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RESEARCH PAPER

Protein Hydrolysates from Bluefin Tuna (*Thunnus thynnus*) Heads as Influenced by the Extent of Enzymatic Hydrolysis

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Abstract Functional properties and antioxidant activities of protein hydrolysates from tuna (*Thunnus thynnus*) heads (THPHs), with different degrees of hydrolysis, obtained by treatment with *Bacillus mojavensis* A21 alkaline proteases and Alcalase, were investigated. Protein content of all freeze-dried THPHs ranged from 73.74 ± 0.5 to 78.56 ± 1.2%. The THPHs had excellent solubility, compared to untreated tuna head proteins and possessed interfacial properties, which were governed by their concentrations. Similarly, at a degree of hydrolysis (DH) of 12 and 15%, > 90% nitrogen solubility was observed at all experimental pH values tested. The emulsifying activity index (EAI) and emulsion stability index (ESI) of both hydrolysates at different DHs decreased ($p < 0.05$) with increasing DH. At low DH (5%), hydrolysates exhibited strong emulsifying properties. All THPHs produced by the A21 proteases generally showed higher antioxidative activity than that of the Alcalase protein hydrolysates. The highest DPPH radical-scavenging activity (78 ± 2.1% at 3 mg/mL) was obtained with a DH of 15%. The IC_{50} value for the β-carotene bleaching assay was 0.5 ± 0.03 mg/mL. Alcalase (DH = 12%) and A21 (DH = 15%) protein hydrolysates contained glutamic acid/glutamine and arginine as the major amino acids, followed by lysine, aspartic acid/asparagine, histidine, valine, phenylalanine, and leucine. In addition, the THPHs had a high percentage of essential amino acids, which made up 50.52 and 50.47%, of the protein hydrolysates obtained by the Alcalase and A21 proteases, respectively. Therefore, THPHs can be used as a promising source of functional peptides with antioxidant properties.

Keywords: protein hydrolysate, tuna heads, protease, antioxidant activity, functional properties

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1. Introduction

Fish processing by-products and under-utilized species are commonly recognized as low-value resources with negligible market value. Additionally, inappropriate disposal is a major cause of environmental pollution. Hydrolysis processes have been developed to convert under-utilized fish and fish by-products into marketable and acceptable forms [1,2], that can be widely used in food rather than as animal feed or fertilizer [3].

Besides nutritional quality, functional properties of proteins are also important for food product formulations. The importance of these properties varies with the type of food product in which the protein is used. As most native proteins do not show functional properties desirable for the food industries, their modification for improvement of these properties, particularly, solubility, need to be addressed [4]. Enzymatic hydrolysis is known to improve functional properties of dietary protein without affecting their nutritive value by converting them into peptides with desired size, charge, and surface properties [4-6].

Besides their functionalities, protein hydrolysates from different sources, such as whey, soy protein [7], egg-yolk [8], prawn [9], tuna cooking juice [10], yellow fin sole frame [11], Alaska Pollack frame [12], herring [13], mackerel [14], and capelin [15] have antioxidant activity.

Many synthetic antioxidants, such as buthylated hydroxyanisole and butylated hydroxytoluene, are used as food

additives to prevent deterioration. Although these synthetic antioxidants show stronger antioxidant activity than that of natural antioxidants such as tocopherol and ascorbic acid, there is concern about their health safety [16]. Therefore, the development of natural antioxidants as an alternative to synthetic antioxidants is of great interest among researchers. Vitamin C, α-tocopherol, and phenolic compounds, which are present naturally in vegetables, fruits, and seeds, reduce oxidative damage associated with many diseases. The ability of phenolic substances including flavonoids and phenolic acid to act as antioxidants has been extensively investigated [17-19].

A number of studies have demonstrated that protein hydrolysates from marine organisms act as potential antioxidants, such as jumbo squid [20,21], oyster [22], blue mussel [21,23], hoki [12,20,24], tuna [25,26], cod [27], Pacific hake [28], capelin [15], scad [29], mackerel [14], Alaska pollack [12,30], conger eel [31], yellow fin sole [11], yellow stripe trevally [32], and microalgae [33].

In the present study, we investigated some functional properties and antioxidant activity of enzymatically prepared tuna heads protein hydrolysates (THPHs) using Alcalase and *Bacillus mojavensis* A21 proteases.

2. Materials and Methods

2.1. Tuna sample preparation

Bluefin tuna (*Thunnus thynnus*) was provided by the Institut Nationale des Sciences et Technologie de la Mer, Centre de Mahdia, Tunisia. It is neither a warm blooded nor a cold-blooded fish and can live for up to 40 years, and grow to over 4 meters in length and 600 kg in weight. The bluefin tuna used in this work were about 2.5 meters long and weighed about 350 kg. Immediately after catch, the heads were removed using an electrical saw. Approximately 2 h later, the heads were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w), and transported to the research laboratory within 30 min. Once received in the laboratory, the fish heads were immediately ground twice using an industrial grinder (FATOSA P 130, Hachoir E130; Technocarne, La Wantzenau, France) at medium speed for 30 min. The mixed heads were frozen again at -20°C for 24 h.

2.2. Enzymes

The enzymes used were *B. mojavensis* A21 proteases [34] and Alcalase from *B. licheniformis* (Novozyme).

