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Original Article

Valorizing fish processing waste: Production of protein hydrolysates from milkfish *(Chanos chanos)* by-products using acid hydrolysis♣

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Abstract

Milkfish (*Chanos chanos*) by-products were used as raw material for the production of protein hydrolysates. Acid hydrolysis was performed at 121 °C for 90 min at 15 psi using various concentrations of hydrochloric acid (4, 6, and 8M). The protein hydrolysates were characterized for the degree of hydrolysis (DH), and antioxidant and other functional food properties. The yield obtained ranged from 5.14±0.42% to 6.08±1.53%. High DH was observed at a high acid concentration with 43.88±9.50% DH for 8M HCl. Regarding the functional food properties, solubility of over 80% over a wide range of pH (2-12) was observed, and emulsifying and foaming properties were found to depend on the pH (2-10). As for the antioxidant activity, 8M exhibited the highest antioxidant activity among the three treatments. The results showed that milkfish by-products have potential to serve as raw material for protein hydrolysates that can be used as ingredients for food formulations.

Keywords: milkfish by-products, protein hydrolysates, circular economy

1. Introduction

Milkfish (*Chanos chanos*), locally known as bangus, is one of the most important fish commodities in the Philippines. It can be cultivated in freshwater, brackish water, and marine environments. Besides being a good source of nutritious proteins, it is popular in aquaculture due to its resistance to diseases (Yap, Villaluz, Soriano, & Santos, 2007). Though locally consumed fresh, milkfish can be frozen, filleted, deboned, smoked, and canned, and so it has many product forms. It is also exported to many countries such as the USA, Canada, Australia, Korea, Singapore, Guam, UAE, and Papua New Guinea. In 2018, exports of milkfish and milkfish processed products reached about 4,200 MT valued at Php 980.94Million (Philippine Statistics Authority, 2019). Additionally, the local production of milkfish is steadily growing due to good farming management, availability of quality fry/fingerlings, and continuous development of production technologies (Santos, Destura, & Ordoñez, 2014). As both production and demand for milkfish and milkfish processed products continue to rise, there is also an increase in generation of processing by-products considered as wastes.

Wastes generated from fish processing industries include heads, fins and tails, scales, skins, bones, livers, roe, and viscera (Bergé *et al.*, 2014). These materials consist of lipids, protein, and other valuable compounds, and can be refined into high-value products such as proteins, oil, amino acids, minerals, enzymes, collagen, gelatin and bioactive peptides (Ghaly *et al.*, 2013; Rustad *et al.*, 2011). According

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to Santos *et al.* (2014), milkfish has the potential to be utilized in bio-factories. However, due to the lack of research and documentation in fish biotechnology in the Philippines, the bio-factory potential of milkfish, most especially for making protein hydrolysates, has yet to be realized.

Fish protein hydrolysates have been gaining interest from food scientists around the world due to their numerous pharmaceutical and nutraceutical applications (Halim, Yusof, & Sarbon, 2016). Protein hydrolysates from marine by-products have been reported with several bioactivities: anti-hypertensive (Elavarasan, Shamasundar, Badii, & Howell, 2016; Nasri *et al.*, 2013), anti-cholesterol (Wergedahl *et al.*, 2004), anti-diabetes (Harnedy-Rothwell *et al.*, 2020), anti-cancer (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2018; Picot *et al.*, 2006), anti-anemia (Dong, Sheng, Fu & Wen, 2005), anti-inflammatory (Giannetto *et al.*, 2020), anti-bacterial (Jemil *et al.*, 2014), antioxidant (Je, Park, & Kim, 2005; Ktari *et al.*, 2012) and cell repair (Fitzgerald *et al.*, 2005) activities. Additionally, they also possess functional properties such as solubility, emulsifying, foaming, water holding, and fat binding capacity; hence they have huge potential for applications as food ingredients (Halim *et al.*, 2016; Idowu *et al.*, 2020; Kristinsson & Rasco, 2000b).

