since Alcalase is endopeptidase capable of hydrolysing proteins with broad specificity for peptide bonds, which cut the generated antioxidant peptides to smaller sizes which cannot have antioxidant power, causing a decrease in antioxidant activity of hydrolysates [22]. Besides, several findings have also suggested that peptide size and solubility, the amino acid composition, sequence and abundance of free amino acids may have a key role in determining the DPPH· radical scavenging capacity.

In the study of Sara Bordbar et al. [22], FRAP values of stone fish tissue proteolysates also decreased after 5 hours of hydrolysis. Previous studies on ferric reducing antioxidant capacity of enzymatic proteolysates concluded that the reducing power was related to some factors including molecular weight of peptides, amino acid sequence of peptides, number of hydrophobic amino acids, and amount of sulphur containing and acidic amino acids. Besides, the presence of some amino acids such as Leu, Lys, Met, Tyr, Ile, His, and Trp has been reported contributing to the strong reducing power of proteolysates. However, it is still not well understood how the composition of peptides influenced their antioxidant capacity.

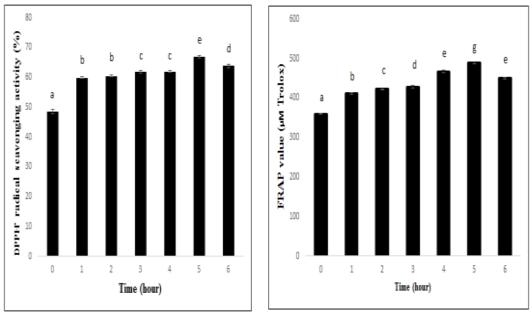
The highest DPPH• scavenging capacity of alcalase proteolysate in this experiment reached 66.845±0.446% and FRAP value reached 488.833±3.283 μM Trolox equivalent after 5 hours of proteolysis. The great FRAP value indicated that hydrolysates could donor the electron to the free radical, leading to the prevention or retardation of oxidation propagation. After 5 hours of proteolysis, the rate reached the steady phase and the prolongation in hydrolysis had no significant effect on the radical scavenging power and reducing potential.

3.3. Effect of enzyme/substrate ratio on antioxidant activity of proteolysate

The relation between the enzyme/substrate ratio and the antioxidant activity of proteolysates measured by DPPH· radical scavenging assay and FRAP method were determined as illustrated in Fig. 2. It could be suggested that enhancing the amount of alcalase led to the increase in the antioxidant activity of proteolysate (P<0.05). The result indicated that peptide bonds were more extensively cleaved in the presence of a higher amount of enzyme or the peptides released were further hydrolysed, producing amino acids and smaller peptides by the enzyme. Changes in size, level and composition of free amino acids and small peptides affect the antioxidant activity [28]. The DPPH radical scavenging activity and FRAP value of the hydrolysate significantly decreased (P<0.05) when the enzyme amount was continuously increased (Fig. 2). Similar result was reported by S. Tanuja et al. (2014) [29] with proteolysate from P. hypophthalmus frame meat. This may be due to the breakdown of antioxidant peptides formed during early stages of the hydrolysis process. In this experiment, with the E/S ratio of 30 U/g protein, the highest DPPH• scavenging potential of 69.561±0.17 % and the highest FRAP value of 612.666±2.517 µMTrolox equivalent were obtained.

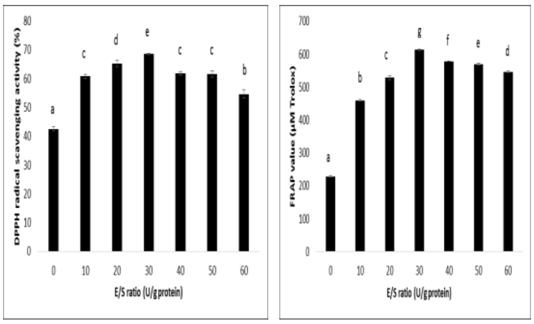
3.4. Effect of temperature on antioxidant activity of proteolysate

