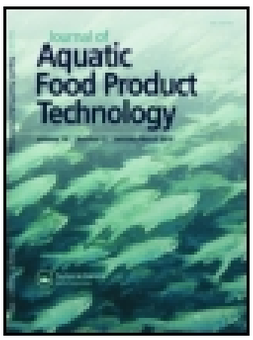

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Bioactivity and Functionality of Gelatin Hydrolysates from the Skin of Oneknife Unicornfish (*Naso thynnoides*)

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ABSTRACT

Skin gelatin of oneknife unicornfish (*Naso thynnoides*) was hydrolyzed using a crude protease from *Bacillus sp*. under optimum hydrolysis conditions. The resulting hydrolysate was subjected to centrifugal ultrafiltration to produce fractions of ≤10 KDa molecular weight. Antioxidant, antihypertensive, and functional properties of the hydrolysate fraction were determined. Results showed that 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (63%) and ferric reducing antioxidant power (25.90 trolox equivalent (mM mg^{-1}) increased with increasing protein concentration in the fraction. Angiotensin-converting enzyme (ACE) inhibitory activity was directly proportional to the protein concentration, with the highest value obtained at 33.97% and IC_{50} determined to be 10.17 µg mL^{-1}. Gel electrophoresis revealed that the gelatin hydrolysates contained mostly peptides, with a molecular weight ranging between 5 KDa and 30 KDa. Amino acid profile of the hydrolysates showed that it is rich in residues of glycine (Gly) (40.70%) and glutamic acid+glutamine (Glx) (25.40%). The hydrolysate was soluble over a wide pH range (79.38–97.12%). Foaming properties increased, while emulsion properties decreased with increasing concentration of the gelatin hydrolysates. The results of the present study revealed the potential of the oneknife unicornfish gelatin hydrolysates as a food ingredient with antioxidant and antihypertensive properties.

KEYWORDS

Fish skin gelatin; hydrolysates; bioactive compounds; functional properties

Introduction

Processing of fish and other marine organisms includes the removal of unneeded parts, such as skin, bones, bone frames, viscera and scales, which are estimated to be about 20–80% of the starting raw material (Caruso 2015; Ghaly et al. 2013; Shaviklo et al. 2017). These by-products account for fish processing discards of approximately 7.3 million tons annually (Ug et al. 2018). These are often processed into fish meal, fish silage, and fertilizers and have low market and economic value (Lopez-Enriquez et al. 2016; Silva et al. 2014).

Efforts to utilize these wastes as a source of functional compounds like collagen and gelatin have been shown in some studies. Approximately 30% of skin and bones from the fish filleting process is collagen, which can be further processed into gelatin (Mahmood et al. 2016). Studies of gelatin production from fish sources have been focused on its potential to substitute bovine and porcine-derived gelatin. Although considered as a healthy alternative, gelatin from fish is just a portion at 1.5% of the total annual world gelatin production from other sources (Hue et al. 2017). This has been attributed to its inferior quality and characteristics (Karayannakidis and Zotos 2016).

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Protein hydrolysate is one of the valuable products that can be derived from fish skin collagen and gelatin. The production of hydrolysates involves controlled hydrolysis treatment to further break down collagen/gelatin molecules that, during the process, cleave the peptide chains into smaller fragments (Petrova et al. 2018). For the production of hydrolysates, gelatin is widely preferred over pure collagen due to high-cost requirements if a large amount of collagenases is needed (Mohammad et al. 2015).

The conversion of fish protein, specifically collagen and gelatin, into hydrolysates involves the hydrolytic action of proteases. Hydrolysis by enzyme action is widely applied, specifically in the food industry, due to its ability to improve and upgrade the nutritional and functional properties of the fish proteins (Chalamaiah et al. 2012). Enzymatic treatment of proteins has several advantages, such as controlled reaction, minimal by-product formation, and milder processing conditions (Mohammad et al. 2015).

Gelatin hydrolysates of marine origin produced through enzyme action have been reported to have biologically active peptides. Collagenous fish by-products from processing plants have been found to be valuable sources of peptide hydrolysates with bioactive properties exhibiting antioxidative, antihypertensive, anti-thrombotic, and immunomodulatory activities (Slizyte et al. 2016). In addition to its bioactivity, hydrolysis of protein improves its functional properties, such as stability, solubility, foaming, and emulsifying capacities, which governs its performance and behavior in food systems during processing, storage, and consumption (Benjakul et al. 2014).

A viable source of fish processing discards for collagen and gelatin is surgeonfish, including unicornfish belonging to the Acanthuridae family, which is highly distributed in the waters of the Indo Pacific area and is one of the commercially important fishes of the Philippine fishing industry (Carpenter et al. 2017). Usually, surgeonfish is processed to produce fillets, leaving behind the skin that could be hydrolyzed enzymatically (Alolod and Nuñal 2018). Thus, this study aims to determine the bioactivities and functional properties of gelatin hydrolysates from the skin of oneknife unicornfish (*Naso thynnoides).*

Materials and methods

Raw material

The skin of oneknife unicornfish was collected from the dried fish processor of Culasi, Antique, Philippines. Samples were frozen and transported to the laboratory to be cleaned, cut into small pieces (approximately 0.5 × 0.5 cm), packed in polyethylene bags, and stored at −20°C until used.