Fish protein hydrolysates can be produced by enzymatic or chemical hydrolysis. The use of enzymes is the most common method used for hydrolysis because the conditions are more controllable; and the use of different enzymes can produce different functional properties in the hydrolysates (Ghaly *et al.*, 2013). However, production on a large scale is then difficult due to the high cost of enzymes, which increases the capital cost for production. Chemical hydrolysis, on the other hand, can be harsh and difficult to control, but it is efficient and considered an economical method (Kristinsson & Rasco, 2000b). Therefore, this study aimed to produce protein hydrolysates through acid hydrolysis from milkfish (*C. chanos*) processing by-product

2. Materials and Methods

2.1 Raw materials and reagents

Milkfish (*C. chanos*) by-products (viscera, fins and bones) were collected from a public market in the Science of City of Muñoz, Nueva Ecija, Philippines. The sample were transported in the laboratory under iced conditions. The milkfish by-products were then washed and cleaned using distilled water, and stored at -20 °C. All reagents used in all experiments were of analytical grade and used as received.

2.2 Fish protein hydrolysate

Acid hydrolysis was carried out using the method of Wisuthiphaet, Kongruang, & Chamcheun, (2015) with minor modifications (Figure 1). The milkfish by-products were thawed at 4 °C for 16 h. A combination of ground viscera (50 g) and ground bones and fins (50 g) were used in this study. Afterwards, the mixture (100 g) was added with 50 mL distilled water in a 250 mL Erlenmeyer flask. Three different hydrochloric acid concentrations (4, 6, and 8M) were used in this study. Fifty milliliters of acid were added to the fish slurry. The mixture was autoclaved at 121 °C under 15 psi for 90 min. The reaction was then terminated by adjusting the pH of the solution to 5 using 6M NaOH. The slurry was then filtered to remove any remaining debris. The filtered liquid was then dried in an oven at 80 °C for 48 hrs. The dried sample was powdered and stored in a desiccator until further analysis.

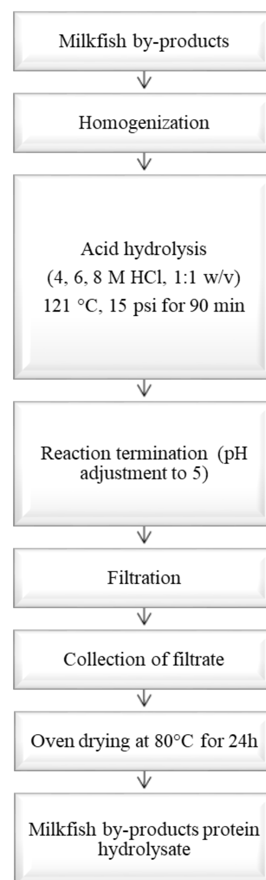

Figure 1. Schematic diagram for the production of protein hydrolysate using acid hydrolysis

2.3 Proximate analysis

Moisture and ash contents of the protein hydrolysates were determined by standard official methods of analysis (AOAC, 1990).

2.4 Degree of hydrolysis

The degree of hydrolysis (DH) was determined through formol titration (Taylor, 1957). Two and a half milliliters (2.5 mL) of sample at pH 8.1 (adjusted using 0.1 N NaOH solution) was added with 1 mL of 35% formaldehyde solution, pH 8.1. The mixture was incubated at room temperature for 1 min. The solution was then titrated with 0.25 N NaOH solution until it reached the potentiometric point of 8.1. The volume of the utilized NaOH solution was recorded. The degree of hydrolysis (% DH) was calculated using the following formula:

$$\%DH = \frac{B \times N_b \times 1.5}{M_p \times h_{tot}}$$

where B refers to the volume of NaOH solution utilized to reach the pH of 8.1, N_b is the normality of the NaOH solution, 1.5 is acquired from 1/α (at pH 8.1 based on the calibration factors for pH-Stat at various temperature), h_{tot} is the number of peptide bonds per unit: 8.6Meq/g (for fish protein concentrate), 8.2 meq/g (for casein), and M_p is the amount of protein in grams.