Fig. 3 demonstrated the effect of temperature on antioxidant activity of protein hydrolysate using DPPH• SM and FRAP assay. From the result, there was a slight increase in the DPPH radical scavenging activity when the temperature increasing from 40^0C to 50^0C and this activity decreased afterwards. The scavenging activity reached highest value of 69.660 ±0.486 % when the temperature reached 55^0C (Fig. 3). The FRAP value significantly raised ($P<0.05$) and reached the highest value of 603.333 ±7.000 µM Trolox equivalent when the temperature reached 50^0C; afterwards, this value decreased. Similar results were also reported in studies of Ren et al. [30], of Satya Sadhan Dey and Krushna Chandra Dora [31], of Lijun You et al. [32]. The decrease in FRAP value may be because at high temperatures in comparison with the optimum temperature, the enzyme tertiary structure may change completely, disabling all activity, and the substrate won't fit the active site. Temperature affects the activity of enzyme by breaking hydrogen bonds, changing the shape of the active site.



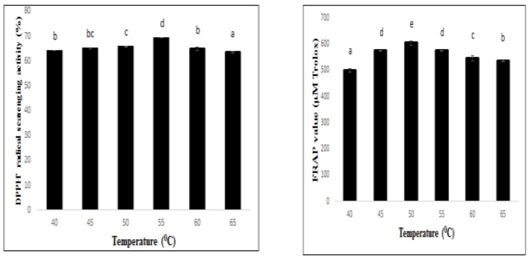
(a) (b)

Figure 1. Effect of proteolysis time on antioxidant activity of the proteolysate. Values represent the mean ± SD of three determinations. Bars with different letters indicate significant differences ($P<0.05$). (a) using DPPH• SM, (b) using FRAP method.



(a) (b)

Figure 2. Effect of enzyme/substrate ratio on antioxidant activity of hydrolysate.Values represent the mean ± SD of three determinations. Different letters indicate significant differences ($P<0.05$). (a) using DPPH• SM, (b) using FRAP method.



(a) (b)

Figure 3. Effect of temperature on antioxidant activity of of hydrolysate. Values represent the mean ± SD of three determinations. Different letters indicate significant differences (P<0.05).(a) using DPPH• SM, (b) using FRAP method.

The optimum temperature will support the formation of an enzyme-substrate complex most efficiently, due to the enzyme active site being the most accurate shape to fit the substrate. At temperatures below the optimum, the tertiary structure and the active site of the enzyme are not altered, slowing the rate of reaction due to less kinetic energy and therefore reduced collisions between the enzymes and substrates.

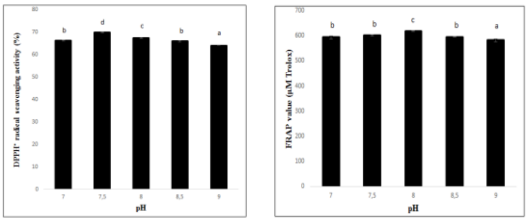
3.5. Effect of pH on antioxidant activity of protein hydrolysate

The result of the pH effect on antioxidant activity of hydrolysate derived from Tra catfish by-product was demonstrated in Figure 4.

As can be seen from Fig. 4, there was a slight change in the DPPH radical scavenging activity and FRAP values with pH ranging from 7 to 9. Similar results were also revealed in the research of Vilailak Klompong et al. (2008) [33]. This can be explained that short chain peptides and amino acids in protein hydrolysate are not much affected by charge modification governed by pH changes [33]. Basically, protein hydrolysate is soluble over a wide pH range, showing low influence by pH, whereas native proteins with tertiary and quarternary structure are affected considerably by pH change [34]. In our study, the DPPH radical scavenging capacity reached the highest value of 69.984 ±0.192 when pH value was 7.5 and the maximal FRAP value of 618.000 ±2.333 was observed with the pH value of 8. It might be suggested that the proteolysate could be used in foods with this pH ranges, in which it could function as the primary antioxidant.