2.3. Production of THPHs using the proteases

Mixed *T. thynnus* heads (500 g), in 1,000 mL distilled water were cooked at 90°C for 20 min to inactivate endogenous enzymes. The cooked head protein sampled was then homogenized in a Moulinex blender for about 2 min. The samples were adjusted to optimal pH and temperature for each enzyme; crude enzyme from A21 (pH 10.0; 50°C), Alcalase (pH 8.0; 50°C). The alkali protease from *B. mojavensis* A21 was prepared in our laboratory according to Haddar *et al*. [34]. Protease activity was measured by the method of Kembhavi *et al*. [35] using casein as a substrate. A 0.5 mL diluted aliquot of protease was mixed with 0.5 mL 100 mM glycine-NaOH (pH 10.0) or Tris-HCl (pH 8.0) containing 1% casein, and incubated for 15 min at 60°C. The reaction was stopped by the adding 0.5 ml trichloroacetic acid (20%; w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 × *g* using a microcentrifuge (MIKRO20, Hettich Zentrifugen, Tuttlingen, Germany) for 15 min to remove the precipitate. The acid-soluble material was estimated spectrophotometrically at 280 nm (T70 UV/VIS spectrophotometer, PG Instruments, Beijing, China). A standard curve was generated using solutions of 0 ~ 50 mg/L tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used. The enzyme was added to the reaction at the same enzyme/substrate ratio (*E/S* = 3U/mg) to compare hydrolytic efficiencies. During the reaction, the pH of the mixture was maintained constant by continuously adding 4 M NaOH solution. After the required digestion time, the reaction was stopped by heating the solution at 80°C during 20 min to inactivate enzymes. The tuna head protein hydrolysates were then centrifuged at 5,000 ×*g* for 20 min to separate insoluble and soluble fractions. Finally, the soluble phase was freeze-dried using a freeze-dryer (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France) and stored at -20°C for further use.

2.4. Determination of the degree of hydrolysis (DH)

The DH, defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds in the substrate (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis [36] as given below:

$$\mathrm{DH}(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where *B* is the amount of NaOH consumed (ml) to keep the pH constant during the reaction, *Nb* is the normality of the base, *MP* is the mass (g) of protein (N × 6.25), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}}$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in the fish protein concentrate was assumed to be 8.6 meq/g [36].

2.5. Chemical analysis

Moisture and ash content were determined according to the AOAC standard methods 930.15 and 942.05, respectively [37]. Total nitrogen content of the fish protein hydrolysates was determined by the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by 6.25. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate. The protein and fat contents were expressed on a dry weight basis.

2.6. Antioxidant activity

2.6.1. DPPH radical-scavenging assay

DPPH radical-scavenging activity of the hydrolysates was determined as described by Bersuder *et al*. [38]. A 500 μL aliquot of each sample at different concentrations (0.5 ~ 3 mg/mL) was mixed with 500 μL of 99.5% ethanol and 125 μL of 0.02% DPPH in 99.5% ethanol. The mixture were shaken and incubated for 60 min in the dark at room temperature. Reduction of the DPPH radical was measured at 517 nm using a UV-visible spectrophotometer (T70, UV/VIS spectrophotometer, PG Instruments Ltd, Beijing, China). The DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = \left[1 - \frac{A_{517} \text{ of sample}}{A_{517} \text{ of control}}\right] \times 100$$

The control was assessed in the same manner, except that distilled water was used instead of sample. A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity. Butylated hydroxyanisole (BHA) was used as a positive standard at different concentrations ranging from 0.5 to 3 mg/mL. The test was carried out in triplicate.

2.6.2. Reducing power assay

The ability of the hydrolysate to reduce iron (III) was determined according to the method of Yildirim *et al.* [39]. An 1 mL aliquot of each hydrolysate sample at different concentrations (0.5 ~ 3 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 30 min, followed by adding 2.5 mL of 10% (w/v) trichloroacetic acid. The mixture was then centrifuged at 1,650 × *g* for 10 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride. After a 10 min reaction, the absorbance of the resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The values are presented as the means of triplicate analyses. BHA was used as the positive standard at different concentrations of 0.5 ~ 3 mg/mL.

2.6.3. Antioxidant assay using the β-carotene bleaching method

The ability of the hydrolysates to prevent bleaching of ß-carotene was determined as described by Koleva *et al.* [40]. Briefly, 0.5 mg β-carotene in 1 mL chloroform was mixed with 25 mg of linoleic acid and 200 mg of Tween-40. The chloroform was evaporated under vacuum at 45°C, then 100 mL distilled water was added, and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. An aliquot (2.5 mL) of the β-carotene-linoleic acid emulsion was transferred to tubes containing 0.5 mL of each sample at different concentrations (0.5 ~ 3 mg/mL). The tubes were immediately placed in a water bath and incubated at 50°C for 2 h. Thereafter, the absorbance of each sample was measured at 470 nm. A control consisted of 0.5 mL of distilled water instead of the sample solution. BHA was used as the positive standard at different concentrations ranging from 0.5 to 3 mg/mL.

2.6.4. Metal-chelating activity

The Fe^{2+}chelating activity was determined using the method of Decker and Welch [41]. One ml of sample solution (2 mg/mL) was mixed with 3.7 mL of distilled water. The mixture was then reacted with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used instead of sample. EDTA was used as the reference. Chelating activity (%) was calculated as follows:

$$\text{Chelating activity (\%)} = \left[1 - \frac{A_{562} \text{ of sample}}{A_{562} \text{ of control}}\right] \times 100$$

2.7. Functional properties of tuna head protein hydrolysates

2.7.1. Peptide solubility (PS)

PS was carried out according to Tsumura *et al.* [42] with

slight modifications. Briefly, 200 mg of THPHs were dissolved in 20 mL deionized distilled water, and the pH of the mixture was adjusted to 2.0 ~ 10.0 using 2 N HCl or 2 N NaOH solutions. The mixtures were stirred for 10 min at room temperature (25 ± 1°C) and then centrifuged at 8,000 × *g* for 10 min. After appropriate dilution, the nitrogen content in the supernatant was determined by the Kjeldhal method. The nitrogen solubility of the THPHs, defined as the amount of soluble nitrogen from the total nitrogen, was calculated as follows:

$$\text{Nitrogen solubility (\%)} = \frac{\text{Supernatant nitrogen concentration}}{\text{Sample nitrogen concentration}} \times 100$$

The solubility analysis was carried out in triplicate.