2.5 Functional properties of fish protein hydrolysate

2.5.1 Solubility

Solubility was determined by applying the method adapted from Taheri, Anvar, Ahari, & Fogliano, (2013) with slight modifications. One percent (w/v) fish protein hydrolysate solution was prepared by dispersing 0.2 g of dried fish protein hydrolysate in 20 mL distilled water. The solution was prepared with varying pH (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12) adjusted using 1 N HCl or 1 N NaOH and stirred at room temperature for 30 min. The samples were centrifuged at 7,500 g for 15 min. The supernatant collected was measured using the Biuret method. The total protein content of the sample was determined by dispersing the same amount of fish protein hydrolysate to 0.25 N NaOH followed by Biuret test. Protein solubility was calculated as:

$$\text{Solubility (\%)} = \frac{\text{Protein content of the supernatant}}{\text{Protein content of the sample}} \times 100$$

2.5.2 Emulsifying activity and stability

Emulsifying properties were evaluated using the method of Klompong, Benjakul, Kantachote, & Shahidi, (2007) with minor modification. Three milliliters (3 mL) of 1% fish protein hydrolysate solution was mixed with 1 mL vegetable oil. The pH of the mixture was adjusted into 2, 4, 6, 8, or 10. The mixture was homogenized for 1 min at 20,000 rpm. After homogenization, 50 µL was collected at the bottom of the container. Another 50 µL was collected from the sample after 10 min. The sample was mixed with 5 mL of 0.1% sodium dodecyl sulfate (SDS) solution and was read using a UV-Vis spectrophotometer at 500 nm (T60UV, PG Instruments Limited, Wibtoft, England). The emulsifying activity index was calculated as follows:

$$\text{EAI } (m^2/g) = \frac{(2 \times 2.303 \times A_0)}{(0.25 \times \text{protein weight (g)})}$$

The emulsion stability index was calculated using:

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

where A_0 is the absorbance of the sample at 500 nm, ΔA refers to absorbance of the sample at 500 nm minus to the absorbance of sample in 10 min, while Δt refers to the 10 min reaction time.

2.5.3 Foaming properties

The foaming capacity and stability of the hydrolysate was evaluated using the method of Klompong *et al.* (2007). A total of 20 mL of 0.5% (w/v) fish protein hydrolysate was prepared with varying pH (2, 4, 5, 8, or 10). The solution was homogenized at 16,000 rpm for 2 min at room temperature. The whipped sample was transferred into a graduated cylinder and the volume was recorded after 30 sec. The foaming capacity was calculated using the formula:

$$\text{Foaming capacity (\%)} = \frac{(A-B)}{(B)} \times 100$$

where A refers to recorded volume after whipping (mL), and B is the volume before whipping (mL).

The whipped samples were left to stand at 20 °C for 3 min; and the volumes of the samples after 3 min were recorded. Foaming stability was calculated using the formula:

$$\text{Foaming stability (\%)} = \frac{(A-B)}{(B)} \times 100$$

where A refers to the recorded volume after 3 min (mL), and B refers to the volume before whipping (mL).

2.5.4 Determination of antioxidant properties

The method of Chan, Lim, & Omar (2007) was adapted with minor modification for the DPPH (2,2-Diphenyl-1-pichyhydrazyl) radical scavenging activity assay. Different concentrations in the samples was prepared through serial dilution (1.0, 0.3, 0.1, 0.01, 0.001, and 0.0001 mg/mL). A total of 1.5 mL of the sample was mixed with 2.5 mL of DPPH solution (6Mg/100 mL methanol). Ascorbic acid was used as a positive control. The mixture was homogenized and left in the dark for 30 min. The samples were read at 517 nm. The DPPH radical scavenging activity assay was calculated as (Rivero-Pino, Espejo-Carpio, & Guadix , 2020):

$$\text{\%DPPH Scavenging} = 1 - \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

The DPPH scavenging activity was later expressed as EC_{50}, the efficient concentration of the protein hydrolysate to scavenge the DPPH radical concentration by 50%.