3.6. Determination of degree of hydrolysis (DH), 50% DPPH• inhibition concentration (IC50) of the proteolysate and comparision with that of vitamin C and BHT

The DH of Tra catfish by-product-derived proteolysate determined using the method mentioned above was 14.6% (at pH 7.5, 55^{0}C) and 14.93% (at pH 8, 50^{0}C). The IC_{50} and FRAP values of the proteolysate, vitamin C and BHT were presented in Table 1. The result revealed that IC_{50} of the proteolysate was 6.775 mg/mL while that of salmon by-product-derived proteloysate using Alcalase preparation was 4.76 mg/mL [35], that of Alaska Pollack skin -derived hydrolysate using Protamex was 2.5 mg/mL [36], that of bigeye tuna head -derived hydrolysate using Alcalase was 1.34 mg/mL [37]. The IC_{50} of the Tra catfish by-product-dedrived proteolysate was 1645-fold higher than that of vitamin C and 17-fold higher than that of BHT. The FRAP value of the proteolysate was 53-fold and 18-fold lower than those of vitamin C and BHT. This can be easily understood by considering the fact that vitamin C and BHT are strong antioxidants, while the hydrolysate composed of different compounds, some of which may have strong antioxidant capacity and others may have weak or no antioxidant activity. Although the antioxidant potential of the proteolysatein our research was lower than those of vitamin C and BHT, it is still promising to be used in the food industry and neutraceuticals as natural alternative antioxidant.



(a) (b)

Figure 4. Effect of pH on antioxidant activity of protein hydrolysate. Values represent the mean ± SD of three determinations. Different letters indicate significant differences ($p < 0.05$).(a) using DPPH• SM, (b) using FRAP method.

Table 1. The IC50 and frap value of the proteolysate compared to those of vitamin C and BHT

Antioxidant activity	**Proteolysate**	**Vitamin C**	**BHT**
IC_{50} (µg/mL)	6775 ± 214	4.083 ± 0.023	395.523 ± 1.009
FRAP value (µMTrolox)	52.12 ± 1.99	2766.8 ± 4	964.4 ± 5.02

6. CONCLUSIONS

In this study, the Alcalase hydrolysis of Tra catfish by-products provided an antioxidant proteolysate. Although antioxidant activity of the proteolysate was lower than those of vitamin C and BHT, it has the potential for use as a natural alternative antioxidant in nutraceutical and functional food industry. The result suggested that Tra catfish by-product is a good natural source for producing antioxidants. Further detailed studies on isolation and purification of peptide fractions from the proteolysate as well as the different mechanisms of their antioxidant activities are needed.

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Khảo sát hoạt tính kháng oxy hóa của dịch thủy phân từ phụ phẩm cá tra sử dụng chế phẩm Alcalase® 2.4L FG ứng dụng như một chất kháng oxy hóa tự nhiên

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TÓM TẮT

Trong nghiên cứu này, ảnh hưởng của nhiệt độ, pH, hàm lượng enzyme, thời gian thủy phân đến hoạt tính kháng oxy hóa của dịch thủy phân từ phụ phẩm chế biến cá tra (Pangasius hypophthalmus) sử dụng chế phẩm Alcalase® 2.4 L FG được khảo sát sử dụng phương pháp nhốt gốc tự do DPPH• và phương pháp khử ion sắt (III) FRAP. Thành phần hóa học của phụ phẩm chế biến cá tra bao gồm độ ẩm 58,5%, hàm lượng protein 33,88%, hàm lượng lipid 50,14% và hàm lượng tro 15.83% (tính theo hàm lượng chất khô). Kết quả hoạt tính kháng oxy hóa của dịch thủy phân cho thấy nồng độ ức chế 50% DPPH• (IC50) đạt khoảng 6775 mg/mL, cao gấp 1645 lần so với vitamin C và 17 lần so với BHT với mức độ thủy phân của dịch thủy phân là 14,6% khi thời gian thủy phân là 5h, tỉ lệ E/S là 30 U/g protein, nhiệt độ thủy phân là 55oC và pH là 7,5. Khả năng kháng oxy hóa của dịch thủy phân sử dụng phương pháp FRAP đạt khoảng 52,12 μMTrolox, thấp hơn 53 lần và 18 lần so với hoạt tính kháng oxy hóa của vitamin C và BHT, khi thời gian thủy phân là 5h, tỷ lệ E/S là 30 U/g protein, nhiệt độ thủy phân 50oC và pH 8. Kết quả nghiên cứu cho thấy dịch thủy phân có hoạt tính kháng oxy hóa từ phụ phẩm cá tra có tiềm năng sử dụng như một chất kháng oxy hóa tự nhiên trong công nghiệp thực phẩm chức năng và dược phẩm.

