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Research Article

Functional properties and antioxidant activities of protein hydrolysates from orangefin ponyfish (*Photopectoralis bindus*)

Ramezani Z.[1]; Rajabzadeh Ghatarmi E.[1]; Hosseini S.F.[2*]; Regenstein J.M.[3]

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Abstract

Amino acid profiles, functionality, and antioxidative potential of orangefin ponyfish (*Photopectoralis bindus*) protein hydrolysates, using 1% alcalase, with diverse hydrolysis times (1, 2, 3 and 4 h), were evaluated. The samples solubility in the pH range of 3-9, emulsifying and foaming properties at various protein contents (2.5, 5 and 10 mg/mL), as well as the scavenging of DPPH, ABTS free radicals and reducing power (at concentrations of 1-5 mg/mL), were studied. The amino acid profile of orangefin ponyfish was used to estimate nutritional value. A high amount of essential amino acids (35%) involved in protein hydrolysates and Glx, Asx, and Gly were the dominant amino acids. Solubility was above 90% in the pH range 3-9 and increased at all hydrolysis times by changing the pH from acidic to alkaline (p<0.05). The emulsifying activity (EAI) and stability (ESI) indices were reduced with increasing hydrolysis; at 2.5 mg protein/mL, 1 h hydrolysates displayed the highest EAI (87±2 m^2/g) and ESI (22±0.4 min), when considered with those possessing higher hydrolysis time. On the other hand, the foam expansion and stability increased with increasing hydrolysate content. Antioxidant activity of hydrolyzed protein increased with increased hydrolysis (up to 3 mg/mL), especially DPPH and ABTS. However, the highest DPPH and ABTS scavenging activity of FPH was about 73% and 93% at 5 mg/mL hydrolysate concentration and hydrolysis time 4 h, respectively.

Keywords: Fish by-catch, Orangefin ponyfish, Fish hydrolysates, Amino acid composition, Emulsifying properties, Foaming attributes, Antioxidant activities

1-Department of Fisheries, Faculty of Marine Natural Resource, Khorramshahr Marine Sciences and Technology University, Khorramshahr, Iran.
2-Department of Seafood Processing, Faculty of Marine Sciences, Tarbiat Modares University, Noor, Iran.
3-Department of Food Science, Cornell University, Ithaca, NY 14853-7201, USA.
*Corresponding author's Emial: hosseinisf@modares.ac.ir

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Introduction

Fish by-catch is the portion of total fish catch that is dumped back into the water (García-Moreno *et al.,* 2014). Non-commercial fish species with low value and damaged fish that are not worth retaining comprise the marine discards. Discards cause environmental problems since they are normally dead when dumped back into the water. Therefore, it may be helpful to develop processes that will allow the development of products with added value from this underutilized material (García-Moreno *et al.,* 2014). Enzymatic hydrolysis of proteins to produce fish protein hydrolysates (FPH) is one of these methods. The antioxidant activities, amino acid profile, and nutritional composition of FPH have been studied and they are being used in some industrial applications such as cosmetics, pharmaceutical products, and human nutrition (Chalamaiah *et al.*, 2012; Rabiei *et al.,* 2019).

On the other hand, the food industry, as well as consumers, has a concern about the lipid oxidation. So, there is an escalating interest in finding natural antioxidants, which may have a less potential hazard in comparison to synthetic ones (You *et al.,* 2009). Additionally, functional attributes of FPH including solubility, emulsification, and foam-forming ability have been reported (Hemker *et al.,* 2020). FPH had better solubility at a high degree of hydrolysis, which, in turn, affects the other functional attributes, like foaming and emulsifying (Ktari *et al.,* 2020).

Orangefin ponyfish (*Photopectoralis bindus*) is a demersal and amphidromous marine fish (Riede, 2004). This species is found in the Persian Gulf and the western part of the Oman Sea (Russell and Houston, 1989), and its maximum length is 11 cm. Orangefin ponyfish may be as much as 55% of the by-catch in the Persian Gulf (Nikoo *et al.,* 2010). Therefore, the production of value-added products like FPH may allow greater use of this species. According to the author's knowledge, this study is the first report regarding functional properties and *in vitro* antioxidant characterization of protein hydrolysate from Orangefin ponyfish of the Persian Gulf. Thus, the present study aimed to make hydrolyzed proteins, and to (i) evaluate the influences of the hydrolysis time and hydrolysate concentrations on the functional attributes of FPH using solubility, foaming, and emulsifying tests, and (ii) to measure their *in vitro* antioxidant action by ABTS, DPPH, and reducing power assays.