2.6 Statistical analysis

The results were subjected to analysis of variance (ANOVA), followed by *post hoc* test (Tukey's HSD) at $p<0.05$ level of significance using Statistical Package for the Social Sciences (SPSS) program. Non-linear regression analyses for EC_{50}, the half maximal effective concentration, were performed using the GraphPad Prism program. All experiments were done in triplicate.

3. Results and Discussion

3.1 Yield and proximate analysis

The average yield and proximate composition of Milkfish (*C. chanos*) fish protein hydrolysates produced through acid hydrolysis using 4, 6 and 8M HCl are presented in Table 1. The use of 6M HCl recorded the highest yield with 5.67±1.98%, but there were no significant differences between using different HCl concentrations. The moisture content ranged within 7.56±0.28% - 8.74±0.48% from oven-drying. Typically, freeze-drying is applied to the drying of protein hydrolysates. The study of Ktari *et al.* (2012) reported a similar moisture content from freeze-dried protein hydrolysates from zebra blenny (*Salaria basilisca*). Additionally, Elavarasan & Shamasundar (2016) stated that oven drying at 80 °C for 48 h can be applied to fish protein hydrolysates with minimal effect on the peptides.

The ash contents measured were 37.69% - 46.44%. These are higher than the prior reported ash content for fish protein hydrolysate, ranging from 0.45% to 27% (Chalamaiah, Dinesh, Hemalatha, & Jyothirmayi, 2012). This high ash content may be due to the formation of salt from the neutralization of the acid, and to some leftover fish bones. According to Kristinsson & Rasco, (2000), one of the disadvantages of acid hydrolysis is the high production of NaCl (salt) that may affect the food functionality of the protein hydrolysates.

3.2 Degree of hydrolysis (DH)

The degree of hydrolysis (DH) measures the extent of protein breakdown (Kristinsson & Rasco, 2000a). The protein hydrolysates obtained from treating milkfish (*C. chanos)* by-products with 8M HCl had the highest DH of 43.88±9.50% (Table 2). The DH of the hydrolysates produced using 8M HCl were significantly higher than with 4M (22.05±9.63%, p=0.033). There was also no significant difference between 8M and 6M (p=0.269) or between 6M and 4M (p=0.284). A linear relationship between the treatment molarity and the degree of hydrolysis was noted— a higher DH was observed with higher HCl concentration. Similar results were found in the study of Wisuthiphaet *et al.* (2015), entitled "Production of fish protein hydrolysates by acid and enzymatic hydrolysis", which obtained 35.56% DH using 8M HCl, the highest among the three treatments. In the same study, the authors concluded that acid hydrolysis was more efficient and an economically appropriate method for protein hydrolysis due to taking a shorter amount of time to hydrolyze protein in a comparison to enzymatic hydrolysis. Fountoulakis & Lahm (1998) stated that hydrochloric acid (HCl) is commonly used for hydrolysis due to its convenient application.

3.3 Functional properties

3.3.1 Solubility

Solubility is considered the most important functional property of protein hydrolysates, as it influences other properties including emulsifying and foaming properties (Jemil *et al.*, 2014). The solubilities of protein hydrolysates produced using three different acid concentrations at pH 2-12 are shown in Figure 2. In general, the protein hydrolysates from 8M HCl treatment showed superior solubility with an average of 88.07% in all pH levels, peaking at pH 6 with 94.26±2.62%. Statistically, 8M HCl solubilities at pH 2-8 had no significant mutual differences (p=0.115). The solubility of the hydrolysates from the 6M HCl treatment ($\bar{x}$=74.13%), though lower compared to 8M, followed a similar trend of highest solubility recorded from pH 2-7 (peaked at pH 5 with 81.89±1.09%). The solubilities of the hydrolysates produced by 4M HCl were significantly lower compared with 6M and 8M ($p<0.05$) with an average of 53.80% solubility in all pH levels, without mutual statistically significant differences with p=0.064.