Từ khóa: *hoạt tính kháng oxy hóa; peptide kháng oxy hóa; dịch thủy phân; phụ phẩm cá tra.*

REFERENCES

[1]. A.Barkia, A. Bougatef, H.B. Khaled and M. Nasri, *Antioxidant activities of sardinelle heads and/or viscera protein hydeolysates prepared by enzymatic treament*, Journal of Food Biochemistry 2010; 34; 303–320.

[2]. G.S. Centenaro, M.S. Mellado and C. Prentice-Hernández, *Antioxidant Activity of Protein Hydrolysates of Fish and Chicken Bones*, Advance Journal of Food Science and Technology 2010; 3(4); 280-288.

[3]. J.-Y. Je, P.-J. Park and S.-K. Kim, *Antioxidant activity of a peptide isolated from Alaska pollack (Theragrachacogramma) frame protein hydrolysate*, Food Research International 2005; 38; 45–50.

[4]. J.-Y. Je, Z.-J. Qian, H.-G. Byun and S.-K. Kim, *Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis*, Process Biochemistry 2007; 42; 840-846.

[5]. J.-I. Yang., H.-Y. Ho, Y.-J. and C.-J. Chow, *Characteristic and antioxidant activity of retorted gelatin hydrolysates from cobia (Rachycentroncanadum) skin*, Food Chemistry 2008; 110(1); 128–136.

[6]. A. Bougatef et al., *Purification and identification of novol antioxidant peptides from enzymatic hydrolysates of sardinelle (Sardinellaaurita) by-products proteins*, Food Chemistry 2010; 118; 559-565.

[7]. C.-B.Ahn, J.-Y. Je and Y.-S. Cho, *Antioxidant and anti-inflammatory peptide fraction from salmon by-product protein hydrolysates by peptic hydrolysis*, Food Research International 2012; 49; 92-98.

[8]. C.-B. Ahn, J.-G. Kim and J.-Y. Je, *Purification and antioxidant properties of octapeptide from salmon by-product protein hydrolysate by gastrointestinal digestion*, Food Chemistry 2014; 147; 78-83.

[9]. J.-Y. Je, Z.-J.Qian, S.-H. Lee, H.-G. Byun and S.-K. Kim, *Purification and Antioxidant Properties of Bigeye Tuna (Thunnusobesus) Dark Muscle Peptide on Free Radical-Mediated Oxidative Systems*, J Med Food 2008; 11(4); 629–637.

[10]. K.-C. Hsu, *Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product*, Food Chemistry 2010; 122; 42-48.

[11]. S.Saidi,A. Deratani, M.-P. Belleville and R. B. Amar, *Antioxidant properties of peptide fractions from tuna dark muscle protein by-product hydrolysate produced by membrane fractionation process*, Food Res Int 2014; 65(Part C); 329–336.

[12]. AE Ghaly, VV Ramakrishnan, MS Brooks, SM Budge and D Dave, *Fish Processing Wastes as a Potential Source of Proteins, Amino Acids and Oils*, Microbial & Biochemical Technology 2013; 5(4); 107-129.

[13]. M. A.Amiza, Y.W. Ow and A. Faazaz, *Physicochemical properties of silver catfish (Pangasius sp.) frame hydrolysate*, International Food Research Journal 2013; 20(3); 1255-1262.

[14]. AOAC, *Methods of Analysis*, Association of Official Analytical Chemists, 15th ed. Washington, DC, 2000.

[15]. N. Bhaskar, and N. S. Mahendrakar, *Physicochemical properties of silver catfish (Pangasius sp.) frame hydrolysate*, International Food Research Journal 2007; 20(3); 1255-1262.

[16]. M.L.Anson, *The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin*, J. Gen. Physiol 1938; 22; 79-89.

[17]. O.H.Lowry, N.J.Rosebrough, A.L.Farr, and R.J.Randall, *Protein Measurement with the Folin Phenol Reagent*, J. Biol. Chem 1951; 193; 265-272.

[18]. N. Hoyle, and J. H. Merrit, *Quality of fish protein hydrolysate from herring*, Journal of Food Science 1995; 59; 4769-4774.