Materials and methods

Materials

Orangefin ponyfish (8±1 cm length) were obtained from the fishing harbor of Khuzestan (Iran) and kept on ice (with a fish/ice ratio of 1:3 w/w) during transportation, which was about 3 h. Once they arrived in the laboratory, whole ungutted fish were minced by industrial mixer (ARSHIA MG135-

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1409, Tehran, Iran), and frozen at -20 °C until use less than a week. Alcalase, DPPH (2,2-diphenyl-1-picrylhydrazyl), BSA (bovine serum albumin), ABTS (2,2-azino bis(3ethylbenzthiazoline)-6-sulfonic acid), potassium persulfate, potassium ferricyanide, and ferrous chloride were obtained from Sigma-Aldrich (St. Louis, Mo., USA). Folin-Ciocalteu and SDS (sodium dodecyl sulfate) were gained from Merck (Darmstadt, Germany). TCA (trichloroacetic acid) and all other chemicals were obtained from Samchun Pure Chemical Co. Ltd. (Pyeongtaek, Korea) as a reagent grade.

Production of orangefin ponyfish hydrolysates

Enzymatic hydrolysis of orangefin ponyfish was done according to the method of Ovissipour *et al.* (2013). Fifty g of the thawed (4°C, overnight) fish mince was heated at 85°C (for 20 min) for the inactivation of endogenous enzymes; then, the cooked mince was dispersed in 0.2 M SPB (sodium phosphate buffer, pH 8.5) at 1:2 (w:v). It was homogenized using a Heidolph DIAX 900 homogenizer (Kelheim, Germany) at 12000 rpm for about 2 min at ambient temperature (25±2°C). Alcalase (1%) was then added to the mixture and the reaction was done with constant agitation (200 rpm) at 55°C (for 1-4 h). The mixture was centrifuged at 8000×g (for 20 min at 4ºC) utilizing a Universal 320R centrifuge (Hettich, Tuttlingen, Germany); the supernatants (hydrolysates) were stored at -80ºC for further assays, a maximum of one week.

Protein determination

Protein content was obtained based on the method of Lowry *et al.* (1951) using BSA assumed to be 100% pure as the standard in the range of 1-1000 µg/mL and assuming that BSA adequately represented the protein content of the fish samples. Using UV-vis spectroscopy (UV-1800, Shimadzu, Kyoto, Japan) the absorbance was quantified at 750 nm. The total protein content at 1, 2, 3 and 4 h was 32±0.3, 31.3±0.5, 31±0.4 and 31±0.2 mg/mL (n=3), respectively.

Hydrolysis degree (DH)

DH was determined according to the procedure described by Ovissipour *et al.* (2009). A sample volume of 500 µL was removed, mixed with 500 µL of 20% (w/v) TCA, and subsequently centrifuged at 6700×g (for 5 min at 4ºC); to determine the soluble amino acids in the supernatant, recognizing that the loss of peptide bonds would decrease the apparent Lowry protein content. The DH was estimated as:

$$\text{DH}\ (\%) = \frac{\text{solubile protein in 10\% TCA}}{\text{total protein in sample}} \times 100$$

Amino acid composition

Detection and quantification of amino acids were measured by high-performance liquid chromatography (HPLC) according to the method of Girgih *et al.* (2015). The freeze-dried sample of hydrolysate (100 mg) was

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dissolved in 7.5 mL of 6 mol/L HCl and the mixture was hydrolyzed in glass tubes under an atmosphere of nitrogen at 110ºC for 24 h. Reverse-phase column (Sphere-Image 80-5 ODS 2, 25 cm, Knauer, Berlin, Germany) were applied for amino acids separation using methanol acetate buffer (as the mobile phase) and a fluorescence detector (Shimadzu, RF-20A). The 16 amino acids were determined from standard curves of each amino acid in the standard mixture based on peak area measurements assuming of response with concentration, using EZChrom Elite software using the Scientific Software Inc. module (SS420x) (Pleasanton, CA, USA), and expressed as mg amino acid/100 g of Lowry protein.

