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PROOF RESEARCH PAPER

Protein Hydrolysates Prepared from the Viscera of Skipjack Tuna (*Katsuwonus pelmamis*): Antioxidative Activity and Functional Properties

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Abstract

Protein hydrolysates from skipjack tuna viscera prepared using Alcalase 2.4 L (VPH) with different degree of hydrolysis (DH: 10%, 20% and 30%) were prepared and determined for their antioxidative activity. VPH with 20% DH had the highest DPPH, ABTS radical scavenging activities and ferrous chelating activity ($P<0.05$). However, ferric reducing antioxidant activity of hydrolysates increased as DH increased ($P<0.05$). When VPH with 20% DH was determined for its pH and thermal stability, ABTS radical scavenging activity remained constant or slightly decreased in a wide pH range (2-11) and during heating (100ºC) for 180 min. Functional properties of VPH (20% DH) at different concentrations were also investigated. The emulsifying and foaming properties were governed by its concentrations used. Hydrolysis by Alcalase at 20% DH increased protein solubility to above 91% over a wide pH range (3-9). Therefore, VPH could be used as a food additive possessing both antioxidant activity and functional properties.

Keywords: Hydrolysates; hydrolysis; functionalities; antioxidant; viscera.

Introduction

Tuna is one of the most economically important groups of fish species. The main forms for international tuna trade are the raw material for canning, tuna for direct consumption (sashimi), smoked tuna and dried tuna (Klomklao, Kishimura, Nonami & Benjakul, 2009). In Thailand, the tuna processing industry has been economically important as an essential income generator. Skipjack tuna (*Katsuwonus pelamis*) has served as the most common species with the largest amount of canned tuna production in Thailand. During processing, a large amount of viscera has been collected and generally used as animal feed with a low market value. Tuna viscera contain valuable protein and lipid fractions (Klomklao, & Benjakul, 2016), but are also an important source of environmental contamination. To maximize the use of those wastes, enzymatic hydrolysis is one of the most efficient methods to recover protein and thus to increase the commercial value of such biomass.

Enzymatic modification of proteins using selected proteolytic enzyme preparations to cleave specific peptide bonds is widely used in the food industry (Ovissipour et al., 2009). It has been implemented to produce protein hydrolysate in order to improve its functional properties and to enhance bioactivities, especially antioxidant activity (Klomklao, Benjakul, & Kishimura, 2013). Proteases from different sources are commonly employed to obtain a more selective hydrolysis due to their specificity for peptide bonds adjacent to certain amino acid residues (Nalinanon, Benjakul, Kishimura, & Shahidi, 2011; Klomklao, Benjakul, & Kishimura, 2010). The most common commercial protease used for the hydrolysis of fish protein is Alcalase (EC 3.4.1.62), due to the high degree of hydrolysis that can be achieved in a relatively short time under moderate pH conditions, compared to neutral or acidic enzymes (Benjakul & Morrissey, 1997; Shahidi, Han, & Synowiecki, 1995). Hydrolysis protein can also improve intestinal absorption (Kristinsson & Rasco, 2000), and be used as a source of peptides, such as peptone, for ingredients in microbial growth media. The protein hydrolysate has been found to possess bioactivity e.g. radical scavenging activity, chelating (Klomklao et al., 2013), antihypertensive (Bougatef et al., 2008) etc. Fish protein hydrolysates such as by-products protein hydrolysate from Persian sturgeon (Ovissipour et al., 2009), threadfin bream (Wiriyaphan, Chitsomboon, &

Yongsawadigul, 2012) or meat protein hydrolysates from round scad (Thiansilakul, Benjakul, & Shahidi, 2007), mackerel (Wu, Chen, & Shiau, 2003) and toothed ponyfish (Klomklao et al., 2013) have been reported to show antioxidative activity. However, no information regarding protein hydrolysates from the viscera of skipjack tuna and their antioxidant activities and functional properties has been reported. Hence, the objective of this investigation was to study the antioxidative activities and functional properties of protein hydrolysate from skipjack tuna viscera.

Materials and Methods

Chemicals

Alcalase 2.4 L (2.4 units/ml) was obtained from Novo Nordisk (Bagsvaerd, Denmark). L-leucine, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine) and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Tris (hydroxymethyl) aminomethane (Tris-HCl), sodium sulfite, potassium persulphate, ferrous chloride and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was procured from Bio-Rad Laboratories (Hercules, CA, USA). All reagents were of analytical grade.

Fish Sample Preparation

Internal organs from skipjack tuna (*Katsuwonus pelamis*) were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. Those samples (5 kg) were packed in a polyethylene bag, kept in ice and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung within 2 h. Pooled internal organs were immediately frozen and stored at -20°C until used.