2.7.2. Emulsifying properties

Emulsifying properties were determined according to the method of Pearce and Kinsella [43]. Vegetable oil (10 mL) and 30 mL of 1% (w/v) THPH solution were mixed, and the pH was adjusted to different values from 2.0 to 10.0. The mixtures were homogenized for 3 min. Aliquots of the emulsion (50 μL) were pipetted immediately and then mixed with 5 mL of 0.1% sodium dodecyl sulfate (SDS) solution after 10 min. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer (T70, UV/VIS spectrophotometer). The absorbances measured immediately (A_0) and 10 min (A_{10}) after forming the emulsion were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows:

$$\text{EAI } (m^2/g) = \frac{2 \times 2.303 \times A_{500}}{0.25 \times \text{protein weight (g)}}$$

Emulsion stability was determined by measuring the absorbance at 500 nm in aliquots of the emulsion 30 min after forming the emulsion.

$$\text{ESI (\%)} = [100 - (EAI_{(t=0)} - EAI_{(t=30min)} / EAI_{(t=0)}] \times 100$$

2.8. Statistical analysis

Statistical analyses were performed with Statgraphics ver. 5.1, professional edition (Manugistics Corp., Bethesda, MD, USA) using analysis of variance . Differences were considered significant at $p < 0.05$.

3. Results and Discussion

Protease digestion of tuna head proteins was conducted under the same conditions except we used different optimum pHs for the A21 proteases and Alcalase. Furthermore, to study the effect of the DH on protein recovery and the evolution of functional properties and antioxidant activities, hydrolysates with DH values of 5, 10, and 15% and 5, 10, and 12% were generated by the A21 proteases and Alcalase, respectively.

The proximate composition of the freeze-dried tuna protein hydrolysates is shown in Table 1. The protein hydrolysate had high a protein content (78%) and could be an essential source of proteins. The high protein content was a result of protein solubilization during hydrolysis, the removal of insoluble undigested nonprotein substances, and the partial removal of lipids after hydrolysis [3]. The percentage of solubilized protein depended on the amount of lipids in the raw material [44]. Raw material containing the highest amount of lipids gave the lowest percentage of solubilized protein [44]. The high ash content of the samples was attributed to the addition of alkali required to adjust the pH and its control during the hydrolytic process.

The hydrolysis curve of the tuna head proteins is shown in Fig. 1. The curve showed a high rate of hydrolysis for the first 1 h. The rate of hydrolysis subsequently decreased and the enzymatic reaction reached a steady state phase when no apparent hydrolysis occurred. The DH reached 15 and 12% after 240 min of hydrolysis for the A21 proteases and Alcalase, respectively. The higher DH observed with the A21 proteases indicates higher proteolytic activity of *B. mojavensis* proteases toward tuna head proteins, compared

Table 1. Composition of undigested tuna heads and its hydrolysates

	Moisture (%)	Lipids (%)	Ash (%)	Protein (%)
Tuna heads	69.4 ± 0.4	10.4 ± 0.7	7.9 ± 0.5	12.1 ± 0.3
A21 protein hydrolysates				
DH 5%	2.88 ± 0.4	1.52 ± 0.1	11.9 ± 0.3	76.23 ± 0.7
DH 10%	2.07 ± 0.6	0.88 ± 0.2	12.2 ± 0.4	77.81 ± 0.6
DH 15%	2.25 ± 0.1	0.22 ± 0.02	14.9 ± 1.1	78.56 ± 1.2
Alcalase protein hydrolysates				
DH 5%	3.52 ± 0.1	2.21 ± 0.3	10.6 ± 0.5	73.74 ± 0.5
DH 10%	3.58 ± 0.7	1.38 ± 0.5	11.9 ± 0.6	74.63 ± 0.2
DH 12%	4.02 ± 0.5	1.12 ± 0.5	13.7 ± 0.9	76.57 ± 1.0

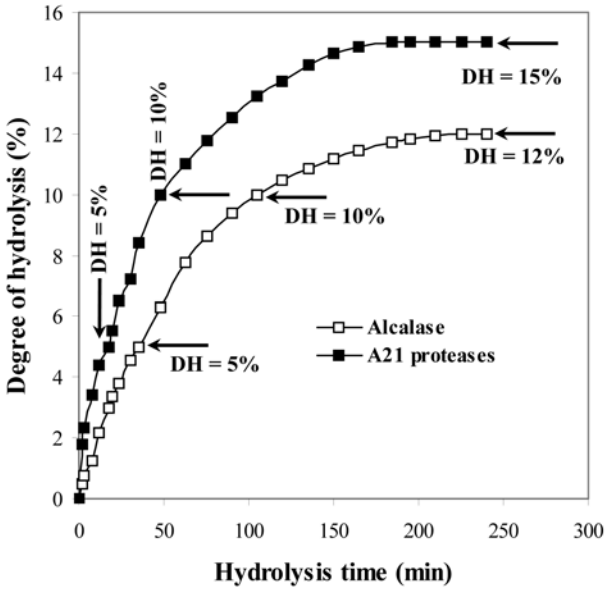

Fig. 1. Hydrolysis curves of tuna heads treated with proteases from *Bacillus mojavensis* A21 and Alcalase.

to that of Alcalase.