Functional attributes of protein hydrolysates

Solubility

For this, hydrolysates (200 mg) were dissolved in 20 mL of deionized water, and pH was adjusted to 3, 5, 7, and 9 with 1 M HCl or 1 M NaOH. The blend was agitated at ambient temperature and centrifuged at 8000×g (for 20 min at 4°C). Supernatant protein was determined and the solubility calculated (Lowry *et al.*, 1951):

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

Emulsifying attributes

The indices of emulsifying activity (EAI) and emulsion stability (ESI) were determined as previously demonstrated by Giménez *et al.* (2009). Soybean oil (2 mL) and different concentrations (2.5, 5 and 10 mg/mL) of FPH solution (6 mL) were homogenized (~20000 rpm, 1 min); a sample volume of 50 µL was diluted by 0.1% SDS, and the absorbance was then quantified at 500 nm. The absorbance (A0 and A10) were utilized to compute the EAI and ESI as follows:

$$\text{EAI } (m^2/g) = \frac{2 \times 2.303 \times 100 \times A}{c \times 0.25 \times 10000}$$

where A = absorbance at 500 nm; c = protein concentration (g/mL).

$$\text{ESI (min)} = \frac{A0 \times 10}{A0 - A10}$$

where A0 and A10 are the absorbance quantified immediately and 10 min after emulsion formation, respectively.

Foaming attributes

FE (foam expansion) and FS (foam stability) were ascertained as delineated by Nalinanon *et al.* (2011). FPH solutions with 2.5, 5, and 10 mg/mL protein were moved into a cylinder and homogenized at 14000 rpm for 1 min at ambient condition. FE and FS were then computed by these equations:

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

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

where V_T is total volume after whipping, V_0 is the initial volume before whipping, and V_t is total volume after 60 min.

Antioxidant activity determination

DPPH assay

The DPPH radical-scavenging capacity was measured using the protocol of Bougatef *et al.* (2010), with some changes. An aliquot of 500 µL of each

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sample (at 1-5 mg/mL) was mixed with 500 µL of 0.15 mM DPPH, and then vigorously mixed and kept in the dark (for 30 min) at room condition. The absorbance of the solution was quantified at 517 nm. The scavenging ability was:

$$\text{DPPH radical-scavenging assay (\%)} = \frac{\text{Ablank}-\text{Asample}}{\text{Ablank}} \times 100$$

ABTS assay

This assay was performed using the modified procedure of Giménez *et al.* (2009). Briefly, a sample volume of 20 µL was mixed with 980 mL of ABTS reagent and absorbance was quantified at 734 nm after 10 min incubation at 30ºC. The scavenging capacity was:

$$\text{ABTS radical-scavenging assay (\%)} = \frac{\text{Ablank}-\text{Asample}}{\text{Ablank}} \times 100$$

Reducing power assay

This assay was done using the method of Bougatef *et al.* (2010). An aliquot of the sample (200 µL) at 1-5 mg/mL was blended with the same quantity of sodium phosphate buffer and potassium ferricyanide solution (1% w/v). The mixture was incubated at 50ºC for 30 min, pursued by the inclusion of 500 µL of TCA (10% w/v); then the mixture was centrifuged at 1650×g (for 10 min at 4°C). Finally, 500 µL of distilled water and 100 µL of ferric chloride (0.1% w/v) were added to 500 µL of supernatant solution. The solution was incubated at room condition for 10 min for color development, and absorbance was read at 700 nm.

Statistical analysis

Statistical analyses of the data were evaluated using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. The threshold for statistical significance was set at $p<0.05$. All experiments were performed in triplicate and the results are presented as means ± standard deviation.

Results

Degree of hydrolysis

The effect of time on the degree of hydrolysis (DH) of orangefin ponyfish protein is illustrated in figure 1. As can be seen from Figure 1, the lowest amount of hydrolysis was 25.61±0.61% and the highest was 28.07 ± 0.31% for the first and fourth hours, respectively.

Amino acid composition

The amino acid composition of both protein hydrolysates, expressed as mg amino acid/100 g protein, is shown in Table 1. The total amino acids recovered from orangefin ponyfish protein hydrolysate was 97.7 mg/100 g (Table 1). Because amino acid residues add water on hydrolysis, the actual amount of amino acids that should be recovered is around 115 to 118 mg/100 g protein. Thus, errors in the protein determinations, the absence of tryptophan, hydroxyproline, and hydroxylysine along with the digestion destroying some amino acids and not

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hydrolyzing some peptide bonds would account for the lack of full recovery. As shown in Table 1, the orangefin ponyfish hydrolysate was rich in Glx (17.8 mg/100 g), Asx, Gly, Val, and Leu.