Extraction of gelatin from fish skin

Gelatin was extracted from the cleaned fish skin following the method of Razali et al. (2015) with slight modification. Approximately 30 g of fish skin was mixed with 120 mL of 0.15 M acetic acid (Macron Fine Chemicals, Thailand) for 1 h at 4°C. The acid was drained, and the skin was rinsed twice with 150 mL distilled water and later mixed with distilled water at a ratio of 1:6 (skin/water). The mixture was stirred for 3 h in a water bath (Kotterman) at 60°C, and the extract was filtered with two layers of cheesecloth. The collected liquid fraction was then freeze-dried.

Preparation of fish skin gelatin hydrolysates

Unicornfish skin gelatin hydrolysates, herein referred to as hydrolysates, were prepared according to the method of Lassoued et al. (2015) with slight modification. For the hydrolysis, crude bacterial protease from *Bacillus sp*. was obtained from the Enzyme Laboratory of the University of the Philippines Los Baños (UPLB), Los Baños, Laguna, Philippines. The enzyme preparation was determined to have 100 U mL^{-1} enzyme activity and a neutral pH optimum.

Optimum conditions for hydrolysis were adapted from Alolod and Nuñal (2018), as follows: 2.11% enzyme concentration (%v/v), 137.45 min hydrolysis time, and 47.60°C hydrolysis temperature, leading to a 29.12% degree of hydrolysis predicted value.

Determination of amino acid composition of the hydrolysates

Analysis of the amino acid composition of the hydrolysates was performed following the method standardized by Shimadzu Corp. (Kyoto, Japan) using Shimadzu LC-10A High-Performance Liquid Chromatograph Amino Acid Analysis system (HPLC-AAA) with 18 amino acid standards. The amino acid composition of the hydrolysate sample (AA%) was expressed as residues per 100 amino acid residues using the equation below:

$$\text{AA\%} = \frac{\text{Concentration of an individual amino acid (mM)}}{\text{Concentration of total amino acids tested (mM)}} \times 100$$

Determination of molecular weight of the hydrolysates

Molecular weight of the hydrolysates was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) using a 4% stacking gel and 10% separating gel. Hydrolysate solution (20 mg mL^{-1}) was added with sample loading buffer (Bio Rad Laboratories, Inc., Hercules, CA, USA) at an equal amount, heat-denatured at 100°C for 5 min and run in a Mini Protean unit (BioRad Mini Protean Tetra System) at 25 mA/gel and 150 V. Protein bands were stained for 2 h using Coomassie Brilliant Blue R-250 staining solution (Bio Rad Laboratories, Inc.). The molecular weight of the hydrolysates was estimated using Precision Plus Protein^{TM} Dual Extra Standards (Bio Rad Laboratories, Inc.).

Fractionation of the hydrolysates

Hydrolysates (2 mg protein mL^{-1}) were fractionated using Amicon® Ultra-15 centrifugal filter devices with <10 KDa molecular weight cut off (MWCO) (Merck Millipore Ltd, Germany) following the method of Choonpicharn et al. (2015). The devices were centrifuged at 805 x g for 30 min. The collected filtrate was used for antioxidant activity, angiotensin–I converting enzyme (ACE-I)-inhibitory activity, and stability assays. The samples for these assays were prepared by diluting the collected fractions to obtain varying protein concentrations (0.92 μg mL^{-1}, 1.83 μg mL^{-1}, 2.75 μg mL^{-1}, 3.67 μg mL^{-1}, and 4.59 μg mL^{-1}) based on the initial concentration determined through the Lowry method.

DPPH radical scavenging activity of the hydrolysates

The scavenging effect on a,a-diphenyl-b-picrylhydrazyl (DPPH) free radical was measured using the method of Sae-Leaw et al. (2016) with slight modification. Trolox (Sigma Corp., St. Louis, MO, USA), a vitamin E derivative, was used as the positive control. Antioxidant activity was expressed as DPPH radical scavenging activity (%RSA) calculated using the following equation, where A is the absorbance of the sample, and B is the absorbance of the blank:

$$\text{Radical Scavenging Activity \%} = [(B - A)/B] \times 100$$

Ferric reducing antioxidant power (FRAP) of the hydrolysates

The capacity of the hydrolysates to convert Fe^{3+} into Fe^{2+} was evaluated using the method of Song et al. (2015) with slight modification. A standard curve of Trolox with a concentration range of 10 to

100 µM was prepared. Antioxidant activity was expressed as mmol Trolox equivalents per mg of protein (TE mM mg^{-1}) sample.