3.1. Effect of DH and enzyme type on functional properties of the tuna protein hydrolysates

3.1.1. Solubility of the protein hydrolysates with different DHs

Good protein solubility is required in many functional applications, particularly for emulsions, foams, and gels. Soluble proteins provide a homogeneous dispersible matrix in a colloidal system and enhance interfacial properties [45]. Our results suggested that the high solubility of the protein hydrolysate was due to the size reduction and the formation of smaller, more hydrophilic and more solvated polypeptide units [36]. Additionally, insoluble protein fractions were removed by centrifugation before the protein hydrolysate was freeze-dried. The high nitrogen solubility of the protein hydrolysate indicates potential applications in formulated food systems by providing an attractive appearance and smooth mouth feel to the product [46].

The solubility profiles of the untreated tuna heads protein (UTHP) and THPH as a function of pH are presented in Fig. 2. UTHP was less soluble than the hydrolysates, with solubility < 30% at pH 2 ~ 10. Hydrolysis of the tuna head proteins with A21 proteases (Fig. 2A) and Alcalase (Fig. 2B) increased their solubility. Moreover, solubility increased as a function of DH. At low DH (5%), the solubility increased up to 80% at pHs of 2 ~ 10. Similarly, at DH of 12 and 15%, > 90% soluble nitrogen was observed at all experimental pH values. The solubilities of the A21 and Alcalase hydrolysates were quite low at pH 4, whereas salmon by-

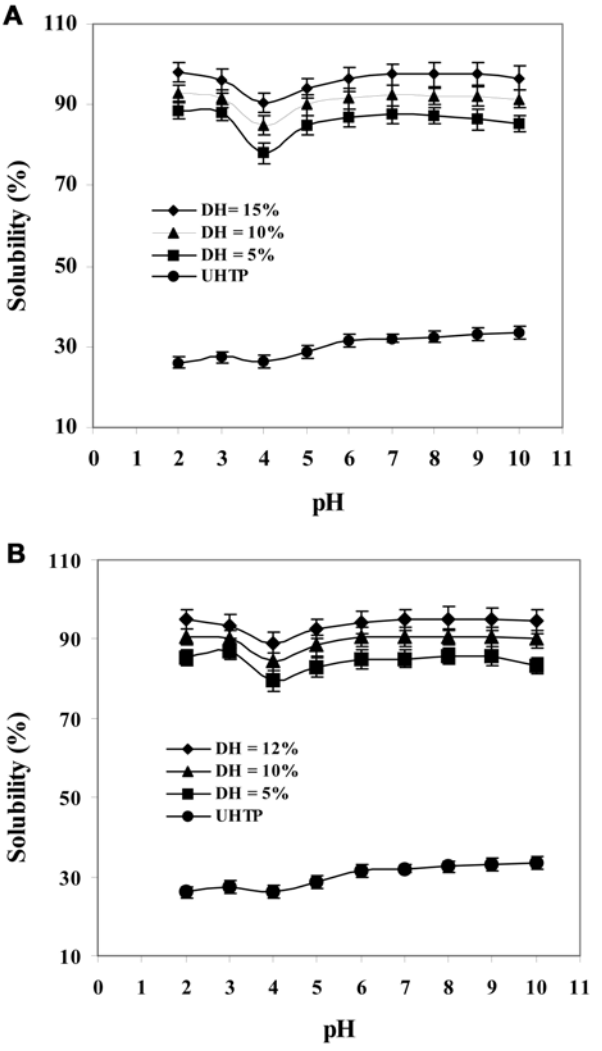

Fig. 2. Solubility profiles of the tuna head protein hydrolysates prepared using proteases from *Bacillus mojavensis* A21 (A) and Alcalase (B) with different degrees of hydrolysis as influenced by pH. UTHP: untreated tuna head protein.

product hydrolysates show the lowest solubility at pH 4 [47]. pH affects the charge on the weakly acidic and basic side chain groups; thus, hydrolysates generally show low solubility at their isoelectric points [48]. Solubility variations could be attributed to both a net charge of peptides that increase as pH moves away from pI, and surface hydrophobicity, that promotes aggregation *via* hydrophobic interaction [49]. The high nitrogen solubility of the protein hydrolysate indicates its potential applications in formulated food systems by providing attractive appearance and smooth mouth feel to the product [46].

3.1.2. Emulsifying properties

The EAI and ESI of both the A21 and Alcalase protein hydrolysates with various DHs are shown in Figs. 3A, 3B, 3C, and –3D. The EAI and ESI of both hydrolysates at