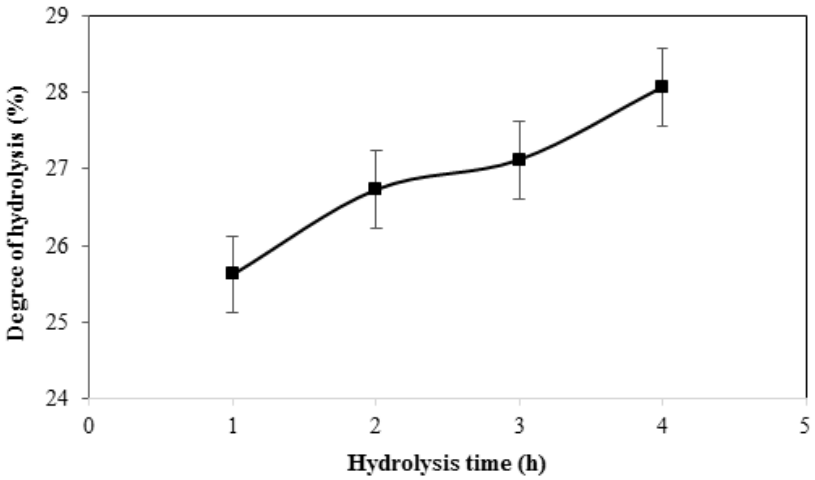

Figure 1: Degree of hydrolysis (DH) of orangefin ponyfish hydrolysates. Error bars represent standard deviation, *n* = 3.

Table 1: Amino acid composition of protein hydrolysates from orangefin ponyfish.

Amino acid	mg amino acid/100 g protein
Aspartic acid (Asx)	11.8
Glutamic acid (Glx)	17.8
Serine (Ser)	7.3
Histidine (His)	0.8
Glycine (Gly)	11.1
Threonine (Thr)	5.1
Arginine (Arg)	3.2
Alanine (Ala)	3.3
Proline (Pro)	3.4
Tyrosine (Tyr)	4.9
Methionine (Met)	2.4
Valine (Val)	9.2
Phenylalanine (Phe)	3.3
Isoleucine (Ile)	4.2
Leucine (Leu)	8.8
Lysine (Lys)	1.1
HAA	50.6
AAA	8.2
EAA	34.9
Total	97.7

Combined total of hydrophobic amino acids (HAA) = Ala, Val, Ile, Leu, Tyr, Phe, Pro, Met, Gly; romatic amino acids (AAA) = Phe, Tyr; essential amino acids (EAA) = Histidine, Ile, Leu, Lys, Met, Phe, Thr, Val.

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Hydrolysates solubility

The solubility of orangefin ponyfish hydrolysates with various hydrolysis time at different pH values are tabulated in Table 2. All samples were soluble over a wide range of pH, in which more than 90% solubility was acquired. Meanwhile, the solubility of hydrolysates at alkaline pH was higher than that of acidic one (p<0.05). The least solubility of FPH was in pH 5.

Table 2: Solubility (%) of hydrolysates from orangefin ponyfish at various pH.

	Hydrolysis time (h)			
pH	**1**	**2**	**3**	**4**
3	94 ± 0.1^{Cc}	97 ± 0.1^{Bb}	98 ± 0.1^{Ab}	98 ± 0.1^{Ac}
5	92 ± 0.1^{Bd}	95 ± 0.2^{Ac}	95 ± 0.1^{Ac}	95 ± 0.1^{Ad}
7	94 ± 0.1^{Cb}	98 ± 0.2^{Ba}	98 ± 0.1^{ABa}	98 ± 0.1^{Ab}
9	96 ± 0.1^{Da}	98 ± 0.2^{Ca}	98 ± 0.1^{Ba}	99 ± 0.1^{Aa}

Values in the same column followed by different lower case letters and in the same row followed by different upper case letters, for each parameter, are significantly different (p<0.05).

Emulsifying properties

Emulsifying characteristics of orangefin ponyfish hydrolysates concerning the emulsion activity (EAI) and stability indices (ESI) with different hydrolysis time at various amounts (2.5, 5 and 10 mg/mL) are presented in Table 3. In general, both EAI and ESI of hydrolysates reduced with raising hydrolysis time and protein concentration, particularly regarding EAI when the protein content increased (p<0.05). At 2.5 mg protein/mL, 1 h hydrolysates displayed the highest EAI (87±2 m^2/g) and ESI (22±0.4 min), when considered with those possessing higher hydrolysis time (p<0.05).