Prior to the hydrolysis process, frozen viscera were thawed overnight in a refrigerator at 4°C. The viscera were ground finely and then heated at 85°C for 20 min to inactivate endogenous enzymes (Guerard, Guimas, & Binet, 2002). The heat treated skipjack tuna viscera were used as a protein substrate for protein hydrolysate preparation. They were kept at 4°C until needed.

Production of Protein Hydrolysate from Skipjack Tuna Viscera

The heat-treated skipjack tuna viscera (20 g) was mixed with 0.2 M borate buffer (pH 10.0) at a ratio of 1:2 (w/v) and pre-incubated at 65°C for 10 min. The enzyme hydrolysis was started by adding Alcalase with the amount required to gain 10%, 20% and 30% DH into 250 ml of mixture, as described by Klomklao and Benjakul (2016). After 20 min of hydrolysis, the enzyme was inactivated by heating at 90°C for 15 min in a water bath. The mixture was then cooled on ice and centrifuged at 5,000×g at 4°C for 10 min to collect the supernatant. Skipjack tuna viscera protein hydrolysate was freeze-dried using a Dura-Top^{TM}µp freeze-dryer (FTS systems Inc., Stone Ridge, NY, USA). The freeze-dried skipjack tuna viscera protein hydrolysate (VPH) obtained was subjected to analyses.

Determination of α-amino Acids and DH

The α-amino acid content was determined according to the method of Benjakul and Morrissey (1997). To diluted hydrolysate samples (125 µl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acid was expressed in term of L-leucine. DH was calculated as follows:

$$DH = [(L_t\text{-}L_0)/(L_{max}\text{-}L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time *t*. L_0 is the amount of α-amino acid in the original skipjack tuna viscera homogenate. L_{max} is total α-amino acid in the original skipjack tuna viscera homogenate obtained after acid hydrolysis with 6 N HCl at 100°C for 24 h.

Determination of Antioxidant Activity

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined as described by Nalinanon et al. (2011) with a slight modification. To the sample (1.5 ml), 1.5 ml of 0.15 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol were added. The mixture was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of resulting solution was measured at 517 nm using a UV-1800 spectrophotometer (Shimadzu). The blank was prepared in the same manner, except that distilled water was used instead of the sample. DPPH radical scavenging activity was calculated according to the following equation (Yen & Wu, 1999):

$$DPPH = \frac{1 - A517\ of\ sample}{A517\ of\ control}\ x\ 100$$

ABTS Radical Scavenging Activity

ABTS radical scavenging activity was determined by ABTS assay, as described by Binsan et al. (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol, in order to obtain an absorbance of 1.1±0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Sample (150µl) was mixed with 2,850 µl of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. ABTS radical scavenging activity was calculated according to the following equation:

$$\text{ABTS} = \frac{1 - A734\ of\ sample}{A734\ of\ control}\ x\ 100$$

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP was assayed according to the method of Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 6H_2O$ solution. A working solution was prepared fresh by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 6H_2O$ solution. The mixed solution was incubated at 37ºC for 30 min and was referred to as the FRAP solution. The sample (150 µl) was mixed with 2,850 µl of FRAP solution and kept for 30 min in the dark. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. Increased absorbance of the reaction mixture indicates the increasing ferric reducing antioxidant power.

Metal Chelating Activity

The chelating activity on Fe^{2+} was measured using the method of Boyer and McCleary (1987) with a slight modification. Diluted sample (4.7 ml) was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then measured at 562 nm. The blank was conducted in the same manner but distilled water was used instead of the sample. The chelating activity was calculated as follows:

$$\text{Chelating activity} = \frac{(1 - \text{A562 of sample})}{\text{A562 of control}}\ x\ 100$$

Effect of Protein Hydrolysate Concentration on Antioxidant Activity

Skipjack tuna viscera protein hydrolysate with 20% DH was dissolved in distilled water to obtain concentrations of 0.5, 1, 2, 3, 4, 5, 7 and 10 mg protein/ml. Antioxidant activity of protein hydrolysates at different concentrations was measured by monitoring the ABTS radical scavenging activity.

pH and Thermal Stability of Antioxidant Hydrolysate

For the pH stability study, skipjack tuna protein hydrolysate (20% DH) was dissolved in distilled water to obtain the concentration of 5 mg protein/ml. The 5 ml of sample solutions were adjusted to pHs 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1 or 6 M HCl and 1 or 6 M NaOH and incubated at room temperature for 30 min. The pHs of sample solutions were then adjusted to 7.0. The final volume of all solutions was brought up to 20 ml using distilled water. The residual antioxidant activities were determined using ABTS assay.