[19]. H. M., Chen, K.Muramoto, F.Yamauchi, K.Fujimoto, & K Nokihara, *Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein*, Journal of Agricultural and Food Chemistry 1998; 46; 49–53.

[20]. I. F. F.Benzie, J. J.Strain, *The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay*. Anal. Biochem 1996; 239; 70–76.

[21]. M.A.Amiza, N. S.Ashikin, and A.L.Faazaz. *Optimization of enzymatic protein hydrolysis from silver catfish (Pangasius sp.) frame*, International Food Research Journal 2011; 18; 751-757.

[22]. S. Bordbar et al. *The Improvement of The Endogenous Antioxidant Property of Stone Fish (Actinopygalecanora) Tissue Using Enzymatic Proteolysis*", BioMed Research International 2013.

[23]. E.Mendis, N.Rajapakse, & S. K.Kim, *Antioxidant properties of a radicalscavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate*, Journal of Agricultural and Food Chemistry 2005; 53; 581–587.

[24]. Chen, H. M., K.Muramoto, F.Yamauchi, & K.Nokihara, *Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein*, Journal of Agricultural and Food Chemistry 1996; 44; 2619–2623.

[25]. C.Wiriyaphan, B. Chitsomboon, and J. Yongsawadigul, *Antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts* ,Food Chemistry 2012; 132(1); 104–111.

[26]. Z. J. Qian, W. K. Jung, & S. K. Kim, *Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, Ranacatesbeiana Shaw*, Bioresource Technology 2008; 99; 1690–1698.

[27]. A. Hirose & K.Miyashita, *Inhibitory effect of proteins and their hydrolysates on the oxidation of triacylglycerols containing docosahexaenoic acids in emulsion*, Journal of the Japanese Society for Food Science and Technology 1999; 46; 799–805.

[28]. H.C.Wu, H.M. Chen, & C.Y. Shiau, *Free amino acids and peptides as related to antioxidant properties in protein hydrolysates*, Food Reseach International 2003; 36; 949-957.

[29]. S. Tanuja et al. *Functional and antioxidative properties of fish protein hydrolysate (FPH) produced from the frame meat of striped catfish Pangasianodon*, Indian J. Fish 2014; 61(2); 82-89.

[30]. J.Y.Ren, et al., *Optimization of antioxidant peptide production from grass carp sarcoplasmic protein using response surface methodology*, Lebensm.-Wiss. Technol 2008; 41; 1624–1632.

[31]. S. S. Dey& K. C. Dora, *Antioxidative activity of protein hydrolysate produced by alcalase hydrolysis from shrimp waste (Penaeusmonodon and Penaeusindicus)*, J Food SciTechnol 2014; 51(3); 449–457.

[32]. L. You, M. J. Regenstein and R. L. *Optimization of Hydrolysis Conditions for the Production of Antioxidant Peptides from Fish Gelatin Using Response Surface*

Methodology, Journal of Food Science 2010; 75; Nr. 6.

[33]. V. Klompong, S. Benjakul, D. Kantachote, K. D. Hayes & F. Shahidi, *Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme*, International Journal of Food Science and Technology 2008; 43; 1019–1026.

[34]. G.A.Gbogouri, M.Linder,J.Fanni, & M.Parmentier, *Influence of hydrolysis degree on the functional properties of salmon byproduct hydrolysates*, Journal of Food Science, vol. 69, pp. 615–622, 2004.

[35]. C.-B.Ahn, J.-G.Kim, and J.-Y.Je, *Purification and antioxidant properties of octapeptide from salmon byproduct protein hydrolysate by gastrointestinal digestion*, Food Chem 2014; 147; 78–83.

[36]. J.Jia, et al., *Enzymatic hydrolysis of Alaska pollack (Theragrachalcogramma) skin and antioxidant activity of the resulting hydrolysate*, J Sci Food Agric 2010; 90(4); 635–640.

[37]. P.Yang, et al., *Antioxidant activity of bigeye tuna (Thunnusobesus) head protein hydrolysate prepared with Alcalase: Antioxidant activity of tuna head hydrolysate*, Int J Food SciTechnol 2011; 46(12); 2460–2466.