Determination of the ACE-I inhibitory activity of the hydrolysates

Angiotensin–I converting enzyme inhibitory activity was assayed following Nasri et al. (2013) with slight modification. ACE-I inhibitory activity was calculated using the equation below, where A is the absorbance of the sample, and B is the absorbance of the blank. IC_{50} value, defined as the hydrolysates' concentration (µg mL^{-1}) required to reduce 50% of ACE-I activity under experimental condition, was also calculated.

$$\text{ACE}-\text{I Inhibition}\,\% = [(\text{A} - \text{B})/\text{B}]\,\text{x}\,100$$

Determination of the pH and thermal stabilities of the hydrolysates

Determination of the stability of the hydrolysates over a range of pH was performed according to Ketnawa et al. (2017) with slight modification. The pH of the hydrolysate fraction (<10 KDa) was adjusted to different values (1, 3, 5, 7, 9, and 11) using 1 M HCl or 1 M NaOH, and samples were incubated at room temp for 1 h. Thereafter, the pH of the solution was adjusted back to neutral. Residual DPPH scavenging and ACE-1 inhibitory activities, following the method described previously, were calculated relative to the activity of the sample without pH adjustment (% Relative Activity).

Determination of the bioactivity of hydrolysate fraction after prolonged exposure to high temperature was done according to Ketnawa et al. (2017). The tubes containing the hydrolysates were placed in boiling water (100°C) for 0, 15, 30, 60, 120, and 240 min. After heat treatment, the tubes were cooled in ice water. Residual DPPH scavenging and ACE-I inhibitory activities were determined and also calculated in terms of % Relative Activity.

Determination of the solubility of the hydrolysates

To determine the solubility of the hydrolysates, 10 mg of the sample was diluted in 8 mL of deionized water (Nalinanon et al. 2011). The pH of the resulting solution pH was adjusted to different values (1, 3, 5, 7, 9, and 11) by adding 1 M HCl or 1 M NaOH solutions. The mixture was stirred at room temp for 30 min, volume made up to 10 mL with distilled water while maintaining the same pH, and centrifuged at 5,000 x g for 15 min. The supernatant was collected, and the protein content was determined by Lowry method (Lowry et al. 1951). Total protein content of the sample was determined after solubilization of the sample in 0.5 M NaOH. The protein solubility was measured using the equation below:

$$\text{Solubility}\,(\%) = \frac{\text{Protein content in supernatant}}{\text{Total Protein content in sample}}\,\text{x}\,100$$

Determination of the emulsifying and foaming capacities of the hydrolysates

Foam expansion (FE) and foam stability (FS) of the hydrolysates was determined following the procedure of Jridi et al. (2014). Hydrolysate sample volume of 20 mL (V_0) at varying concentrations (0.1%, 0.5%, and 1%) (w/v) was homogenized at 16,000 rpm for 1 min at room temp to incorporate air. After which, the whipped sample was immediately transferred into a 50 mL graduated cylinder, and the total volume was measured (V_1). Foam capacity was expressed as foam expansion after homogenization and measured using the equation FE % = (V_1-$V_{0/}V_0$) x 100. Foam stability was determined as the volume of remaining foam after 30 min at room temperature (V_2), calculated as FS % = (V_2-$V_{0/}V_0$) x 100.

The hydrolysates' emulsion activity index (EAI %) was determined according to Razali et al. (2015). Briefly, the working solution was prepared by mixing 50 mL of hydrolysates (0.1%, 0.5% and 1.0% w/v) and 50 mL sunflower oil. The mixture was homogenized (WiseTis homogenizer, Humanilab Instrument Corp.) for 2 min at a speed of 16,000 rpm to form an emulsion. It was then centrifuged at 89 x g for 5 min. Emulsion activity index was measured following the equation: EAI% = (V_a/V_b) x 100, where V_a is the height of the emulsified layer, and V_b is the height of the total tube content.

Emulsion stability index (ESI%) of the hydrolysates was determined by subjecting the samples to heat treatment at 80°C for 30 min. The samples were then cooled down in the water for 15 min, centrifuged again at 89 x g for 5 min, and the volume was recorded. ESI % was calculated using the equation: ESI % = (V_c/V_b) x 100, where V_c is the height of the emulsified layer after heating, and V_b is the height of the total content before heating.

Statistical analysis

Statistical test was performed using the SPSS 16.0 computer program (SPSS Statistical Software, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was carried out for DPPH radical scavenging activity, FRAP, ACE-I inhibitory, pH and thermal stabilities, protein solubility, foaming properties, and emulsion properties. The difference of means between pairs was resolved by means of confidence intervals using Tukey-b test at a level of significance of $p < .05$.

Results and discussion

Fish skin gelatin yield

Prior to hydrolysis, the gelatin powder yield from unicornfish skin was determined to be 12.99%, which is within the 6–19% range of fish gelatin extraction yield reported in several studies (Alolod and Nuñal 2018; Mahmood et al. 2016). This result is relatively lower than some reported values, which can be attributed to the intrinsic properties of the fish skin raw material since physical and structural characteristics of gelatin are different among fish species (Razali et al. 2015). Also, the variation of the yield also depends on the extraction method used, which includes prior treatment of the raw material, concentration of pre-treatment solvent, pre-treatment time, and temperature (Firdayanti and Supraytino 2016). Furthermore, the lower yield can be due to the possible leaching out of extracted collagen after a series of washing and incomplete hydrolysis (Nurul and Sarbon 2015).