A linear relationship of solubility to DH was apparent: higher solubility was observed in hydrolysates with higher DH (8M HCl). According to Liu *et al.* (2014), excellent solubility may be due to the degradation of large protein molecules to smaller peptides, which are responsible for the increase in the solubility of the hydrolysates. Similar results were obtained from the study of Gbogouri, Linder, Fanni, & Parmenter (2004) on salmon hydrolysates.

Table 2. Degree of hydrolysis (DH) of the fish protein hydrolysate (n=3)

Treatment	Degree of hydrolysis (%)
4M HCl	22.05±9.63[b]
6M HCl	32.82±0.07[ab]
8M HCl	43.88±9.50[a]

*Values with different superscripts are significantly different at p <0.05.

Table 1. Average yield and proximate composition of milkfish (*Chanos chanos*) fish protein hydrolysates produced through acid hydrolysis at three different concentrations

Proximate analysis	Treatment		
	4M HCl	6M HCl	8M HCl
Yield (%)	5.40±0.20[a]	5.67±1.98[a]	4.22±0.78[a]
Moisture content (%)	8.74±0.48[a]	8.18±0.05[ab]	7.56±0.28[b]
Ash (%)	37.69±1.28[c]	44.42±0.08[b]	46.44±0.22[a]

*Values in the same row with different superscripts are significantly different at p <0.05.

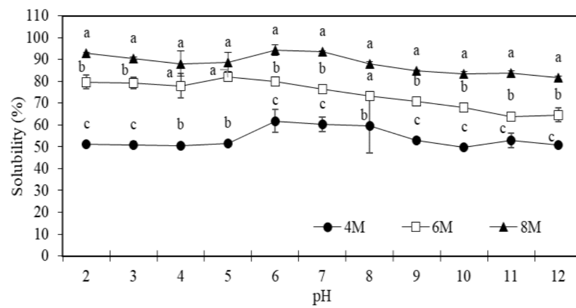

Figure 2. Solubility by pH of fish protein hydrolysates obtained using three different acid concentrations: 4M HCl (●), 6M HCl (□), and 8M HCl (▲). Data are expressed as means with error bars for SD (n=3). Different letters at the same pH indicates significant statistical differences ($p<0.05$).

The pH also influences solubility of the protein hydrolysates. This study revealed the lowest solubility from pH 4, 8-12 for 8M and pH 9-12 for 6M: both concentrations recorded the highest solubility at pH 5-8. According to (Kristinsson & Rasco, 2000b), low solubility at isoelectric points (pI) was noted for protein and protein hydrolysates. These variations in the solubility of protein hydrolysates may be attributed to the peptide's net charge, which increases as the pH moves away from the pI, and to surface hydrophobicity, which causes aggregation through hydrophobic interactions (Taheri *et al.*, 2013). Overall, protein hydrolysates from 8M and 6M HCl exhibited high solubility over a wide range of pH, indicating that fish protein hydrolysates from acid hydrolysis can be used easily in food formulations.

3.3.2 Emulsifying properties

The ability of protein hydrolysates to stabilize food emulsions is considered an important property, since interactions between protein and lipids are essential in many food systems applications (Chalamaiah, Jyothirmayi, Diwan, & Dinesh, 2015). The emulsion activity index (EAI) and emulsion stability index (ESI) of protein hydrolysates at pH 2, 4, 6, 8 and 10 are shown in Figure 3a and 3b. EAI refers to the units of the area of the interface that is stabilized per unit weight of protein. This can be measured by the turbidity of the emulsion at 500 nm absorbance (Liceaga-Gesualdo & Li-Chan, 1999). The 8M and 6M HCl produced hydrolysates with an average of 39.97 m^2/g and 38.97 m^2/g, respectively. The EAI of the hydrolysates produced by 8M HCl peaked at pH 8 with 53.51±3.16M^2/g, while that of the hydrolysates obtained from the 6M HCl treatment peaked at pH 10 with 52.86±8.67 m^2/g. No significant differences were found for either concentration across the pH levels ($p>0.05$). The hydrolysates obtained from the 4M HCl treatment, with an average of 28.18M^2/g across all pH, showed significantly lower EAI at pH 8 and 10 compared to the samples from 6M and 8M HCl treatments ($p<0.05$). However, EAI of the hydrolysates obtained from the 4M HCl treatment at pH 2-6 had no significant difference from those of the hydrolysates produced by 6M and 8M HCl ($p>0.05$). In terms of ESI, the hydrolysates produced by 8M HCl got an average of 34.04Min emulsion stability, peaking at pH 6 at 43.83±4.65 min. At pH 4, the 4M HCl hydrolysates recorded the significantly highest ESI of 31.98±3.19 min ($p<0.05$).