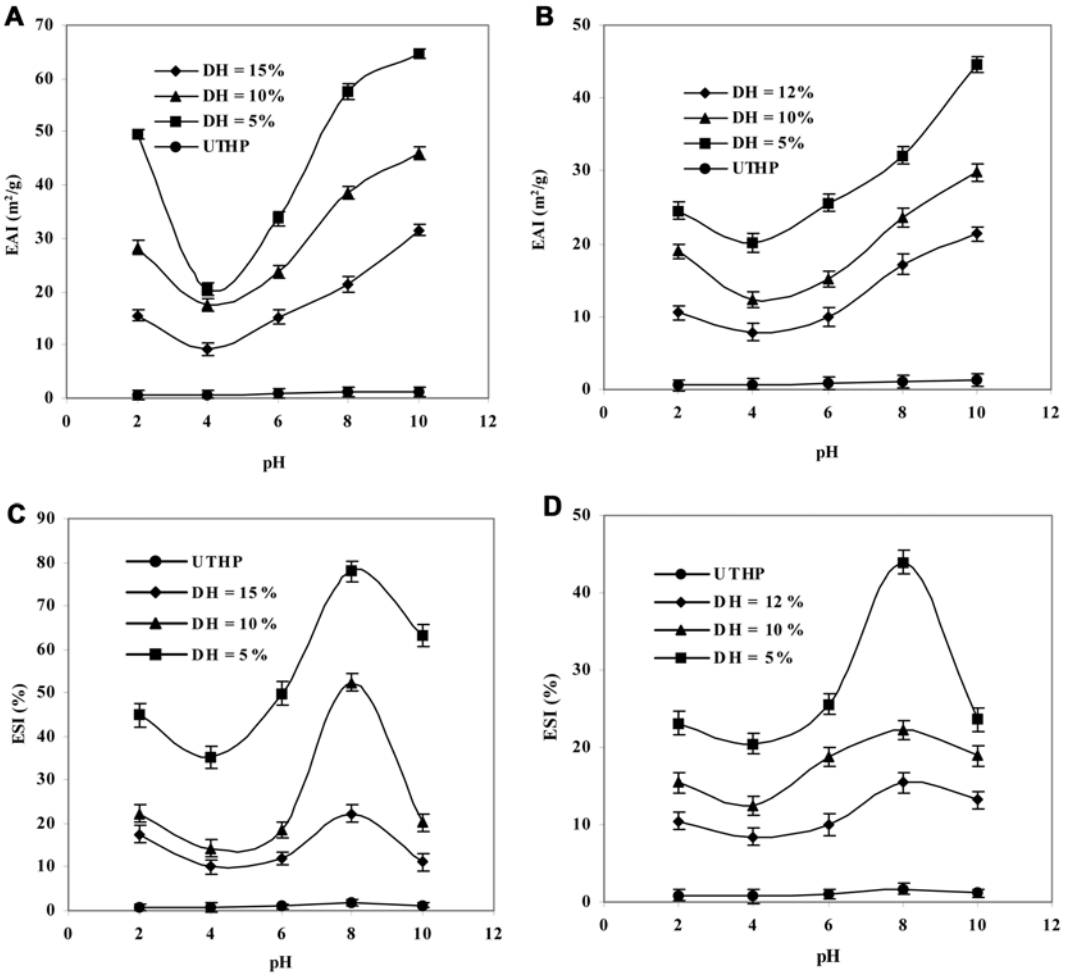

Fig. 3. Emulsifying activity index (EAI) and emulsion stability index (ESI) of the tuna head protein hydrolysates prepared using proteases from *Bacillus mojavensis* A21 (A) and Alcalase (B) at different degrees of hydrolysis as influenced by pH. UTHP: untreated tuna heads protein.

different DHs decreased ($p < 0.05$) with increasing DH. At low DH (5%), the hydrolysates exhibited strong emulsifying properties. With a limited DH, hydrolysates have exceptional emulsifying activity and stability [5]. A higher content of larger molecular weight peptides or more hydrophobic peptides contribute to emulsion stability [50]. In contrast, excessive hydrolysis results in the loss of emulsifying properties [2,5,47]. The mechanism to generate the emulsion system is attributed to the adsorption of peptides on the surface of freshly formed oil droplets during homogenization and the formation of a protective membrane that inhibits oil droplet coalescence [51]. Hydrolysates are surface-active materials and promote an oil-in-water emulsion because of their hydrophilic and hydrophobic groups with their associated charges [47,52]. Thus, hydrolysates with a higher DH had a poorer EAI and ESI due to their smaller peptide size. A direct relationship between surface activity and peptide length was reported by Jost *et al*. [53] and it is generally accepted that a peptide should have a minimum length of 20 residues to possess good emulsifying and interfacial properties. These results are in accordance with those reported by Klompong *et al.* [32] and Wasswa *et al*. [54] who found that the EAI decreased with increasing protein hydrolysis and that this decrease could be attributed to the presence of smaller peptides, which are less effective for stabilizing emulsions. The results in Figs. 3A and 3B also show that the EAI increased with increasing pH. Generally, the EAI increased as the pH moved from pH 4. A higher EAI of the hydrolysates was associated with higher solubility. Hydrolysates with high solubility can rapidly diffuse and adsorb at the interface.

Enzymatic hydrolysis of fish proteins generates a mixture of free amino acids, di-, tri-, and oligopeptides, while increasing the number of polar groups and the solubility of the hydrolysate, and, therefore, modifies the functional characteristics of the proteins, and improves some of their functional qualities and bioavailability. The choice of substrate and proteases employed and the degree to which

the protein is hydrolyzed affects the physicochemical properties of the resulting hydrolysates [55]. Enzyme specificity is important to peptide functionality, because it strongly influences the molecular size and hydrophobicity of the hydrolysate [56]. As reported by Haddar *et al.* [34] the crude extract from *B. mojavensis* A21 showed at least six clear zones on casein zymography, indicating that at least six major proteinases are produced by the strain. These endoprotease and exoprotease activities may offer the ability to achieve a higher DH in tuna heads proteins than Alcalase, which is a serine type endo-protease with very broad substrate specificity.

When considering the effect of pH on the EAI and ESI, the lowest EAI and ESI were found at pH 4, with a coincident decrease in solubility (Fig. 2). As the lowest solubility occurred at pH 4, peptides could not move rapidly to the interface. At the same DH, A21 protein hydrolysates had a better EAI than that of the Alcalase hydrolysates.

3.2. Effect of DH and enzyme type on antioxidant activity

Antioxidative activity of protein hydrolysates depends on the proteases and hydrolysis conditions employed [10,11]. A wide variety of smaller peptides and free amino acids are generated during hydrolysis, depending on enzyme specificity. Changes in size, level, and composition of free amino acids and small peptides affect antioxidative activity [14].

3.2.1. DPPH radical-scavenging activity

DPPH is a stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical is scavenged and absorbance decreases [57]. This radical-scavenging assay is quick, convenient, and reproducible and, thus, is widely used to predict the antioxidant activities of compounds. Figs. 4A and 4B shows the results of DPPH radical scavenging activity of the A21 and Alcalase THPHs at various concentrations. The A21 protein hydrolysate with a 15% DH exhibited the highest DPPH radical-scavenging activity ($p < 0.05$) (87% at 3 mg/mL) (Fig. 4A). As the DH increased, DPPH radical scavenging activity of the THPHs increased ($p < 0.05$). At low DH (5%), the A21 hydrolysate exhibited a better DPPH radical scavenging activity than did the Alcalase hydrolysate. However, Klompong *et al*. [32] found no differences in DPPH radical scavenging activities for a yellow stripe trevall protein hydrolysate prepared with Flavourzyme at different DHs ranging from 5 to 25%. Hydrolysates contain peptides and proteins, which are hydrogen donors that react with radicals to convert them to more stable products, thereby terminating the radical chain reaction [58].