To determine thermal stability, skipjack tuna protein hydrolysate at a concentration of 5 mg protein/ml was prepared using distilled water as a medium. The 5 ml of sample solutions were transferred into a screw-capped test tube. The tube was capped tightly and placed in a boiling water bath (100ºC) for 0, 15, 30, 60, 90, 120, 150 and 180 min. The treated samples were suddenly cooled in iced water. The sample without incubation (25ºC) was used as the control. The residual antioxidant activities were determined using ABTS assay.

Functional Properties of Protein Hydrolysate

The VPH with 20% DH was subjected to functional properties analyses.

Solubility

To determine protein solubility, VPH with 20% DH (200 mg) were dispersed in 20 ml of deionized water and pH of the mixture was adjusted to 3, 5, 7 and 9 with either 1 M HCl or 1 M NaOH. The mixture was stirred at room temperature for 30 min. The volume of solutions was made up to 25 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5,000×g for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry, Rosebrough, Fan, & Randal, 1951), using bovine serum albumin as a standard. Total protein content in the same was determined after solubilization of the sample in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \; x\,100$$

Emulsifying Properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were used to measure the emulsifying properties of VPH (20% DH). EAI and ESI were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 ml) and VPH solutions (5%, 10%, 15% and 20%, 6 ml) were homoginized (Model T25 basic; IKA Labortecnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipetted from the middle portion of the container at 0 and 10 min after homogenization and subsequently diluted 100-fold using 0.1% sodium dodecyl sulphate (SDS) solution. The mixture was mixed thoroughly for 10 s using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY). A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formulae:

$$\text{EAI } (m^2/g) = (2 \times 2.303 \times A \times DF) / l\phi C$$

where A = A_{500}, DF = dilution factor (100), l = path length of cuvette (m), ϕ = oil volume fraction and C = protein concentration in aqueous phase (g/m^3);

$$\text{ESI (min)} = A_0 \times \Delta t/\Delta A$$

where $\Delta A = A_0 - A_{10}$ and Δt = 10 min.

Foaming Properties

Foam expansion (FE) and foam stability (FS) of hydrolysate solutions were determined according to the method of Shahidi et al. (1995) with a slight modification. Hydrolysate solutions (20 ml) with 5%, 10%, 15% and 20% protein concentrations were transferred into a 100-ml cylinder. The solutions were homogenized at 13,400 rpm for 1 min at room temperature. The samples were allowed to stand for 0, 30 and 60 min. FE and FS were then calculated using the following equations:

$$\text{FE (\%)} = (V_T/V_0) \times 100$$
$$\text{FS (\%)} = (V_t/V_0) \times 100$$

where V_T is total volume after whipping; V_0 is the original volume before whipping and V_t is total volume after leaving at room temperature for different times (30 and 60 min).

Protein Determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical Analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

Results and Discussions

Antioxidant Activities of Protein Hydrolysates with Different DHs

The antioxidant properties of VPH having different DHs (10-30%) were evaluated using different assays (DPPH and ABTS radical scavenging activities, chelating activity and FRAP).

DPPH and ABTS Radical Scavenging Activity

DPPH and ABTS radical scavenging activities of VPH with different DHs are illustrated in Figure 1A and Figure 1B, respectively. The differences in activities determined by both assays were found amongst VPH with different DHs. The highest DPPH and ABTS radical scavenging activities were obtained in VPH with 20% DH (P<0.05) (Figure 1A and Figure 1B). As the DH increased from 20% to 30%, a decrease in DPPH and ABTS radical scavenging activities was observed (P<0.05). DPPH and ABTS radical scavenging activities of VPH with 10% DH were 38.18% and 93.08%, respectively, while, that of 30% DH had activities of 30% and 94.81%, respectively. Hence, peptides in VPH with varying DH might differently scavenge two different radicals, DPPH and ABTS radicals. This might be governed by a difference in chain length, amino acid composition, amino acid side chain and hydrophobicity (Intarasirisawat, Benjakul, Visessanguan, & Wu, 2012). Chalamaiah, Dinesh kumar, Hemalatha, and Jyothirmayi (2012) reported that the amino acid composition of protein hydrolysate plays a significant role in antioxidant activities. The higher contents of hydrophobic and aromatic amino acids facilitated the radical scavenging of protein hydrolysate fractions from dark muscle of skipjack tuna (Chi, Hu, Wang, Li, & Luo, 2015). Generally, DPPH radical scavenging activity is used to determine the hydrogen-donating ability of protein hydrolysate (Thiansilakul et al., 2007). The stable DPPH radical shows a maximum absorbance at 517 nm in ethanol. When DPPH encounters a hydrogen-donating substance, such as an antioxidant, the radical is