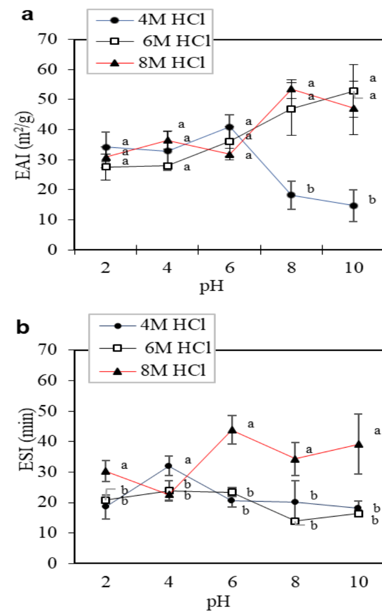

Figure 3. Emulsifying activity index (EAI) (a), and emulsion stability index (ESI) (b) of milkfish by-product based protein hydrolysates at various pH (2, 4, 6, 8 & 10) prepared using different HCl concentrations: 4M HCl (●), 6M HCl (□), and 8M HCl (▲). Data are presented as means with error bars representing SD (n=3). Different letters for values at the same pH indicate significant differences at $p <0.05$.

However, the hydrolysates produced by 8M HCl had the highest ESI at all other pH levels ($p<0.05$), except for pH 4.

Though recorded with a high DH, the 8M cases still showed excellent emulsifying properties (both EAI and ESI). Several studies have reported the loss of emulsifying properties in protein hydrolysates with higher DH (He, Franco, & Zhang, 2013; Thiansilakul, Benjakul, & Shahidi , 2007; Wasswa, Tang, Gu, & Yuan , 2007). Klompong *et al.* (2007) explained excessive DH results by the formation of a smaller peptide size that can negatively affect the emulsifying properties. Peptides with high molecular weight can hold and stabilize an emulsion system due to their hydrophobicity. During homogenization, hydrophobic peptides adsorbed to the surface of oil droplets, and this formed a protective membrane that can inhibit the conglomeration of oil droplets, thus creating an emulsion system. Peptides with a low molecular weight may not be amphiphilic enough to be adsorb to the surface of oil droplets and have a low efficiency in forming and stabilizing an emulsion system. However, similar results with a positive relationship between DH and emulsifying activity were reported by Chalamaiah *et al.* (2015) with high DH (30%) protein hydrolysates showing higher EAI and ESI at pH 6, 8 and 10. The study of Balti *et al.* (2010) showed identical results in protein hydrolysates with high DH exhibiting high EAI at pH 10. According to Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suárez, (2008), who obtained results in agreement with this study, these differences may be due to the amino acid composition and its distribution in the produced poly-peptides. Kristinsson & Rasco, (2000b) noted that emulsifying properties are influenced by the enzymes used. This was observed in the study of Klompong *et al.*

(2007) with protein hydrolysates from flavourzyme and alcalase, which had the same DH but produced different EAI and ESI. Similarly, Liu *et al.* (2014b) showed a better EAI and ESI from hydrolysates prepared using alcalase compared to protamex with the same DH (20% and 30%). Finally, Khan *et al.* (2020) stated that several factors, including the manufacturing method and the physicochemical properties of protein such as size, hydrophobicity, pH, and surface charge, can influence the emulsifying properties of protein hydrolysates.