Molecular weight distribution of the gelatin hydrolysates

The molecular weight profile by SDS-PAGE (in triplicate) shown in Figure 1 reveals that the hydrolysates contain bands corresponding to polypeptides below 30 KDa. The band fractions may consist of proteins such as tropomyosin (32–38 KDa) and myosin light chain (25–10 KDa) (Arias-Moscoso et al. 2014; Khiari et al. 2014). This result is in agreement with the molecular weight profile of gelatin/collagen hydrolysates from fan-bellied leatherjacket (Ky et al. 2018) at <28 KDa, Spanish mackerel (Chi et al. 2014) with 6 peptide fractions with molecular weight distribution of 5.04–28.77 KDa, and squid pen (Shavandi et al. 2017) with peptide range of <4.5–9 KDa. Distribution of the molecular weight of the peptides in the hydrolysates in this study was observed to be at 5–30 KDa range. Further, there are slightly visible peptide band smears observed in the range of 20–25 KDa in all replicates.

Unclear peptide bands observed in the electrophoretic profile of the gelatin hydrolysates may be due to the hydrolysates being composed of a wide range of polydisperse products with varying degrees of hydrolysis. These are mostly small peptides that cannot be accurately detected under analytical electrophoresis conditions (Arias-Moscoso et al. 2014). As such, the less intense peptide bands validate the passage of the smallest peptides in the electrophoresis gel.

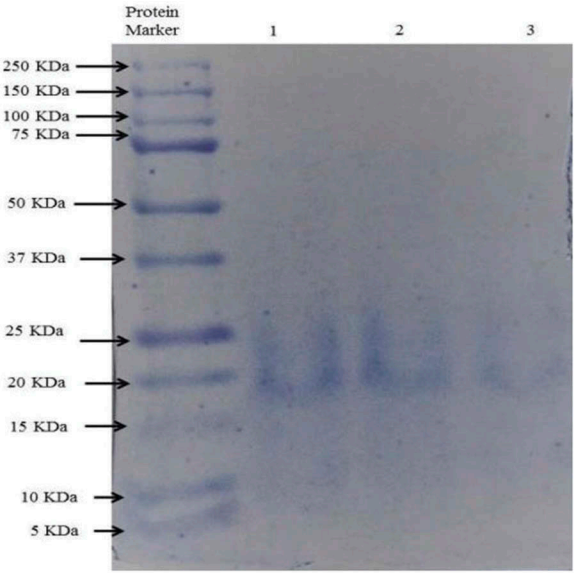

Figure 1. Molecular weight profile of gelatin hydrolysates at 20 mg ml-1 concentration in triplicate (SDS-PAGE).

Amino acid composition

The amino acid composition of gelatin hydrolysates is expressed as residues per 100 amino acids, as shown in Table 1. The amino acid composition of the hydrolysates is a mixture of hydrophilic and hydrophobic amino acid and is rich in residues of glycine (Gly) and glutamic acid+glutamine (Glx), with a considerable percentage of alanine (Ala), proline (Pro), aspartic acid+asparagine (Asx), and cysteine (Cys). In this study, glycine is the amino acid with the highest concentration at 40.70%. This finding was expected since gelatin is primarily composed of the tripeptide Glycine-Proline-Hydroxyproline in which glycine occupies the third position. This makes the helical structure closely packed and results in effective helix formation and stabilization of the spiral structure (Carvalho et al. 2018). This result is also in agreement with the glycine levels (>30%) of the gelatin hydrolysates produced from Amur sturgeon (Nikoo et al. 2015), cuttlefish (Jridi et al. 2014), and milkfish gelatin hydrolyzed by Flavourzyme (Huang et al. 2018)

The second most abundant amino acid (25.40%) in the hydrolysates is Glx, a combination of glutamic acid and glutamine. In the process of HCl pre-treatment, amino acids glutamine and asparagine are converted to glutamic acid and aspartic acid, respectively (Samek et al. 2013). On the other hand, a low amount of imino acid proline was detected (4.36%). Proline is generally lower in fish gelatin as compared to mammalian gelatin, with its presence mainly due to its participation in the helix structure of collagen (Mahmood et al. 2016). These results suggest that the amino acid profile of oneknife unicornfish gelatin hydrolysates resembles the amino acid composition of most fish-derived gelatin and its hydrolysates.

DPPH radical scavenging activity

Gelatin hydrolysates with a molecular weight of <10 KDa produced by ultracentrifugation showed protein concentration-dependent DPPH scavenging activities ranging from 5.5% to 63.53%. As protein concentration increases, radical scavenging activity (%RSA) also increased, with significant differences ($P < .05$) observed among values (Figure 2a). The significantly highest scavenging activity ($P < .05$) was exhibited in a protein concentration of 4.59 μg mL^{-1}. An increasing trend of the radical scavenging activity may be due to the increase in the

Table 1. Amino acid composition (%) of oneknife unicornfish skin gelatin hydrolysates.