Foaming characteristics

Foam expansion (FE) and foam stability (FS) of orangefin ponyfish hydrolysates with various hydrolysis times at various contents (2.5, 5 and 10 mg/mL) are shown in Table 3. At each hydrolysate concentration, insignificant decreases in FE and FS were noticed when hydrolysis time was raised (p<0.05). At 10 mg protein/mL, 1 h hydrolysates displayed the highest FE (75±1%) and FS (50±0.3%), when considered with those possessing higher hydrolysis time (p<0.05).

Antioxidant activities

DPPH-scavenging ability

DPPH-scavenging capacity of orangefin ponyfish hydrolysates with different hydrolysis time at various amounts (1-5 mg/mL) are presented in Table 4. At these concentrations, a significant increase in radical-scavenging capacity was observed when hydrolysis time increased from 1 to 4 h (p<0.05), except at concentration 1 (hydrolysis time 2-4

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h) and 2 (hydrolysis time 2-3 h) mg/mL. As can be seen from Table 4, the highest DPPH scavenging activity of FPH was about 73% at 5 mg/mL hydrolysate concentration and hydrolysis time 4 h.

Table 3: Emulsifying (emulsifying activity index, EAI; emulsifying stability index, ESI) and foaming properties (foam expansion, FE; foam stability, FS) of hydrolysates from orangefin ponyfish at various concentrations.

Protein oncentration (mg/mL)	Hydrolysis time (h)	EAI (m^2/g)	ESI (min)	FE (%)	FS (%)
2.5	1	$87 \pm 2^{A*a**}$	22 ± 0.4^{Aa}	29 ± 0.2^{Ca}	18 ± 0.4^{Ca}
	2	80.3 ± 0.1^{Ab}	20 ± 0.3^{Ab}	25 ± 1^{Cb}	13 ± 0.4^{Cb}
	3	77.2 ± 0.2^{Ac}	19 ± 0.2^{Abc}	23 ± 1^{Ab}	9 ± 0.3^{Cb}
	4	72 ± 0.2^{Ad}	18 ± 0.2^{Ac}	15 ± 0.3^{Cc}	3 ± 0.3^{Cc}
5	1	34.4 ± 0.3^{Bb}	19 ± 0.4^{Aa}	44 ± 1^{Ba}	31 ± 0.3^{Ba}
	2	37 ± 1^{Ba}	19 ± 0.1^{Aa}	38 ± 2^{Bb}	25 ± 0.3^{Bb}
	3	28 ± 0.4^{Bc}	18 ± 0.5^{Aa}	38 ± 2^{Ab}	21 ± 1^{Bc}
	4	25 ± 0.4^{Bd}	17 ± 0.2^{Bb}	34 ± 1^{Bc}	18 ± 0.3^{Bd}
10	1	18 ± 0.3^{Ca}	16 ± 0.3^{Ca}	75 ± 1^{Aa}	50 ± 0.3^{Aa}
	2	17 ± 0.2^{Cb}	14 ± 0.1^{Bb}	72 ± 1^{Aa}	48 ± 0.3^{Aa}
	5	15 ± 0.2^{Cc}	14 ± 0.4^{Bbc}	63 ± 0.4^{Ab}	40 ± 0.4^{Ab}
	4	15 ± 0.3^{Cc}	13 ± 0.1^{Cc}	60 ± 0.5^{Ab}	38 ± 0.4^{Ac}

*Different capital letters in the same column within the same hydrolysate concentration indicate significant differences (p<0.05).

**Different lower case letters in the same column within the same hydrolysis time indicate significant differences (p<0.05).

ABTS radical-scavenging capacity

The ABTS-scavenging ability of orangefin ponyfish hydrolysates with various hydrolysis times at different concentrations (1-5 mg/mL) is tabulated in Table 4. According to the results, with increasing concentrations, the scavenging effects of hydrolysate on ABTS radical increased around 93% in 5 mg/mL in FPH concentration and hydrolysis time 4 h.

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Reducing power activity

Table 4 shows the reducing power abilities of orangefin ponyfish hydrolysates at different concentrations (1-5 mg/mL) and different hydrolysis time. The reducing power was found to be very low for all the fractions and exhibited a concentration dependency. Reducing power of hydrolysates significantly increased (p<0.05) with increasing concentrations (1 to 5 mg/mL).