The IC_{50} values were determined. A lower IC_{50} indicates higher free radical-scavenging ability. The hydrolysate at 15% DH obtained by treatment with the A21 proteases was the most active radical-scavenger ($IC_{50} = 0.7 \pm 0.02$ mg/mL) followed by the Alcalase protein hydrolysate at 12% DH ($IC_{50} = 2 \pm 0.12$ mg/mL) (Fig. 4B). These results suggest that the THPHs probably contained peptides, which are electron donors that could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

3.2.2. Reducing power

The presence of antioxidants in tested samples results in

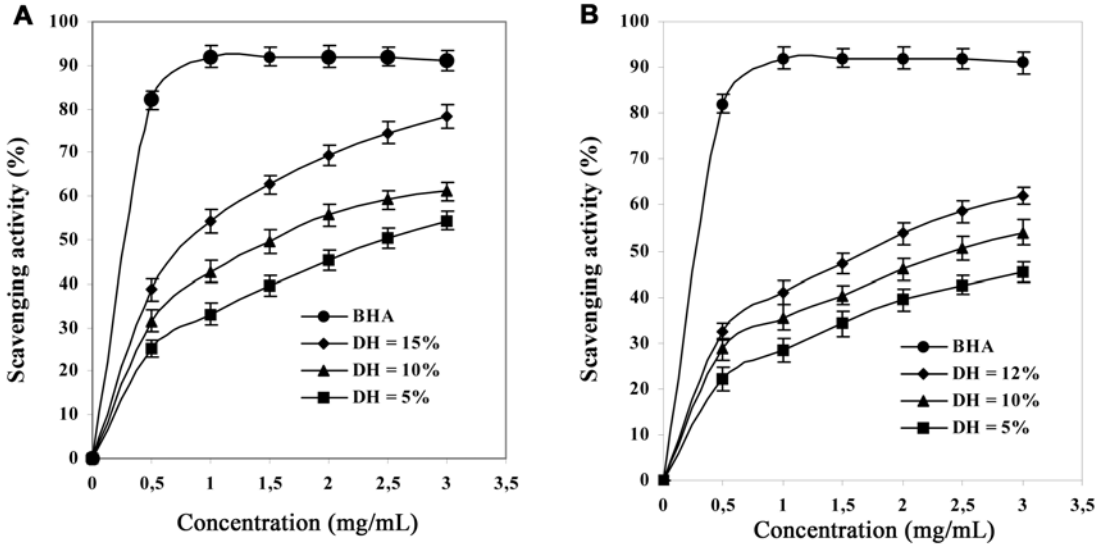

Fig. 4. DPPH scavenging activity of the tuna head protein hydrolysates prepared using proteases from *Bacillus mojavensis* A21 (A) and Alcalase (B) at different concentrations as influenced by the degree of hydrolysis. BHA was used as a positive control.

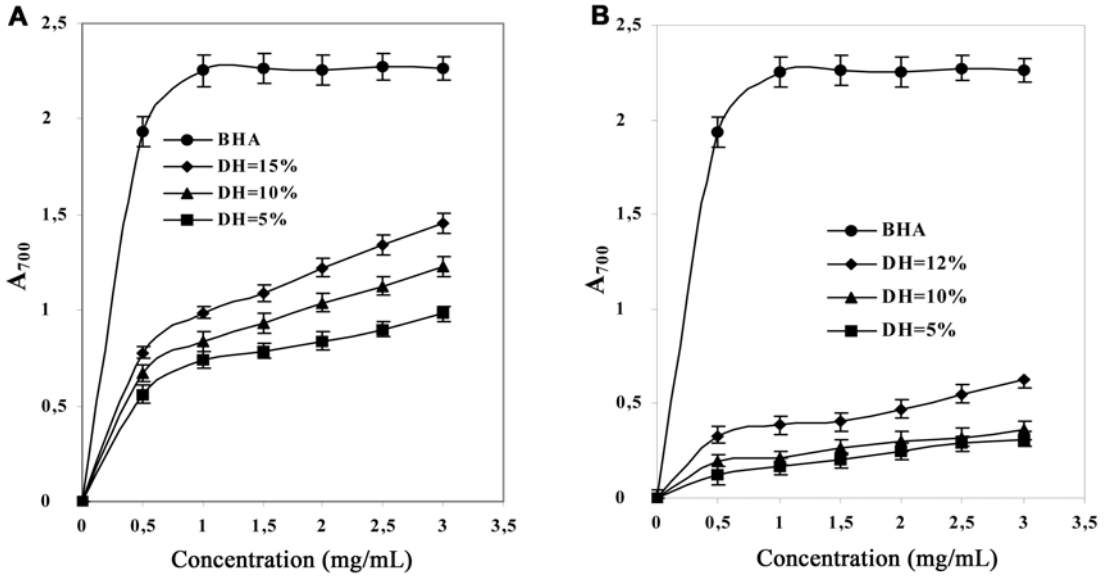

Fig. 5. Reducing power of the tuna protein hydrolysates prepared using proteases from *Bacillus mojavensis* A21 (A) and Alcalase (B) at different concentrations as influenced by the degree of hydrolysis. BHA was used as a positive control.

reducing the Fe^{3+}/ferricyanide complex to the ferrous form and, thus, can be used to evaluate potential antioxidant activity [59]. Figs. 5A and 5B shows that the A21 and Alcalase protein hydrolysates exhibited a dose-dependent increase in reducing power. The reducing power was correlated with the DH. The highest activity (1.45 ± 0.005 at 3 mg/mL) was observed for the A21 protein hydrolysate with a DH of 15%, whereas the lowest (0.72 ± 0.012 at 3 mg/mL) was exhibited by the Alcalase hydrolysate at a DH of 5%. Within the concentration range of 0.5 ~ 3.0 mg/mL, the reducing power of the Alcalase hydrolysate at different DHs was lower than that of the A21 hydrolysate. Thus, the protein hydrolysate from tuna heads obtained by the A21 treatment functioned by donating electrons to the free radicals.