Name	Residues/ 100 amino acids (%)
Asx	3.56 ± 0.07
Thr	2.12 ± 0.04
Ser	0 ± 0.00
Glx	25.40 ± 0.49
Pro	4.36 ± 0.08
Gly	40.70 ± 0.70
Ala	8.34 ± 0.19
Cys	6.12 ± 1.73
Val	0.29 ± 0.01
Met	0.58 ± 0.01
Ileu	0 ± 0.00
Leu	1.06 ± 0.02
Tyr	0 ± 0.00
Phe	0.06 ± 0.05
His	0.87 ± 0.00
Lys	0.77 ± 0.28
Arg	6.05 ± 2.93
Trp	0.38 ± 0.08
HAA	21.19

ASX = aspartic acid + asparagine; GLX = glutamic acid + glutamine; hydrophobic amino acids (HAA) alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, proline, tryptophan, methionine and cysteine.

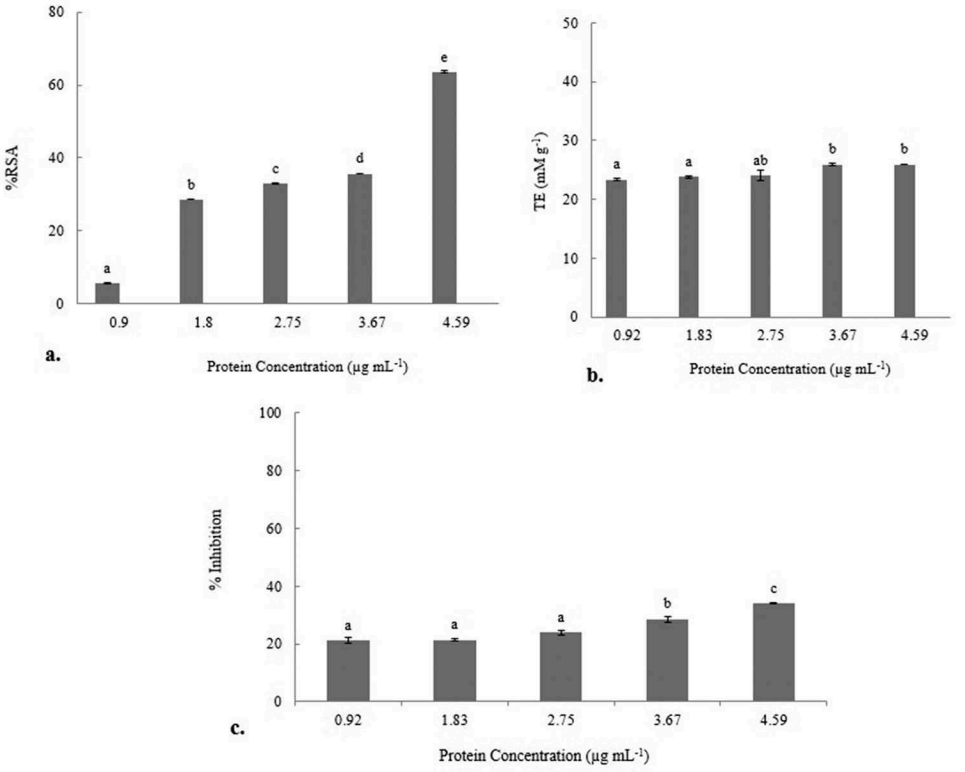

Figure 2. Bioactivity of gelatin hydrolysate fraction (<10 KDa) at increasing protein concentrations; a.) DPPH radical scavenging activity (%RSA), b.) ferric reducing antioxidant power (TE mM mg-1 and c.) ACE-I inhibitory activity.

concentration of active low molecular weight peptides (<10 KDa) present in the fraction sample (Sae-Leaw et al. 2016). The increasing DPPH radical scavenging activity of the hydrolysate fraction with respect to protein concentration may be attributed to the increasing levels of Pro, His, Ala, Val, Leu, Met, and Cys present in the sample, which were reported to enhance the activity of the antioxidative peptides by donating protons into the DPPH free radical (Song et al. 2015). The results of this study are comparable to the findings of Piyadhammaviboon et al. (2012), who reported radical scavenging activity of threadfin bream hydrolysates at a range of 40–55% with 2–3-h hydrolysis time. Also, this is in agreement with Noman et al. (2019), who found that scavenging activity of sturgeon protein hydrolysates increased as protein concentration increased. The results suggest that the DPPH radical scavenging activity of the gelatin hydrolysates is dependent on its protein concentration, as was found in several previous studies (Razali et al. 2015; Sabeena-Farvin et al. 2014) and that its amino acid composition is an important factor for its antioxidant activity (Ulagesan et al. 2018).

Ferric reducing antioxidant power

The ability of the hydrolysate fraction to reduce Fe^{3+} to Fe^{2+} was determined and expressed as reducing power in comparison with Trolox standard. Results showed that there was an increase in the reduction of ferric ions indicated by the increase in the Trolox equivalents (mM g^{-1}) in increasing concentrations of the sample (Figure 2a). It was also observed that the values remain constant (25.90 TE (mM g^{-1}) at the two highest protein concentrations. A significant difference in the values was observed between 0.9 and 1.83 μg mL^{-1} fraction sample concentrations and 3.67–4.59 μg mL^{-1} ($P < .05$).