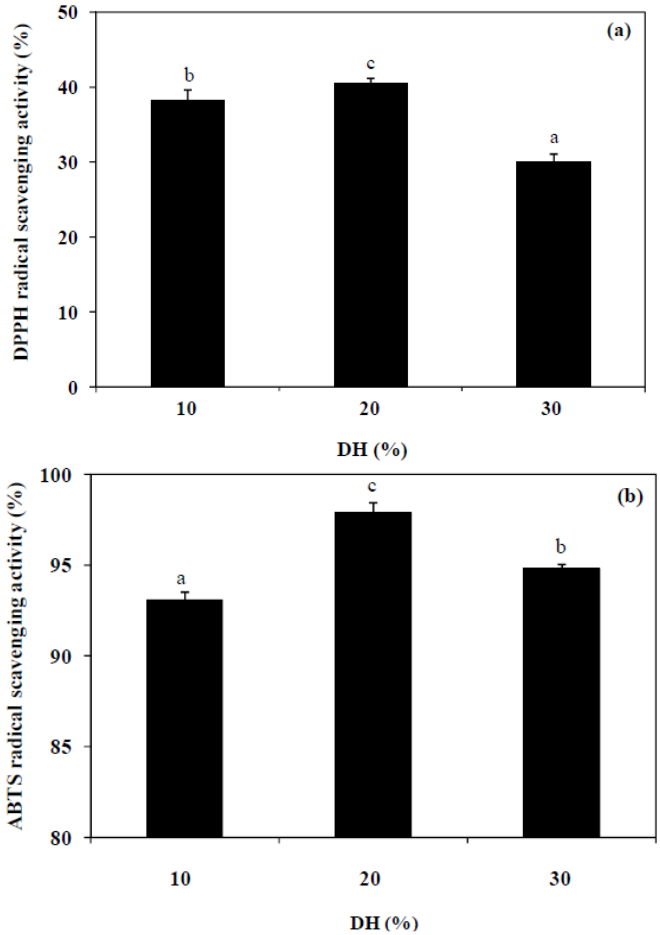

Figure 1. DPPH radical (**a**) and ABTS radical (**b**) scavenging activities of protein hydrolysate from skipjack tuna viscera with different DHs. Bars represent standard deviation from triplicate determinations. Different letters indicate significant differences (P<0.05).

scavenged, as visualized by changing its color from purple to yellow, and the absorbance is reduced (Shimada, Fujikawa, Yahara, & Nakamura, 1992; Nalinanon et al., 2011). ABTS radical scavenging activity is based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals, by converting it to the non-radical species (Binsan et al., 2008). The pre-formed radical monocation of $ABTS^{+}$ is generated by oxidation of ABTS with potassium persulphate and is reduced in the presence of hydrogen-donating antioxidants and chain breaking antioxidants (Binsan et al., 2008). The ABTS radical scavenging activity assay can be applied to both lipophilic and hydrophilic compounds, and has been widely used as an antioxidant activity assay (Senphan & Benjakul, 2014). With high ABTS radical scavenging activity, it was postulated that antioxidative compounds were most likely hydrophilic. The results suggest that the VPH, especially at 20% DH, had the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction.

Ferric Reducing Antioxidant Power (FRAP)

As the DH increased, FRAP increased (P<0.05). VPH with 30% DH had the highest activity (P<0.05) (Figure 2). Increases in reducing power of protein hydrolysate with increasing DH have been reported in round scad protein hydrolysate treated with Flavourzyme, seabass skin hydrolysate prepared by protease from Pacific white shrimp hepatopancreas (Thiansilakul et al., 2007; Senphan & Benjakul, 2014). The greater reducing powder indicated that VPH could donate an electron to free radicals, leading to the prevention or retardation of propagation (Klomklao et al., 2013). Generally, FRAP is used to determine the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Binsan et al., 2008). Thus, the hydrolysis by Alcalase most likely increased FRAP of the resulting hydrolysate via the enhancement of reducing powder towards free radicals. As a result, propagation step could be terminated in the presence of hydrolysate with appropriate DH.