3.2.3. Antioxidant activity measured by the β-carotene bleaching method

The antioxidant assay using the discoloration of β-carotene is widely used to measure the antioxidant activity of

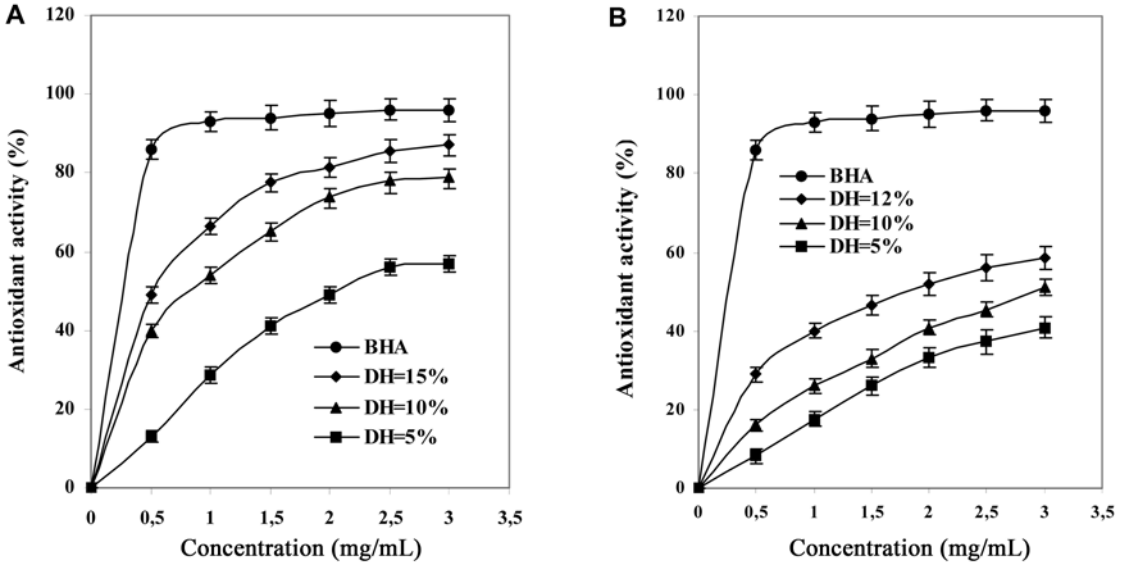

Fig. 6. Determination of antioxidant activity using the β-carotene bleaching method of tuna protein hydrolysates prepared using proteases from *Bacillus mojavensis* A21 (A) and Alcalase (B) at different concentrations as influenced by the degree of hydrolysis. BHA was used as the positive control.

bioactive compounds, because β-carotene is extremely susceptible to free radical-mediated oxidation of linoleic acid [60]. Furthermore, β-carotene is used as a coloring agent in beverages, and discoloration markedly reduces the quality of these products. In this test, β-carotene undergoes rapid discoloration in the absence of antioxidant, which results in a reduction in absorbance of the test solution over time. The presence of antioxidant hinders the extent of bleaching by neutralizing the linoleic free radical and linoleic hydroperoxyl radicals formed. The antioxidant activities of the THPHs as measured by β-carotene bleaching are shown in Figs. 6A and 6B. When treated with the A21 proteases at different DHs, the THPHs showed a higher ability to prevent β-carotene bleaching of than that of the Alcalase protein hydrolysates.

In addition, among the A21 hydrolysates resulting from various DH, the highest antioxidant activity was observed in hydrolysates with a 15% DH (IC_{50} = 0.5 ± 0.03 mg/mL) (Fig. 6A). Furthermore, inhibition of β-carotene bleaching by all hydrolysates was lower than that obtained with BHA (92.5%). These results indicate that the THPHs had strong effects against β-carotene discoloration. The results indicate that THPHs obtained by treatment with A21 proteases exhibited antioxidant activities against β-carotene discoloration.

3.2.4. Metal chelating activity

Metal chelating activities of the THPHs at various DHs were determined. The chelating activity of the hydrolysates increased with hydrolysis time (Figs. 7A and 7B). A21-hydrolyzed tuna head proteins showed high chelating activity ($p < 0.05$), and the 15% DH hydrolysate exhibited the highest metal-chelating activity. Metal-chelating activity could be increased through hydrolysis with certain enzymes. Similar results were reported by Klompong *et al*. [32]. A higher degree of peptide bond cleavage rendered hydrolysates with higher metal-chelating activities. Hydrolyzed protein from capelin also possessed antioxidant activity [15]. Peptides in hydrolysates chelate the pro-oxidants, leading to decreased lipid oxidation. Transition metals, such as Fe, Cu, and Co in foods affect the rate of autoxidation and breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as single-electron donors to form the alkoxyl radical [61]. Therefore, chelation of transition metal ions by antioxidizing peptides retards the oxidation reaction [62]. It was about 78.8 ± 1.5% and 67.5 ± 2.78% mg/mL, respectively, for the A21 and Alcalase protein hydrolysates, at a DH of 15%. This finding suggests that the sequence and composition of amino acids in the peptides of the A21 and Alcalase hydrolysates might be different, leading to differences in the resulting peptides at a particular pH. Ranathunga *et al.* [31] reported that smaller sized peptides derived from muscle