Table 4: *In vitro* antioxidant activity of protein hydrolysates from orangefin ponyfish at various concentrations.

Protein concentration (mg/mL)	Hydrolysis time (h)	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	Ferric reducing power (OD at 700 nm)
1	1	$39 \pm 0.4^{B*e**}$	45 ± 1^{Cc}	0.1 ± 0.01^{Be}
	2	41 ± 0.3^{Ad}	52 ± 1^{Bd}	0.1 ± 0.01^{Be}
	3	41 ± 0.2^{Ad}	52 ± 0.1^{Bd}	0.1 ± 0.001^{Be}
	4	42 ± 0.4^{Ae}	56 ± 1^{Ad}	0.1 ± 0.01^{Ae}
2	1	56 ± 1^{Cd}	80 ± 1^{Cb}	0.2 ± 0.002^{Cd}
	2	61 ± 0.2^{Bc}	82 ± 0.3^{Bc}	0.2 ± 0.002^{Bd}
	3	62 ± 1^{Bc}	82 ± 1^{Bc}	0.2 ± 0.002^{Bd}
	4	65 ± 0.3^{Ad}	83 ± 0.2^{Ac}	0.2 ± 0.01^{Ad}
3	1	62 ± 0.4^{Cd}	89 ± 0.2^{Ca}	0.2 ± 0.002^{Cc}
	2	64 ± 0.3^{Cb}	90 ± 0.2^{Bb}	0.2 ± 0.003^{Cc}
	5	66 ± 1^{Bb}	90 ± 0.1^{ABb}	0.2 ± 0.002^{Bc}
	4	68 ± 0.1^{Ac}	91 ± 0.2^{Ab}	0.2 ± 0.003^{Ac}
4	1	63 ± 0.2^{Db}	90 ± 0.3^{Ca}	0.2 ± 0.003^{Bb}
	2	66 ± 1^{Cab}	91 ± 0.1^{Bab}	0.2 ± 0.01^{Bb}
	3	68 ± 0.4^{Ba}	91 ± 0.2^{Bab}	0.2 ± 0.001^{ABb}
	4	71 ± 0.3^{Ab}	92 ± 0.1^{Ab}	0.2 ± 0.003^{Ab}
5	1	65.3 ± 0.3^{Da}	90 ± 0.1^{Da}	0.3 ± 0.003^{Ca}
	2	67 ± 1^{Ca}	91 ± 0.2^{Ca}	0.3 ± 0.002^{Ba}
	3	69 ± 1^{Ba}	92 ± 0.1^{Ba}	0.3 ± 0.001^{Ba}
	4	73 ± 0.3^{Aa}	93 ± 0.1^{Aa}	0.3 ± 0.001^{Aa}

*Different capital letters in the same column within the same hydrolysate concentration indicate significant differences (p<0.05).

**Different lower case letters in the same column within the same hydrolysis time indicate significant differences (p<0.05).

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Discussion

Degree of hydrolysis (DH), defined as the level of protein hydrolysis degradation, is the most extensively applied index for differentiation among various proteins (Bougatef *et al.,* 2010; Raftani Amiri *et al.,* 2016). The results revealed that the DH increased with increasing time of hydrolysis (Fig. 1); the hydrolysis curve acquired in the present study is alike to those delineated earlier for hydrolysates from Chinese sturgeon (*Acipenser sinensis*) (Noman *et al.,* 2019), protein hydrolysates obtained from discarded Mediterranean fish species (García-Moreno *et al.,* 2014), and hydrolysates of defatted salmon backbones (Slizyte *et al.,* 2016). Noman *et al.* (2018) reported that a longer incubation time would allow the enzyme to act more extensively on the protein, thus resulting in an increment in the DH.