Metal Chelating Activity

The ferrous (Fe^{2+}) chelating activity of VPH as affected by DH is shown in Figure 3. The chelating

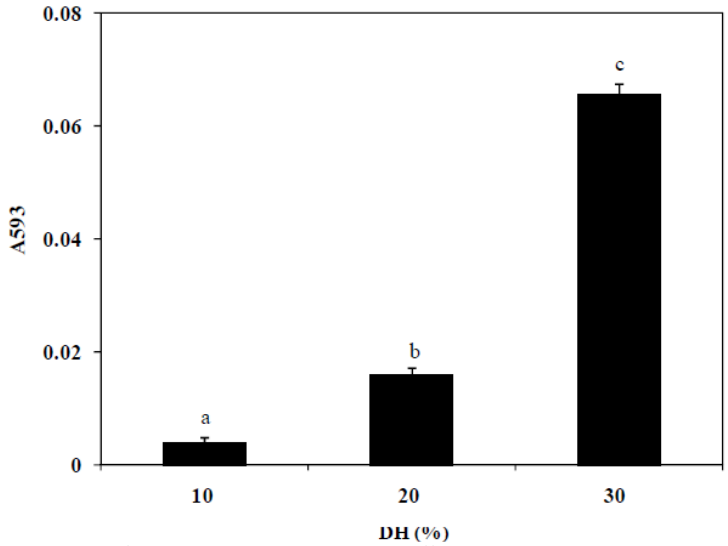

Figure 2. FRAP of protein hydrolysate from skipjack tuna viscera with different DHs. Bars represent standard deviation from triplicate determinations. Different letters indicate significant differences (P<0.05).

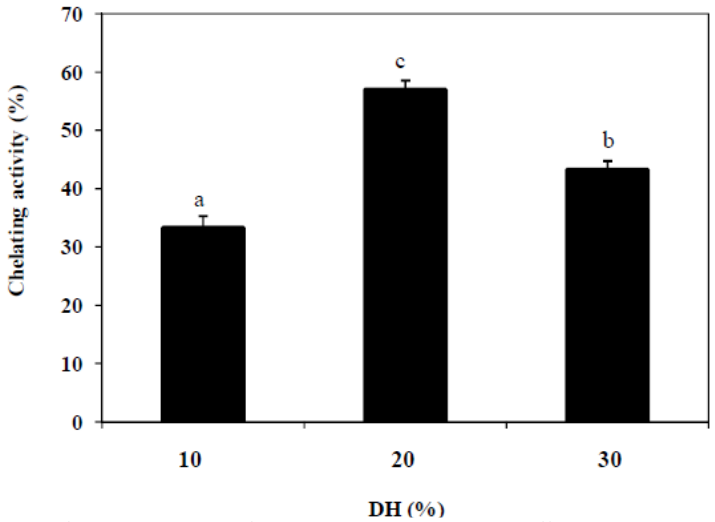

Figure 3. Metal chelating activity of protein hydrolysate from skipjack tuna viscera with different DHs. Bars represent standard deviation from triplicate determinations. Different letters indicate significant differences (P<0.05).

activity increased with increasing DH up to 20% (P<0.05). Furthermore, the decrease in the metal chelating activity was observed when DH was 30% (P<0.05). The ferrous chelating activity of VPH with % DH of 10, 20 and 30 was 33.30, 57.04 and 43.33%. The shorter chain of peptides might lose their ability to form the complex with Fe^{2+}. The results indicated that the limited hydrolysis of viscera protein resulted in the enhanced ferrous chelating activity, compared with the excessive hydrolysis. Intarasirisawat et al. (2012) reported that the metal chelating activity of roe protein hydrolysate from skipjack tuna treated with Alcalase was affected by DH and its metal chelating activity increased with increasing DH up to 40%. Ferrous ion (Fe^{2+}) is the most powerful pro-oxidant among metal ions (Khantaphant & Benjakul, 2008), leading to the initiation and acceleration of lipid oxidation by interaction with hydrogen peroxide in a Fenton reaction to produce the reactive oxygen species, hydroxyl free radical ($OH^{\bullet}$) (Klomklao et al., 2013). Therefore, metal ions chelation by peptides in protein hydrolysates could retard the oxidative reaction.

It was noted that the VPH with 20% DH exhibited the highest radical scavenging activity and Fe^{2+} chelating activity, whilst the VPH with 30% DH showed the highest FRAP. It was speculated that different peptides with different sizes as well as amino acid sequence might have different modes of action. Hence, VPH with appropriate DH could be produced, to maximize their functions as radical scavengers, metal chelators and reducing agents.