In all protein concentrations of the hydrolysate fraction, reducing power was in the range of 23.36–25.90 mM TE mg^{-1} protein. The high volume of the sample fraction corresponds to a high concentration of peptides with amino acids acting as electron donors to the free radicals producing much more stable products, thus stopping radical chain reactions (Sai-Ut et al. 2014). The hydrolysis of gelatin may have led to the exposure of hidden amino acid residues and to the release of the sequence of peptides that may contain antioxidant properties (Xu et al. 2017). It has also been reported that the ferric reducing ability and the quantity of acidic and sulphur containing amino acids have a positive relationship (Song et al. 2015). With this premise, the presence of Met, Cys, Asx, and Glx in the fraction sample might have induced its ferric reducing antioxidant power. The results suggest that the ability of the reductants in the <10 KDa fraction of gelatin hydrolysates is concentration-dependent and that the amino acid composition of the hydrolysates influence its ferric reducing antioxidant capacity.

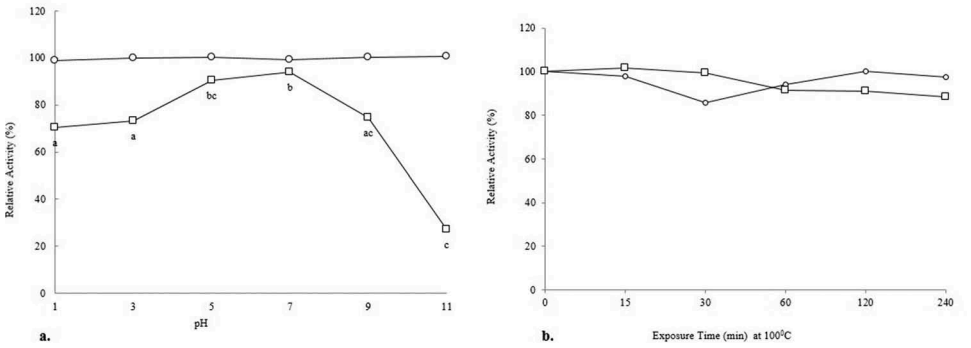

Figure 3. pH (a.) and thermal (b.) stabilities of ACE-1 inhibitory (open square) and DPPH radical scavenging (open circle) activities of gelatin hydrolysate fraction (<10 KDa).

ACE-I inhibitory activity

The ability of the gelatin hydrolysates fraction to inhibit the conversion of Angiotensin I to Angiotensin II is shown in Figure 2c. Percentage inhibition (% inhibition) increased as the protein concentration of the sample increased, with values ranging from 21.19% to 33.97%. No significant differences ($P > .05$) were observed in the inhibition when 0.92, 1.83, and 2.75 μg mL^{-1} protein concentrations were used. At higher concentrations (3.67 μg mL^{-1} and 4.59 μg mL^{-1}), however, significantly higher inhibition ($P < .05$) was observed.

IC_{50} value or the concentration of the fraction required to inhibit 50% of the angiotensin-converting enzyme activity was estimated at 10.18 μg mL^{-1}. This is much lower than the value reported by Mahmoodani et al. (2012) for skin gelatin hydrolysates of Pangasius catfish with same <10KDa molecular weight cut off (124 μg mL^{-1} concentration), Nasri et al. (2013) for goby (*Zosterissesor ophiocephalus*) with 730–1330 μg mL^{-1}, Thuanthong et al. (2017) for Nile tilapia (*Oreochromis niloticus*) gelatin hydrolysates (1200 μg mL^{-1}) and its five fractions (approximately 800 μg mL^{-1}), Lin et al. (2017) for pepsin hydrolyzed tilapia (*Oreochromis spp.*) frame proteins FPHPe 5–10 KDa fraction (830 μg mL^{-1}), and Huang et al. (2018) for milkfish (*Chanos chanos*) gelatin hydrolysates (472–762 μg mL^{-1}). On the other hand, this was much higher than the values presented by Wu et al. (2015) for lizardfish (*Saurida elongata*) hydrolysate fraction. The ACE-1 inhibitory activity of the hydrolysate fraction is due to the presence of shorter peptides (<10KDa) having hydrophobic amino acids (Lee and Hur 2019). In this study, it is emphasized that the lower amount of short peptides present in the sample can efficiently inhibit 50% ACE-I conversion. The obtained results suggest that low molecular weight (<10 KDa) fish skin gelatin hydrolysates from oneknife unicornfish have potent inhibitory peptides and amino acid composition that can inhibit ACE-I activity and that the inhibitory activity is concentration-dependent.

Stability of optimized fish skin gelatin hydrolysates

pH stability

The effect of pH on DPPH radical scavenging and ACE-I inhibitory activities of the hydrolysate fraction is shown in Figure 3a. DPPH radical scavenging activity was stable from acidic to basic pH