The nutritional value of protein hydrolysates (as flavor enhancers and functional ingredients) relies on the composition of their small/large peptides and free amino acids (dos Santos *et al.,* 2011). FPHs showed a difference in their amino acid composition that influenced by various elements e.g. source of the enzyme and fish species (Nasri *et al.,* 2013). As shown in Table 1, the orangefin ponyfish hydrolysate was rich in Glx (17.8 mg/100 g). Determination of Glx as the most prevalent amino acid in fish protein hydrolysate has been addressed in some previous researches (Ovissipour *et al.,* 2009; Noman *et al.,* 2018). As shown in Table 1, the orangefin ponyfish protein had a high amount of HAA (hydrophobic amino acids) (50.6 mg/100 g), providing structural features that can enhance interactions with lipid foods (Girgih *et al.,* 2015). Additionally, the protein hydrolysate had aromatic amino acids (AAA), such as Phe (3.3 mg/100 g) and Tyr (4.9 mg/100 g). Sarmadi and Ismail (2010) described that AAA can donate electrons to convert radicals to stable molecules while maintaining their own stability via resonance structures thus increasing the radical-scavenging activities of the amino acids (Rajapakse *et al.,* 2005). Based on the amino acid profile, orangefin ponyfish hydrolysates showed a high nutritional value and, therefore, maybe a good dietary supplement to balance other dietary proteins.

Generally, the hydrolysates were more soluble in alkaline pH, in contrast with acidic one (Table 2). According to Ktari *et al.* (2020), the reduction of the secondary structure of FPH and the release of smaller polypeptide units from the protein resulted in an increase in solubility of hydrolysates. Similarly, yellow stripe trevally (*Selaroides leptolepis*) hydrolysates with various DHs (5-25%), also displayed high solubility (>85%) in the pH range of 2-12 (Klompong *et al.,* 2007). In the all hydrolysis time, the lowest solubility was observed at pH 5, which is consistent with the results reported by Nalinanon *et al.* (2011) for hydrolysate obtained from ornate threadfin bream

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and Noman *et al.* (2019) for sturgeon hydrolysates. These authors demonstrated that high molecular weight (MW) proteins and/or peptides were precipitated at this pH, which was near the isoelectric points (pI) of myofibrillar proteins. Furthermore, since the net charge of the proteins is minimized near the pI, more protein-protein interaction and less protein-water interaction occur (Noman *et al.*, 2019). Consequently, the protein solubility decreases, whenever, the pH moves away from this point, increases protein-water interactions At pH 9, the solubility of samples was increased with increasing hydrolysis time from 1 to 4 h. According to Nalinanon *et al.* (2011), the dissimilarities in FPH solubility with various DH could be ascertained by the peptides size, the peptides charge group, and the hydrophobic-hydrophilic balance.

Regarding emulsifying characteristics, both EAI and ESI of hydrolysates reduced with raising hydrolysis time and protein concentration (Table 3). Klompong *et al.* (2007) postulated that superfluous hydrolysis causes significant loss in the emulsifying attributes; this may be due to the low MW peptides which not being amphiphilic sufficient to display suitable emulsifying attributes (Nalinanon *et al.*, 2011; Hemker *et al.*, 2020). A decrease in emulsifying activity with increasing protein concentration had been described for other fish proteins such as ornate threadfin hydrolysates (Nalinanon *et al.*, 2011) and sturgeon hydrolysates (Noman *et al.*, 2019). Noman *et al.* (2019) demonstrated that this event may be also owing to a decrease in protein-protein interactions, contributing a lower protein concentration at the interface of oil/water. At high protein concentration, the activation energy barrier does not allow protein migration to take place in a diffusion-dependent manner, leading to protein aggregation in the aqueous phase; while, at low protein concentrations, protein adsorption at the oil-water interface is diffusion-controlled (Nalinanon *et al.*, 2011).

The feature of proteins to form stable foams is principal in the preparation of a variety of foods (Zayas, 1997); egg white, gelatin, casein, soy proteins, and gluten are the most commonly used protein as foaming agents. The foaming properties strongly depend on the transportation, penetration, and rearrangement of molecules at the air-water interface (Noman *et al.*, 2018). FPH has better foaming properties, thus it may be applied in the construction of foam-based products (Barrow and Shahidi, 2007). According to Table 3, insignificant decreases in foaming attributes at each hydrolysate concentration were noticed when hydrolysis time was raised. Similar findings were reported by Nalinanon *et al.* (2011) in ornate threadfin muscle protein hydrolysates and Liu *et al.* (2014) in hydrolysates from surimi processing by-products, which they