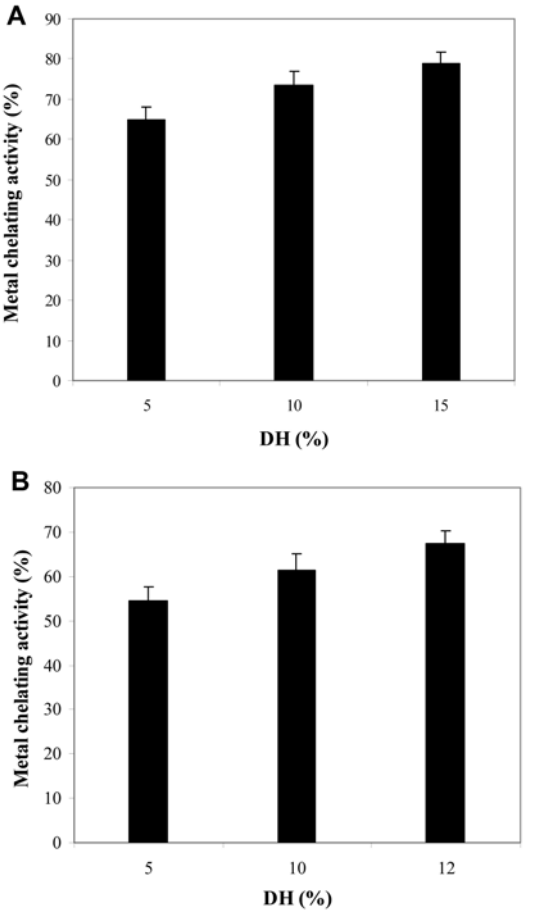

Fig. 7. Metal chelating activity of the tuna protein hydrolysates prepared using proteases from *Bacillus mojavensis* A21 (A) and Alcalase (B) at different concentrations as influenced by the degree of hydrolysis. BHA was used as the positive control.

of conger eel (*Conger myriaster*) may have contributed to higher antioxidative activity in a lipid peroxidation model system. This was expected due to the higher possibility of smaller antioxidant molecules interacting more effectively with free radicals and inhibiting the lipid peroxidation propagation cycles.

3.3. Amino acid composition

The amino acid compositions of the THPHs are shown in Table 2. Both hydrolysates contained glutamic acid/glutamine and arginine as the major amino acids, followed by lysine, aspartic acid/asparagine, histidine, valine, phenylalanine, and leucine. Based on total amino acid content, essential amino acids comprised 50.52 and 50.47% of the Alcalase and A21 protein hydrolysates, respectively. Therefore, these hydrolysates could serve as an excellent source of useful nutrients. The differences in amino acid composition between the hydrolysates depended on the differences in enzyme specificity and the hydrolysis conditions [63]. As presented in Table 2, the total of hydrophobic amino acid

Table 2. Amino acid composition (% mole) of the tuna head protein hydrolysates (THPHs) using Alcalase and the *Bacillus mojavensis* A21 proteases

Amino Acid[a]	Alcalase-THP	A21-THP
Histidine[b]	9.52	9.71
Isoleucine[b]	4.83	4.90
Leucine[b]	6.48	6.06
Lysine[b]	10.23	10.35
Methionine[b]	3.62	3.78
Phenylalanine[b]	6.18	6.16
Tyrosine	5.44	5.29
Threonine[b]	2.17	2.11
Tryptophan	nd	nd
Cysteine	nd	nd
Arginine	11.53	11.42
Valine[b]	7.49	7.40
Aspartic acid	9.91	9.97
Glycine	3.32	3.51
Alanine	2.88	2.94
Serine	5.18	5.09
Glutamic acid	11.22	11.31
TAA[c]	100	100
THAAc	34.8	34.75
TEAA/TAA %	50.52	50.47

THP: Tuna heads protein.
[a]The aspartic and glutamic acid contents included asparagine and glutamine respectively.
[b]Essential amino acids.
[c]TAA, Total amino acids.
THAA Total hydrophobic amino acids.
TEAA Total essential amino acids.
n.d, tryptophan and cysteine were not determined.

content of the THPHs obtained at DHs of 12 and 15% with Alcalase and the A21 proteases was higher and accounted for 34.8 and 34.75% of the total amino acids, respectively. Amino acids in THPHs are possibly involved in antioxidative activity. Amino acids exhibit antioxidant activity; tryptophan and histidine show high antioxidative activity in comparison with methionine, cysteine, glycine, and alanine [64]. Antioxidative activity of histidine or a histidine containing peptides may be attributed to the chelating and lipid radical-trapping ability of the imidazole ring, whereas the tyrosine residues may act as potent hydrogen donors [12]. Aromatic amino acids are generally considered to be effective radical scavengers, because they donate protons easily to electron-deficient radicals. At the same time, their antioxidative stability remains *via* the resonance structure [21]. The THPHs had high nutritional value, based on their amino acid profiles.

4. Conclusion

The antioxidant activity of THPHs varied with DH and enzymes used. The solubility and emulsifying properties of the protein hydrolysates were also dictated by both these factors. Additionally, the functionality of the hydrolysates was affected by pH.

Our results indicate that *B. mojavensis* proteases are suitable for preparing protein hydrolysates from tuna heads by-products. About 80% nitrogen solubilization and a 15% DH were achieved after 4 h of hydrolysis. The A21 protein hydrolysate exhibited high antioxidant activity and notable functional properties.

We conclude that the antioxidative THPHs may be useful ingredients for food and nutraceutical applications.

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