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Tropical Agricultural Research

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Bioactive Properties of Fish Protein Hydrolysates from Amazon Sailfin Catfish (*Pterygoplichthys pardalis*) from the Victoria Reservoir in Sri Lanka

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ARTICLE INFO

Article history:
Received: 12 August 2020
Revised version received: 7 November 2020
Accepted: 17 January 2021
Available online: 1 April 2021

Keywords:
Bioactive properties,
Enzymatic hydrolysis,
Fish protein extract (FPE),
Fish protein hydrolysate (FPH)

Citation:
Wijesinghe, S. K. D., Abeyrathne, E.D.N.S., Vidanarachchi, J.K. and Rajapakse, R.P.N.P. (2021). Bioactive Properties of Fish Protein Hydrolysates from Amazon Sailfin Catfish (*Pterygoplichthys pardalis*) from Victoria Reservoir in Sri Lanka. Tropical Agricultural Research, 32(2): 219-228.

DOI: http://doi.org/10.4038/tar.v32i2.8469

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ABSTRACT

Pterygoplichthys pardalis is an underutilized by-catch fish species considered as an invasive fish, which is a potential threat to Sri Lankan freshwater fishery industry. This study attempted to produce bioactive fish protein hydrolysates (FPH) using *P. pardalis*. Acid-aided aqueous extract of fish protein was produced and the best protein extraction (PE) was selected. The lyophilized extracts were hydrolyzed using pepsin, papain and trypsin enzymes (1:100), and were subjected to analyze antioxidant properties by DPPH scavenging assay and TBARS assay, metal chelation activity and antimicrobial activity by agar well diffusion method. The 15% citric acid sample showed the highest ($p<0.05$) PE yield (1.95%±0.04 w/w). The pepsin (0 hour) treated hydrolysate showed the highest ($p<0.05$) radical scavenging activity (79.71%±5.19). None of the hydrolysates exhibited antioxidant properties against TBARS and showed no significant difference ($p>0.05$) in the ferrous chelating activity (pepsin (0 hour): 52.31%±0.93, papain (9 hours): 50.95%±1.17). Antibacterial activity against *E. coli* was positive in all hydrolysates, where pepsin (0 hour) hydrolysate showed the highest activity against *E. coli*. This concludes, FPHs of acid-soluble protein of *P. pardalis* contain antioxidant properties, iron-binding activity and antibacterial activity.

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INTRODUCTION

Fish is a highly nutritious food, which has a high demand among consumers (Ojutiku *et al*., 2009). It is a rich source of balanced and easily digestible protein particularly sulphur containing amino acids, omega-3 long chain polyunsaturated fatty acids, vitamins and a low caloric density food compared to terrestrial animal meat (Tacon and Metian, 2013). It provides about 15% of the average per capita intake of animal protein of more than 4.5 billion consumers (Béné *et al*., 2015).

Only few species of total landed fish are commercially valuable where, significant proportion of total catch remains unused. Inherent characteristics such as unattractive flavor, color, heavy bony structure, high fat content and texture are the reasons for no market value of some fish species called by-catch or underutilized fish species. By-products generated in fish processing industries (e.g. heads, fins, skin, trimmings, bones, frames and viscera) and the fish species with no commercial value are generally discarded (Venugopal *et al*., 1995). However, in addition to fish flesh of edible fish species, value added products are developed using fish processing leftovers and the underutilized fish species as raw materials. Such value-added products include fish protein hydrolysates, pet food, fish silage, fertilizer, fish oil *etc*. (Ramírez-Ramírez *et al*., 2008).

Pterygoplichthys pardalis, Amazon sailfin catfish (Common called Scavenger fish) is a native fish species to the Amazon river basin of Peru and Brazil. Though they are usually bottom dwellers, they can breathe air from the water surface during dry periods and when live in waters of low dissolved oxygen. This fish species is popular around the world as an aquarium pet due to its characteristic attractive appearance and also as an aquarium tank cleaner to remove moss (Rao and Sunchu, 2017).