Effect of Concentration of Protein Hydrolysate on Antioxidant Activity

The antioxidant activity as measured by ABTS radical scavenging activity of VPH with 20% DH at various concentrations is shown in Figure 4. The marked increase in ABTS radical scavenging activity was noticeable in the concentration range of 0-5 mg/ml. Thereafter, a slight increase in ABTS radical scavenging activity was found up to 10 mg/ml. Klomklao et al. (2013) reported that the antioxidant activity of toothed ponyfish protein hydrolysate was dose-dependent. Intarasirisawat et al. (2012) also found that DPPH and ABTS radical scavenging activities increased as the concentration of the roe hydrolysate from skipjack tuna increased. Therefore, VPH with 20% DH showed the ABTS radical scavenging activity in a concentration dependent manner.

pH and Thermal Stability of Antioxidant Hydrolysate

The effects of pH on ABTS radical scavenging activity of VPH with 20% DH is depicted in Figure 5a. ABTS radical scavenging activity of VPH was quite stable over the pH range of 3-11. At pH 2, and 12, ABTS radical scavenging activity slightly decreased, possible due to the changes of charges in peptides, particularly at N- and C- terminus at high acidic and alkaline pHs. The results suggested that VPH showing ABTS radical scavenging activity might lose their activity to some extent at low and high pHs. Due to the stability over a wide pH range, VPH with 20% DH had a potential for application in any food system over pH range of 3-11.

For thermal stability, thermal stability of antioxidant activity of VPH with 20% DH monitored by ABTS radical scavenging activity is illustrated in Figure 5b. The activities were stable up to 180 min of heating, where activities of more than 96% were retained. A small loss in ABTS radical scavenging activity might be due to either degradation or aggregation of some antioxidant peptides, caused by heat treatment, leading to the protein aggregation and the exposure of hydrophobic domain (Sikorski & Naczk, 1981). Zayas (1997) reported that smaller size peptides were more stable to aggregation at high temperatures. These results indicate that VPH with 20% DH could be incorporated in any food subjected to thermal processed at 100°C for up to 180 min.

Functional Properties

Solubility

Solubility is one of the most important physiochemical and functional properties of protein hydrolysate (Klomklao et al., 2013). Good solubility of proteins is required in many functional applications, especially emulsions, foams and gels, because soluble proteins provide a homogeneous dispersibility of the molecules in colloidal systems and enhance the interfacial properties (Zayas, 1997). The solubility of VPH with 20% DH in the pH range of 3-9 is shown in Table 1. VPH with 20% DH was soluble over wide pH range with solubility of 91-100%. Solubility can be increased by hydrolysis process (Klomklao et al., 2013). Nalinanon et al. (2011) reported that protein hydrolysates from ornate threadfin bream muscle, hydrolyzed by pepsin from skipjack tuna also showed the high solubility (>71%) in the pH range of 3-9. The lowest solubility was observed at pH 3. It was plausibly owing to the lowest repulsion of proteins at their pI. The enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolysates (Intarasirisawat et al., 2012). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility (Gbogouri, Linder, Fanni, & Parmentier, 2004). From the results, VPH (20% DH) with high solubility over a wide pH range can be applied widely in formulated food systems.

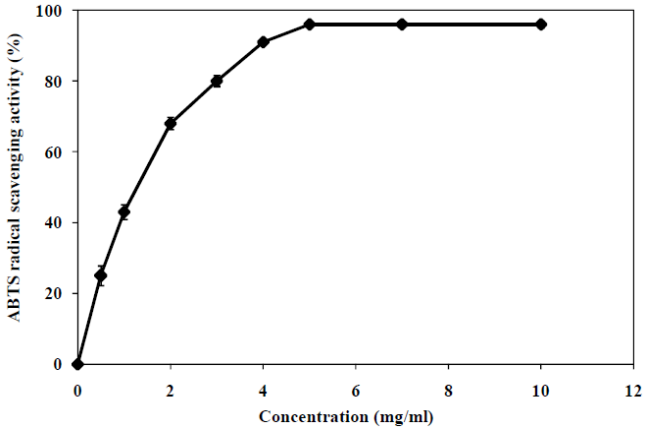

Figure 4. ABTS radical scavenging activity of protein hydrolysate from skipjack tuna viscera with 20% DH at varying concentrations. Bars represent standard deviation from triplicate determinations.