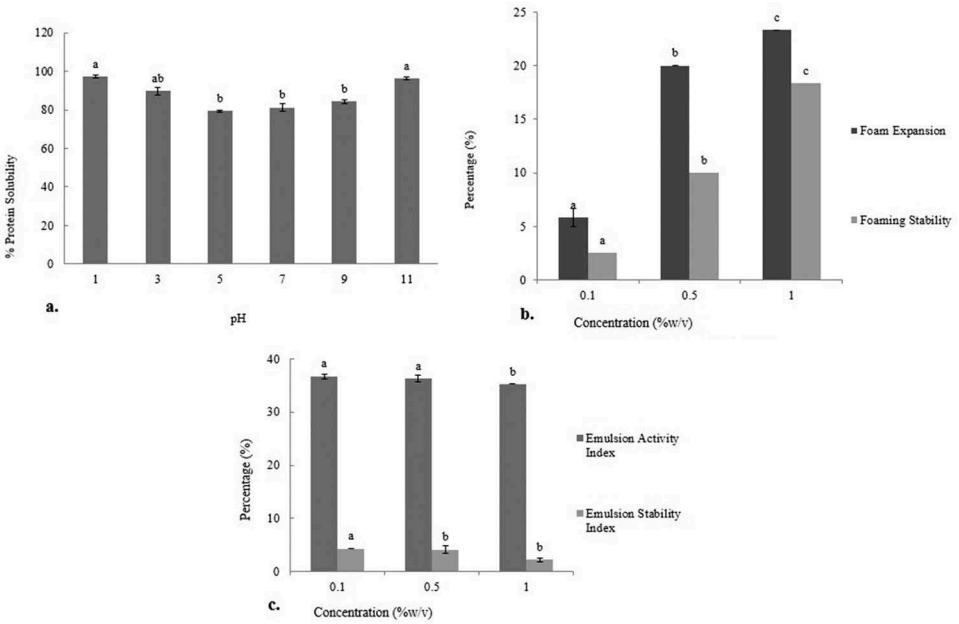

Figure 4. Functionality of gelatin hydrolysates; a.) protein solubility, b.) foaming properties and c.) emulsifying properties.

levels (pH 1–11), with values in the range of 98.17% −100.50% compared to the sample without pH adjustment. There were no significant differences ($P > .05$) in the relative DPPH radical scavenging activities of the sample fraction influenced by pH variation.

These findings are in agreement with the results of Ketnawa et al. (2017) showing that the DPPH radical scavenging activity of giant catfish skin gelatin hydrolysates in the pH range 1–11 was not significantly different ($P > .05$) from that of the sample without pH adjustment. In the present study, the enhanced antioxidant activity of the gelatin hydrolysates fraction can be attributed to the changes in the peptides, particularly at C- and N-termini by pH adjustment (Kittiphattanabawon et al. 2012; Nasri et al. 2013). Thus, this result suggests that fish skin gelatin hydrolysates with low molecular weight peptides can be potentially applied in any food system over pH 1–11 with very minimal loss in its antioxidant activity.

In terms of the ACE-I inhibition, the relative activity (%) of the gelatin hydrolysates fraction significantly increased ($P < .05$) when incubated at pH 1–pH 5 (70.40%- 90.0%), reaching the highest activity of 94.04% at pH 7. However, a further increase in the pH resulted in a significant reduction ($P < .05$) of ACE-1 inhibitory activities. At pH 11, inhibitory activity was significantly decreased ($P < .05$) to 27.13%. Results suggest that the efficiency of the ACE-inhibition of the fraction sample from fish skin gelatin hydrolysates is potent from slightly acidic to neutral pH, which can be incorporated into food products requiring the same pH range during processing.

Thermal stability

The results shown in Figure 3b indicate that the DPPH radical scavenging activity of the sample is minimally lost by 14.29% relative to the control as thermal incubation time is increased up to 30 min. It was also found that radical scavenging activity is slightly increased after 60 min of heating and became stable up to 240 min. This observation may result from the changes in the active amino acids and molecular weight of the hydrolysates during thermal treatment (Ketnawa et al. 2017). Also, the minimal loss in the radical scavenging activity might be attributed to either degradation or aggregation of some antioxidant peptides caused by heat treatment (Yarnpakdee et al. 2014). This is because protein molecules are vulnerable to heat treatment, resulting in aggregation of protein and exposure of hydrophobic domain (Wang et al. 2014). However, smaller size peptides like the <10 KDa fraction sample of fish skin gelatin hydrolysates were found to be more stable to aggregation at high temperatures (Nalinanon et al. 2011; Yarnpakdee et al. 2014). There were no significant differences ($P > .05$) observed among relative activities at differing exposure at 100°C, indicating that the resultant hydrolysate fraction radical scavenging activity is stable at high temperature with prolonged exposure up to 240 min.

Compared to the sample without thermal treatment, there was a calculated insignificant ($P > .05$) loss of 11.48% in the ACE-I inhibition of the fraction sample after heat treatment for 240 min. This suggests that the low molecular hydrolysate fraction satisfactorily maintained stability after heat treatment at prolonged exposure time up to 2 h. This observation is consistent with the work of Fu et al. (2015) on bovine collagen hydrolysates, suggesting similar ACE-inhibitory activity after being subjected to 100°C heat treatment for 2 h. The bioactivity of the resultant bioactive hydrolysates after heat treatment can point its potential incorporation to heat-processed food products.