3.3.3 Foaming properties

The foaming capacity of the protein hydrolysates is presented in Figure 4a. The protein hydrolysates from three treatments showed different foaming capacities from 15.0±8.7% to 233±7.6% at pH 4-10. None of the three treatments produced foam at pH 2. On an average, both 6M ($\bar{x}$=141.67%) and 8M ($\bar{x}$=142.67%) HCl produced hydrolysates with foaming capacity exceeding 140%. All samples exhibited the highest foaming capacity either at pH 8 or 10, though the hydrolysates produced by 4M HCl had foaming capacity that was statistically lower ($p<0.05$).

Taheri *et al.* (2013) stated that foam formation may be attributed to the three factors transportation, penetration, and reorganization of molecules at the air-water interface. Pacheco-Aguilar *et al.* (2008) added that proteins with good foaming properties show rapid surface denaturation and possess certain molecular characteristics including good surface balance and molecular hydrophobicity, net charge, and charge distribution. The foam stability of the protein hydrolysates was recorded by monitoring the foam after whipping. All treatments were found to be most stable at pH 8 and 10 (Figure 4b). The hydrolysates produced by 6M HCl showed the highest foaming stability ($p<0.05$).

The results showed the influence of pH on the foaming properties. All treatments were found to have high foaming capacity and stability at pH 8 and 10. The decrease in foam stability at very low pH may be due to the ionic repulsion of peptides (Klompong *et al.*, 2007). Similar findings were obtained from the study of Halim & Sarbon (2020) on the protein hydrolysate from Asian swamp eel (*Monopterus sp.*) in which the foaming property increased with the pH. In contrast, the results of this study were higher than the foaming properties of protein hydrolysates from round scad (*Decapterus maruadsi*) (Thiansilakul *et al.*, 2007) and freshwater carp *(Catla catla*) (Elavarasan, Naveen Kumar, & Shamasundar, 2014). The high foaming capacity of milkfish by-product based protein hydrolysates could have various applications in the food industry.

3.3.4 Antioxidant properties

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the DPPH reduction in the presence of a hydrogen-donating antioxidant (Jeevitha, Mohana Priya, & Khora, 2014). In this study, the EC_{50} value, or the concentration of antioxidant that causes a decrease in the DPPH absorbance by half, was estimated. Different concentrations (1.0, 0.3, 0.1, 0.01, 0.001, and 0.0001 mg/mL) were tested aside from the control, and Figure 5 displays the dose-response curves of the protein hydrolysates and a known

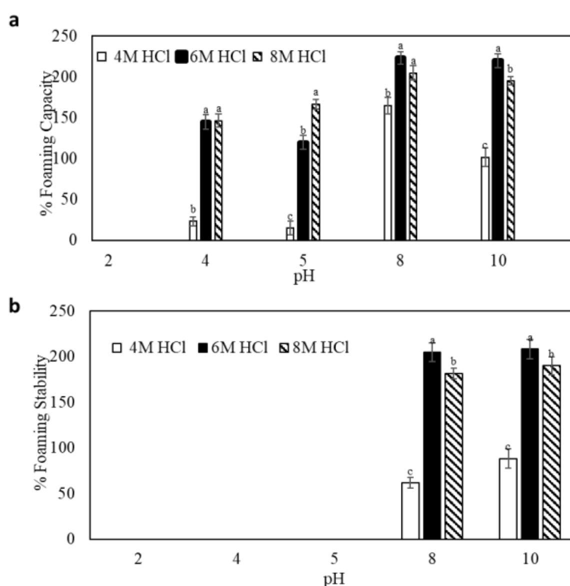

Figure 4. Foaming capacity (a), and stability (b) of fish protein hydrolysates obtained using three treatments (4M, 6M and 8M HCl) at various pH (2, 4, 5, 8, & 10). Data are presented as means with error bars for SD (n=3). Different letters for values at the same pH indicate significant differences at $p<0.05$.