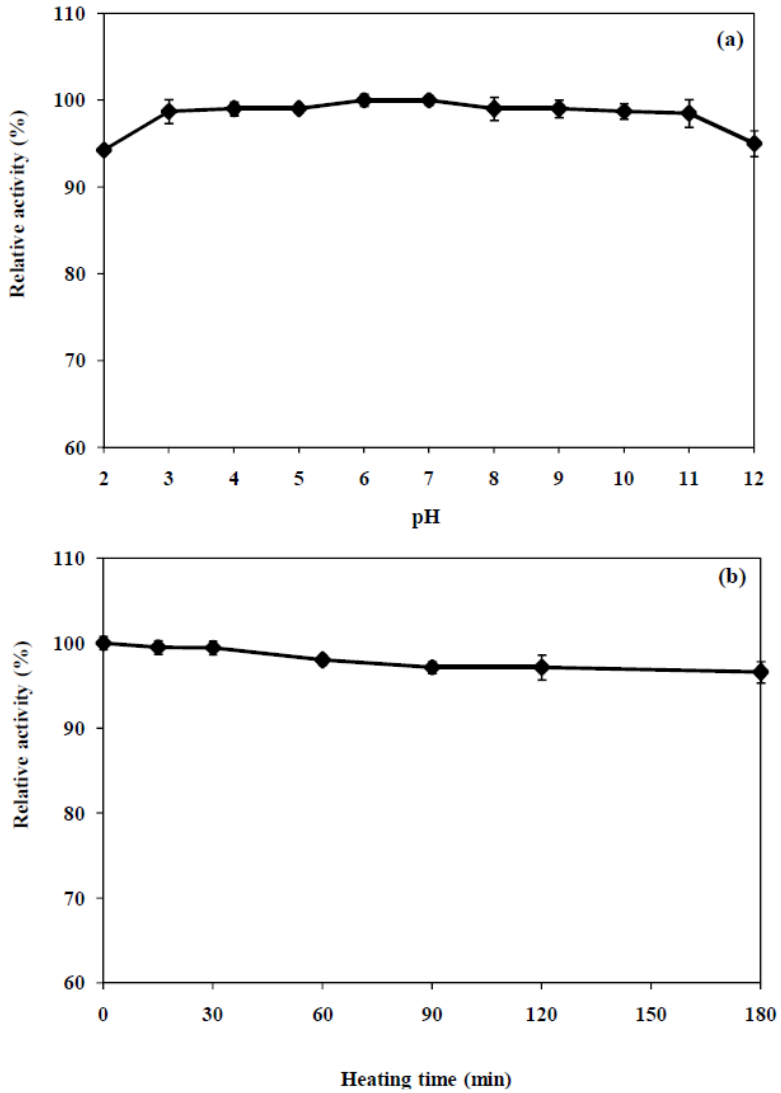

Figure 5. Effect of pH (**a**) and heating time (**b**) on ABTS radical scavenging activity of protein hydrolysate from skipjack tuna viscera with 20% DH. Bars represent standard deviation from triplicate determinations.

Table 1 Solubility of protein hydrolysate from skipjack tuna viscera with 20%DH at various pHs

pH	Solubility (%)*
3	91.89 ± 0.51a**
5	96.39 ± 0.09b
7	97.65 ± 0.12c
9	100.00 ± 0.23d

*Mean ± SD from triplicate determinations.
**Different letters in the same column indicate significant differences (P<0.05).

Table 2 Emulsifying properties of protein hydrolysate from skipjack tuna viscera with 20%DH at various concentrations

Hydrolysate concentrations (%)	Emulsifying activity index* (m^2/g)	Emulsifying stability index* (min)
5	37.95 ± 1.01d**	35.29 ± 1.02c**
10	34.09 ± 0.75c	27.01 ± 0.66b
15	25.96 ± 0.87b	14.97 ± 3.19a
20	20.14 ± 1.19a	14.65 ± 2.98a

*Mean ± SD from triplicate determinations.
**Different letters in the same column indicate significant differences (P<0.05).

Emulsifying Properties

Table 2 shows emulsifying activity index (EAI) and emulsion stability index (ESI) of VPH with 20% DH at different concentrations (5%, 10%, 15% and 20%). EAI of VPH decreased when the protein concentration increased (P<0.05). For ESI, the decrease in ESI of VPH was found when the concentration of hydrolysate increased up to 15% (P<0.05). However, no difference in ESI was observed when the VPH concentration was above 15% (P>0.05). Thiansilakul et al. (2007) found that both EAI and ESI of hydrolysates from round scad meat decreased with increasing hydrolysate concentration. Nalinanon et al. (2011) also reported that EAI of protein hydrolysates from ornate threadfin bream muscle prepared by pepsin from skipjack tuna decreased as the concentration increased. Protein hydrolysates are surface-active materials and promote an oil-in-water emulsion because of their hydrophilic and hydrophobic groups and their charge (Kristinsson & Rasco, 2000; Klomklao et al., 2013). At low protein concentration, protein adsorption at the oil-water interface is diffusion controlled. At high protein concentration, the activation energy barrier does not allow protein migration, the activation energy barrier does not allow protein migration to take place in a diffusion dependent manner, leading to the accumulation of proteins in the aqueous phase (Thiansilakul et al., 2007). Therefore, proteins or peptides were most likely localized in the aqueous phase and a lower amount of proteins or peptides migrated to the interface (Nalinanon et al., 2011). The increase in protein-protein interaction resulted in a lower protein concentration at the interphase, in which a thinner film stabilizing the oil droplet is formed. Thus, emulsifying properties of VPH with 20% DH were governed by the concentration employed.