Protein solubility

The protein solubility of the hydrolysates at various pH (1, 3, 5, 7, 9, and 11) is shown in Figure 4a. The hydrolysates were soluble in water at a wide pH range, in which more than 70% was obtained at a range of 79.38%-97.12% (7.94–9.71 mg mL^{-1}). The observed high solubility over a wide range of pH can be attributed to the enzymatic hydrolysis of gelatin, resulting in major structural change producing low molecular size peptides. The unfolding of gelatin via hydrolysis leads to the exposure of ionizable amino and carboxyl groups of amino acid increasing hydrophilicity resulting in improved solubility (Thamnarathip et al. 2016). Further, the exposed polar amino acids may bind

to water through hydrogen bonding and electrostatic interactions, which could also increase the solubility of the hydrolysates (Muhamyankaka et al. 2013).

Decrease in the solubility was observed from pH 1–5; thereafter, solubility increased again with increasing pH up to pH 11. The hydrolysates were mostly soluble in both extremely acidic (97.12%) and alkaline pH (96.34%), with significant differences ($P < .05$) detected in these pH values. Increase in the protein solubility of the hydrolysates at these pHs may be due to the presence of more ionized protein functional groups that change the pH away from the isoelectric point (pI) of the fish skin gelatin, enhancing water interactions (Galla et al. 2012). The lowest solubility observed at pH 5 may be fish gelatin's isoelectric point, where most of its amino acids are uncharged and therefore precipitated out of the solution, since most proteins have isoelectric point range of 4.5–5.5 (Chaterjee et al. 2015; Noman et al. 2019). Thus, the findings of this study suggest that the pH of the solution is an important factor affecting the behavior of the hydrolysates when it interacts with water, since it showed the lowest solubility at its isoelectric point.

Foaming properties

Foamability of the hydrolysates at varying concentrations (0.1%, 0.5%, and 1%) significantly increased ($P < .05$) from 5.83% to 23.33% when sample concentration increased (Figure 4b). High foam expansion with increasing concentration of protein hydrolysates may be due to a higher number of short peptides having the ability to associate with the air and liquid interface, lowering surface tension and leading to better adsorption (Charoen et al. 2017).

Evaluation of the foam stability after 30 min is presented in Figure 5b. Results showed that there was increasing foam stability (2.5% to 18.33%) when sample concentration also increased. Foaming stability at 1% (w/v) was significantly higher ($P < .05$) among the concentrations tested. The higher foaming stability with increased hydrolysate concentration may be attributed to the formation of stiffer foams (Klomklao and Benjakul 2018).

In this study, the values for the foaming stability are generally low, which can be attributed to the action of enzymatic hydrolysis. Protein hydrolyzed to a limited degree such as the fish skin gelatin hydrolysates improves its foaming expansion. However, the foaming stability was reduced in the present study, which may indicate that the smaller peptides in the hydrolysates may lack the ability to stabilize air cells in the foam (Chaterjee et al. 2015).

Emulsion capacity

Emulsion activity and stability indices of the hydrolysates at various concentrations are shown in Figure 4c. The emulsion activity index (EAI) of the oil-sample solution mixture decreased with increasing hydrolysate concentration and falls within the range of 35.25–36.73%. Emulsifying capacity of the hydrolysates at 1.0% concentration was significantly lower ($P < .05$) than the EAI at higher concentrations. Similarly, emulsion stability (ESI%) after thermal treatment decreased when the concentration of the hydrolysates increased up to 1%. Statistical analysis revealed that ESI% for 0.1% hydrolysate concentration was also significantly lower ($P < .05$). This result is in agreement with the study of Noman et al. (2019) stating that EAI and ESI decrease as protein concentration increases.

Generally, hydrolysates are surface-active materials promoting oil-in-water emulsion because of the presence of hydrophilic and hydrophobic groups with their associated charges (Muhamyankaka et al. 2013). However, emulsion formation and stability of the hydrolysates are mainly governed by the degree of hydrolysis and rate of diffusion of the peptides at the oil–water interface but dependent on the ionic characteristics of the peptides (Chaterjee et al. 2015). The results obtained in this study are in agreement with the findings in eel protein hydrolysates (Jamil et al. 2016).

The deteriorating emulsion activity of the hydrolysates may be attributed to the increase of protein interactions and interferences at the oil–water interface at higher concentrations (Rajabzadeh et al. 2017). Also, enzymatic hydrolysis yielding smaller peptides that are hydrophilic

in nature is more likely to be localized in the aqueous phase, and thus cannot be adsorbed in the interface (Intarasirisawat et al. 2012). These results suggest that emulsion properties of oneknife unicornfish skin gelatin hydrolysates may be influenced by molecular properties, particularly the concentration and size of the peptides.

Conclusion

In this study, bioactivity and functional properties of the hydrolysates produced from the gelatin of the oneknife unicornfish (*Naso thynnoides*) skin were evaluated. Antioxidant and antihypertensive properties of the hydrolysate fraction (<10 KDA MWCO) increased with increasing sample concentration. The hydrolysates have excellent solubility at varying pH foaming and emulsion properties for food and beverage applications. These results suggest that the skin of oneknife unicornfish can be a viable source of gelatin hydrolysates that can be potentially utilized as an ingredient with antioxidant and antihypertensive capacities for developing functional foods, dietary supplements, and pharmaceuticals.

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Conflict of interest

The authors declared that they have no conflict of interest.

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