Numerous exotic fish species have been introduced either accidentally or intentionally into Asian freshwaters and some have been considered as alien invasive species (AIS) due to their potential threats to the freshwater biodiversity in the region (Welcomme, 1988). In Sri Lanka, inland fishery was initiated in 1951 with the introduction of *Oreochromis mossambicus* (Tilapia). However, at present, it has been confined to man-made lakes called reservoirs and is basically dependent on exotic cichlid species (Fernando, 2000). *P. pardalis* is an invasive, exotic and underutilized by-catch fish species introduced through the ornamental fish trade. Currently, it has become a potential threat to Sri Lankan freshwater fishery. *P. pardalis* can be found in many inland reservoirs including Victoria reservoir, Polgolla reservoir, Kala wewa, Balalu wewa, Kandalama wewa and Usgala Siyambangamuwa wewa. (Kumudinie and Wijeyaratne, 2005). Infestation of *P. pardalis* in reservoirs of Sri Lanka makes a substantial negative impact on the commercial fishery by entangling as a by-catch fish in commercial gillnets while making gillnets less effective for catching target species.

Hydrolysis methods are developed to convert fish protein into most acceptable and marketable forms (Halldorsdottir *et al*., 2014). Enzymatic hydrolysis is known to be an efficient method of producing bioactive peptides from fish proteins. Production of fish protein hydrolysates (FPH) and use of peptides as an ingredient of functional foods is a recent technology which is gaining popularity. The potential bioactive properties linked with them include antimicrobial, antioxidant, immunomodulatory, antihypertensive and neuroactive properties (Alasalvar *et al*., 2002). It has been proved that, under controlled enzymatic hydrolysis, the bioactive peptides are released from the protein, yielding functional hydrolysates. The substrate, proteases used, and the degree of hydrolysis are the main factors that govern functional properties of resulting hydrolysate. The type of enzyme employed affects on the cleavage patterns of the peptide bonds in protein. (Jun *et al*., 2004).

Though a considerable amount of information is available on functional properties such as antioxidant, antimicrobial and metal chelation of peptides derived from FPH, the potential use of these bioactive peptides in pharmaceutical and food industry needs to be widely investigated. Therefore the current study aimed to produce FPH from *P. pardalis* using food grade proteases (trypsin, pepsin, papain), and to investigate the process condition (hydrolysis time) that led to high bioactive properties (antioxidant activity, metal chelating activity and antimicrobial activity).

METHODOLOGY

Sample collection

Female *P. pardalis* (average body weight: 250-350 g, average body length: 25- 30 cm) were collected from Victoria reservoir in Digana (Central province, Sri Lanka). Newly harvested fish samples were transported under frozen condition and stored at -21 °C until experiment was carried out. Olive oil was purchased from a local supermarket.

Preparation of fish protein extract (FPE)

Processed fish samples, after removing head, skin and gut, were chopped into small pieces and minced (mixer grinder, model: HL1643/04, India) before taking samples to prepare FPE. Distilled water was added to (DW: Mince 4:1 (w/w)) minced sample (Perera *et al.*, 2018) and homogenized (mixer grinder, model: HL1643/04, India) for 60 s. Citric acid monohydrate powder was added to homogenate at five different concentrations (0, 5, 10, 15, and 20% (w/w)) and blended using a mixer grinder with medium power. Then, the samples were kept in an open-air shaker (Model: OS-2000, Thermofisher Scientific, Germany) at 150 rpm min^{-1} for 1 h with continuous stirring (at 4 ºC), followed by incubation for overnight at 4 ºC in a refrigerator while allowing maximum solubilization of proteins. Incubated extracts were centrifuged (model: SORVALL ST 40R, Thermofisher Scientific, Germany) at 3400 rpm, 4 ºC for 20 minutes and the supernatants were collected. Method of acid aided solubilization of proteins described by Choi and Park (2002) was slightly modified in preparing FPE. Entire procedure was carried out below 10 °C. The pH of the supernatants was adjusted to neutral pH (pH 7) with 3M NaOH to neutralize acidic pH and samples were lyophilized for 48 h using freeze drier (model: 05512, iLShinBioBase Co.Ltd., Korea).