Foaming Properties

Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air-water interface

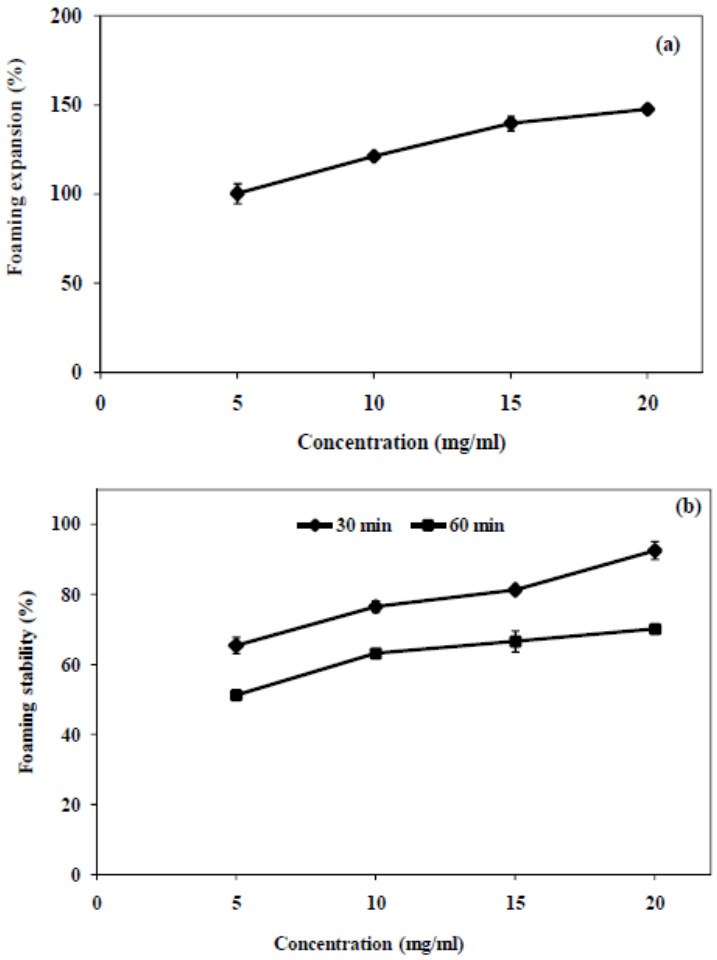

Figure 6. Foam ability (**a**) and foam stability (**b**) of protein hydrolysate from skipjack tuna viscera with 20%DH at varying concentrations. Bars represent standard deviation from triplicate determinations.

(Klomklao et al., 2013). Generally, proteins, which rapidly adsorb at the newly-created air-liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, show better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). The foam expansion (FE) and foam stability (FS) after standing at room temperature for 30 and 60 min of VPH with 20% DH at different concentrations are illustrated in Figure 6. FE indicated the foam abilities of VPH, which increased from 100.24% to 147.58% when the concentration of VPH increased from 5% to 20% (P<0.05) (Figure 6a). Thiansilakul et al. (2007) also reported that FE at 0 min after whipping of round scad protein hydrolysate increased with increasing hydrolysate concentration. For FS, FS of VPH after whipping for 30 min and 60 min were improved by increasing concentration (P<0.05). An increase in foam stability with increasing concentration was a result of formation of stiffer foams (Lawal, 2004). Phillips and Whitehead (1994) reported that FS is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness. A similar result was also reported in protein hydrolysates from ornate threadfin bream muscle treated by pepsin from skipjack tuna (Nalinanon et al., 2011).

Conclusion

Production of protein hydrolysates from skipjack tuna viscera, exerting good functionalities and antioxidative activities, could be achieved by Alcalase hydrolysis. The antioxidant activity of VPH varied with DH. Antioxidative activity of VPH with 20% DH was stable in a wide pH range and to heat treatment at 100ºC up to 180 min. Therefore, it might serve as a potential source of natural antioxidant to prevent lipid oxidation in various foods.

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