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ascribed to a lesser alignment of small peptides at the air/water interface. The decrease in FE may also be related to the aggregation of proteins that interfere in the interactions between proteins and the water needed for the formation of foam (Noman *et al.,* 2018). However, at the same hydrolysis time, foam expansion of hydrolysates showed a significant increase with increasing protein concentration. The same tendency was observed by Thiansilakul *et al.* (2007) with round scad mince hydrolysates, where increased protein concentration led to higher foam expansion. Foam stability (FS) showed a slight decrease with increasing hydrolysis time (Table 3). A similar trend was also observed in ornate threadfin hydrolysates with 10-30% DH (Nalinanon *et al.,* 2011). According to Nalinanon *et al.* (2011), peptides with low MW could not keep a well-ordered orientation of the molecule at the interface. Low FS possibly also arises from the formation of free amino acids during hydrolysis (Noman *et al.,* 2018). Nevertheless, at the same hydrolysis time, FS of hydrolysate was enhanced by increasing content, resulting from the formation of stiffer foams (Lawal, 2004).

The ability of orangefin ponyfish hydrolysates to scavenge the free radicals was tested by their ability to scavenge the stable DPPH radical (Ramezanzade *et al.,* 2017). Generally, a significant increase in radical-scavenging capacity was observed when hydrolysis time increased from 1 to 4 h. Wu *et al.* (2003) showed that the scavenging effect of the hydrolysates slightly increased in the first 5 h, and remained constant throughout the extended time of hydrolysis. At all hydrolysis times, DPPH-scavenging capacities increased with increasing FPH. Similarly, Noman *et al.* (2019) showed that the DPPH radical inhibitory percentage of sturgeon hydrolysates increased with increasing protein concentrations. Pires *et al.* (2013) also reported that there was a positive relationship between scavenging capacity and protein hydrolysate concentration of Cape hake (*Merluccius capensis*), in which the inhibiting percent increased with increasing protein concentration. Also, the high level of DPPH radical scavenging ability of orangefin ponyfish hydrolysates is associated with a high amount of hydrophobic amino acids (50.6% in this study) (Ramezanzade *et al.,* 2018).

To determine the antioxidant ability of hydrogen-donating constituents the ABTS assay is appropriate (Hosseini *et al.,* 2017; Joghataei *et al.,* 2019). Generally, the protein hydrolysates were able to quench ABTS radicals. Although the hydrolysis time had no significant influence on ABTS scavenging ability, the effect of protein concentrations from 1-3 mg/mL was significant, which is supported by previous reports (Ramezanzade *et al.,* 2018; Ktari *et al.,* 2020). The protein concentration can affect antioxidant activity, but excessive concentration has

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the opposite effect. ABTS results showed that orangefin ponyfish protein hydrolysates probably contain amino acids that act as electron donors, reacting with free radicals to give rise to more stable products (Musso *et al.*, 2019).

The reducing power assay was also applied to evaluate the in vitro antioxidant activities of protein hydrolysate since some research has indicated a direct correlation between antioxidant activities and the reducing power of the peptides (Bougatef *et al.*, 2010; Hosseini *et al.*, 2018). This assay is based on the ability of antioxidants to reduce ferricyanide (Fe^{+3}) complex to the ferrous (Fe^{+2}) form (Hosseini *et al.*, 2019). Generally, a significant increase in the reducing power was found with increasing protein concentration (Table 4), which is consistent with the report of Noman *et al.* (2019), who found a linear trend between the increases of the reducing power with increasing sturgeon hydrolysates. However, different hydrolysis times (1-4 h) had no significant influence on reducing power of orangefin ponyfish hydrolysates. Klompong *et al.* (2007) demonstrated that with increasing DH from 5 to 23%, no significant increase in the reducing power of the yellow stripe trevally hydrolysates was observed. Contrary to the present study, Noman *et al.* (2019) demonstrated that reducing power increased with increased DH of sturgeon protein hydrolysates, which was attributed to a higher content of amino acids.

In conclusion, the protein hydrolysates produced from orangefin ponyfish maybe serve as a good source of beneficial peptides and amino acids. This study showed that it is possible to obtain fish hydrolysates having strong *in vitro* antioxidant capacities as well as good functionalities when using the main discarded species (orangefin ponyfish) from the Persian Gulf (~55%) as a raw material. The results established that functional and antioxidant attributes of the hydrolysates were determined by the hydrolysis times as well as protein concentrations. The protein hydrolysates may be applied in food systems as a natural additive with emulsifying and foaming functionalities and antioxidant properties. They would probably inhibit lipid oxidation using radical scavenging activity. More research should be performed to purify and identify the antioxidative peptides in orangefin ponyfish hydrolysate.

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