The best citric acid concentration for the acid-aided protein extraction was analysed using 10% Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) (QNX-700, C.B.S. Scientific) according to Sambrook and Russell (2006) and yield analysis of FPE. The yield of FPE was calculated using the equation 1.

$$\text{Yield } \% = \frac{W_1}{W_0} \times 100 \quad \text{---------- --(1)}$$

where, W_1 is the final weight of lyophilized sample and W_0 is the initial weight of FPE

Production of fish protein hydrolysates (FPH)

Fish protein hydrolysates (FPH) were produced by enzymatic hydrolysis of the FPE according to the method described in Ramakrishnan *et al.* (2013). FPE (20 mg/ mL solution) were enzymatically hydrolyzed by three exogenous enzymes; trypsin (pH 7.8), pepsin (pH 2.5), and papain (pH 6.5) at their optimum pH conditions (pH meter; model: PL-700PV, EZDO, Taiwan). The enzyme to sample ratio was 1:100 (w/v) and mixtures were incubated at 37 ℃ for six different time lags; 0, 3, 6, 9, 12 and 24 h. Then, the enzymes were heat inactivated at 100 °C for 15 min using a dry heat block before adjusting the pH to neutral and lyophilizing for 48 h using freeze drier. Best hydrolysates of each enzyme were determined using 15% SDS-PAGE (Sambrook and Russell, 2006) and selected hydrolysates were used for the determination of bioactive properties.

Analysis of bioactive properties of FPH

Determination of antioxidant properties

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity assay was conducted according to Liu *et al.* (2010) with slight modifications, to measure the antioxidant activity of FPH. The assay was carried out by making a mixture of 700 µL of hydrolyzed sample (20 mg FPH dissolved in 1 mL DW) and 300 µL of DPPH solution which was prepared by dissolving 4 mg of DPPH (Sigma Aldrich-Germany) powder in 100 mL of methanol (Sigma Aldrich-USA), then shaking thoroughly and incubating for 30 minutes in dark conditions. Absorbance was determined at 517 nm using UV-Spectrophotometer (model: UV-2005, Thermofisher Scientific, Germany). The free radical scavenging activity was calculated using equation 2.

$$\text{SA } (\%) = \left[1 - \frac{As - A1}{A0}\right] \times 100 \quad \text{---------- (2)}$$

where, SA is the free radical scavenging activity, As is the absorbance of the sample, A1 is the absorbance of the sample with Methanol, and A0 is the absorbance of the DPPH-Methanol solution (Control).

Lipid peroxidation level was determined by Thiobarbituric acid reactive substances assay (TBARS) according to Abeyrathne *et al.* (2014) with some modifications as described here. An oil-in-water emulsion was prepared by homogenizing (Model: D-500, SCILOGEX, USA) 1 g of olive oil, 100 µL of Tween-20 and 100 mL of distilled water for 2 minutes in an ice bath. The emulsion was incubated at 37 °C for few minutes (Model: YCW-010E). Then, 8 mL of the emulsion was added to 1 mL of lyophilized FPH (dissolved in distilled water (20 mg/ mL)) and 1 mL of distilled water in 15 mL falcon tube, and incubated at 37 °C for 16 h. After incubation, 1 mL of sample was transferred to 50 mL falcon tube, and 2 mL of TBA/TCA (Sigma Aldrich-USA) (20 mM TBA/15% TCA) solution and 50 µL of 10% Butylated Hydroxyanisole in 90% ethanol were added and vortex mixed (Model: ZX3,

VELP Scientifica, USA). The mixture was incubated at 90 °C in a water bath for 15 min to develop the color. Later, sample was cooled in an ice bath for 10 min and centrifuged (model: ST-40R) at 3000 rpm, 5 °C for 15 min. The absorbance of the solution was measured at 532 nm against the blank prepared with 2 mL of TBA/TCA solution, 50 µL of 10% Butylated Hydroxyanisole in 90% ethanol and 1 mL of distilled water. The TBARS value was expressed as milligrams of malondialdehyde per liter of emulsion (MDA mg/ L).

Metal chelation activity of FPH

Metal chelation properties of FPH were measured using Fe (II) chelating activity. It was analyzed using ferrozine method described in Abeyrathne et al. (2014) with slight modifications. The protocol involved vortex mixing of 100 µL of FPH (20 mg FPH/ 1 mL DW), 900 µL of distilled water, and 1 mL of 10 ppm Fe^{2+} ($FeSO_4$) in a 15 mL Falcon tube. Then, the mixture was incubated for 5 minutes at room temperature before adding 900 µL of 11.3% TCA and centrifuging at 2500 × g for 10 min to remove proteins and peptides present in the sample. Then, 1 mL of the supernatant was transferred to a disposable culture tube, and 1 mL of distilled water, 800 µL of 10% ammonium acetate (Fisher Scientific), and 200 µL of Ferroin color indicator were added before vortex mixing. After 5 min of incubation at room temperature, the absorbance of each sample was measured at 562 nm. The Fe (II) chelating activity of FPHs were calculated using the equation 3.

$$\text{CA}\ (\%) = \left[1 - \frac{\text{As}}{\text{Ab}}\right] \times 100 \quad \text{---------- (3)}$$

where, CA is the Fe(II) chelating activity, As is the absorbance of the sample, Ab is the absorbance of the blank.