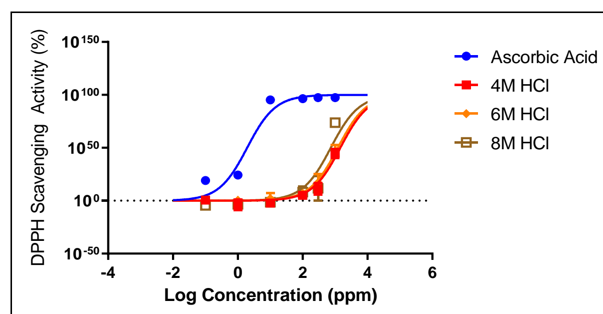

Figure 5. Dose-dependent inhibition of DPPH by fish protein hydrolysates produced by treating mixed milkfish processing by-products with different concentrations of HCl.

antioxidant, ascorbic acid. The curve for ascorbic acid is found at the leftmost side and the estimated EC_{50} is 1.94±0.08Mg/mL (Table 3). Next to it is the curve for the hydrolysates produced by 8M HCl, which has an EC_{50} of 737.90±0.09 mg/mL (Table 3). Among the three hydrolysates, the one produced by 8M HCl had the highest antioxidant activity. However, its activity was not as high as that of the ascorbic acid.

Figure 6 displays the DPPH scavenging activity of the protein hydrolysates and ascorbic acid at 1 mg/mL. The protein hydrolysates from 8M HCl showed the highest scavenging activity (73.8±2.2%). However, it was still significantly lower compared to the activity of ascorbic acid ($p<0.05$). Antioxidant activities of protein hydrolysates have been reported previously. Zebra blenny (*Salaria basilisca*) protein hydrolysates had a scavenging activity of 76.56% at 6 mg/mL concentration (Ktari *et al.*, 2012). A study by

Table 3. Half maximal effective concentrations or EC_{50} for DPPH scavenging by fish protein hydrolysates and ascorbic acid (positive control)

Treatment	EC_{50} (mg/ml)
4M HCl	1,451.0±0.04
6M HCl	1,217.0±0.03
8M HCl	737.90±0.09
Ascorbic Acid	1.941±0.08

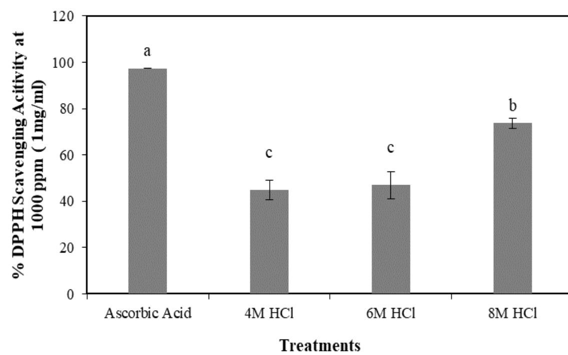

Figure 6. Antioxidant activities of protein hydrolysates obtained from three HCl concentrations and the positive control, ascorbic acid, at 1 mg/mL. Different letters indicate significant differences at $p<0.05$.

Elavarasan *et al.* (2014) showed that at a concentration of 2 mg/mL, the protein hydrolysates from freshwater carp (*Catla catla*) produced using different proteases had the following antioxidant activities: bromelain (77.92±0.59%), flavorzyme (70.45±0.35%), protamex (67.9±0.42%), and alcalase (64.6±1.5%). The sturgeon protein hydrolysates obtained using papain and alcalase caused about 40% DPPH scavenging activity at 1 mg/mL (Anwar, Abdelmoneim, Wedad Qasim, & Wenshui, 2020). The reported highest scavenging activity reached 78.45% and 81.42% at 5 mg/mL. The authors noted an increase in DPPH scavenging activity when the protein concentration was increased from 1 mg/mL to 5 mg/mL. This suggests that protein concentration affects the DPPH scavenging activity.

4. Conclusions

Milkfish (*C. chanos)* by-products can be converted into protein hydrolysates using acid hydrolysis, with certain bioactivities and food functional properties. Oven-drying can be used as an alternative drying method for protein hydrolysates. Furthermore, the researchers recommend a thorough study of acid hydrolysis, using different acids and varying reaction times, to assess the effects on the functional food properties of protein hydrolysates.

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