Antibacterial properties of FPH

Antibacterial properties of hydrolyzed samples were examined using agar well diffusion method (Bendjeddou *et al.,* 2016). Locally isolated food borne *Escherichia coli* (*E. coli*) bacterial culture was used to determine antimicrobial properties. Isolated *E. coli* bacteria culture was first inoculated in EMB agar plates and incubated at 37 ºC for 48 h. A loop full of this cultured isolate was then inoculated in a previously prepared EC broth and incubated at 37 ºC for 24 h.

A concentration series were prepared using each of the FPH as 10,000, 5,000, 2,500, 1,250 and 625 ppm. Initially, 15-20 mL of nutrient agar was poured into the incubated petri plates and allowed to solidify. Then, the bacterial culture was inoculated by streaking them on agar surface using a sterile cotton bud. Afterwards, agar was punched with sterile cork bored of 4 mm size and 100 µL of sample was micro-pipetted into that bore (well), kept for 30 min and plates were incubated at 37ºC for 48 h. Finally, the plates were observed for bacterial inhibition zones to analyze the antimicrobial activity. Augmentin was antibiotic used as the positive control whereas autoclaved distilled water was used as a negative control. The concentration of the positive control was equivalent to the lowest concentration of the series.

Statistical analysis

Experimental design was Complete Randomized Design (CRD) with five replicates. Data were analyzed with SAS statistical software by employing one-way ANOVA and Tukey's method ($p<0.05$).

RESULTS AND DISCUSSION

Acid-aided solubilization of FPE

In the present study, organic acid (citric acid) was used as a replacement to hydrochloric (HCl) and sulfuric (H_2SO_4) acids for the solubilization of proteins. In acid-aided solubilization of fish protein, typically HCl or H_2SO_4 is used. This could lead to significant loss in its functional properties over the alkali-aided solubilization and leave health concerns (Kristinsson and Rasco, 2000). Hence, there is a trend of substituting HCl and H_2SO_4 acids with an organic acid such as citric acid, acetic acid in acid-aided protein recovery studies.

Yield analysis of FPE

According to the yield analysis, citric acid concentration has significantly affected on the protein yield ($p < 0.05$). The protein extract yields are shown in Figure 1. Soluble protein yield in the supernatant has increased with the increase of citric acid concentration up to 15% citric acid and slightly decreased. The lowest yield was observed in the control (0% citric acid) sample though there was no significant difference of the yield between control and 5% citric acid contained samples, whereas, the highest yield was observed in 15% citric acid contained sample. Consequently, the

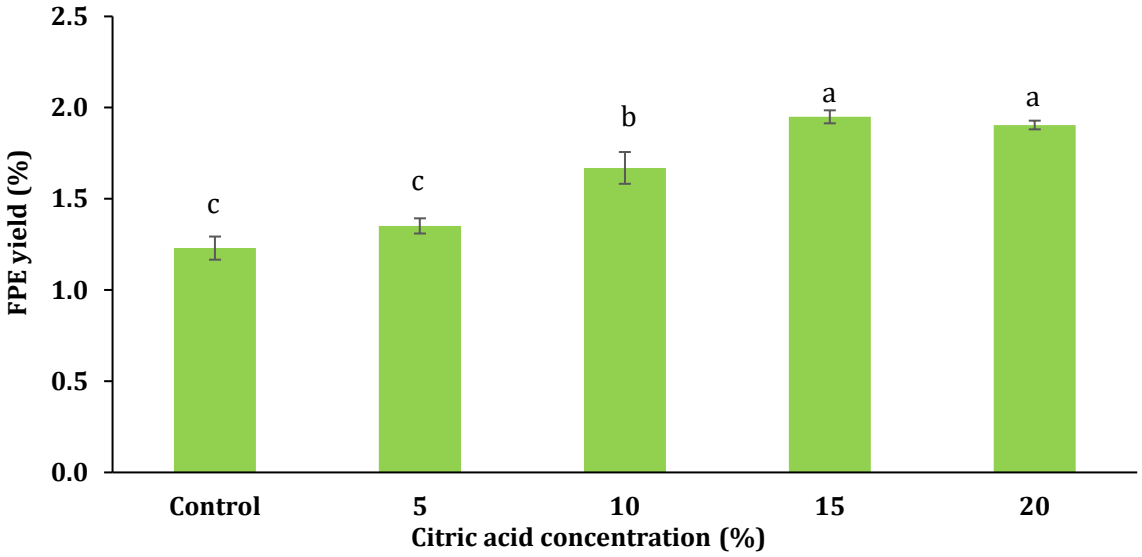

Figure 1. Yield analysis of acid-aided fish protein extract (% w/w)

citric acid concentration (15%) that produced the highest yield was selected as the best acid concentration to prepare FPE based on the yield analysis.According to Hrynets *et al.* (2011), the highest protein solubility of mechanically separated turkey meat with citric acid was revealed at pH 2.5 among the different acidic conditions investigated. The protein solubility was slightly high when extractions were performed at higher citric acid concentrations (low acidic pH) than at lower concentrations (high acidic pH, ~3.5 pH). In the current study, pH of the citric acid treated samples were ranged between pH 2.5 to 2.9. Moreover, the efficiency of acid-aided solubilization of protein might be due to the additional recovery of sarcoplasmic proteins along with the myofibrillar proteins under low pH condition. These results clearly demonstrate the basic principle of the pH-shifting method of protein recovery, where an additional extraction of sarcoplasmic proteins occurs (Ingadottir, 2004).

The band patterns of SDS-PAGE (10%) of FPE were also analyzed to determine the best citric acid concentration to prepare FPE. According to Plate 1, which illustrates the gel electrophoretic patterns of extracted lyophilized proteins with different acid concentrations, all five acid extracted protein samples exhibited protein bands with a similar pattern indicating that proteins extracted from *P. pardalis* might be of same types. The thickness of the protein bands slightly increased with the increasing concentration of acids. Due to the additional recovery of sarcoplasmic proteins along with the myofibrillar proteins under low pH condition, thickness of the protein bands and smears were slightly increased with the increase of citric acid concentration (with the reduction of pH). There was no visually difference found between bands of 15% and 20% citric acid treated samples. Accordingly, 15% citric acid sample was selected for protein extracts preparation.

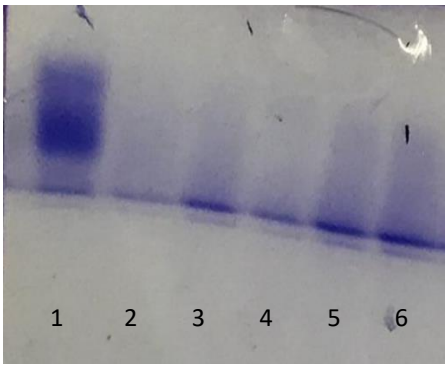

Plate 1. 10% SDS-PAGE image of FPE. Note: Lane 1: Broad range protein molecular weight marker (10 µL), Lane 2: Control (0% Citric acid) FPE, Lane 3: 10% Citric acid added FPE, Lane 4: 5% Citric acid added FPE, Lane 5: 15% Citric acid added FPE, Lane 6: 20% Citric acid added FPE

Enzymatic hydrolysis of acid soluble fish proteins

Before hydrolysis, all the FPE samples were opaque in nature. Later, all the FPH produced by enzymatic hydrolysis were converted to clear light brown color solutions. It showed a slight coagulation at the bottom of Eppendorf tube after the heat denaturation process due to the thermal denaturation of proteins (Boye *et al.*, 1997). Since

coagulation occurred in almost all the FPH samples, it could have not influenced the decision of selecting the best time lag for each enzymatic hydrolysis. In the enzymatic hydrolysis, fish proteins breakdown into soluble peptides and amino acids, and insoluble compounds by proteolytic enzymes (Damodaran et al., 2008). Enzymatic hydrolysis is the efficient method of protein recovery from the animal and fish in food processing industries (Bhaskar et al., 2008).

The Plates 2, 3 and 4 demonstrate the 15% SDS-PAGE gel images for FPH of trypsin, pepsin and papain enzymes, respectively.

The shred (Lane 1; control) which is well matched with FPE has produced a protein band in all three SDS-PAGE images, since it has not subjected to hydrolysis process and remained as the protein itself. In Plate 2, peptide bands could be observed in trypsin treated FPH with all the time lags, which is compatible with control (FPE) suggesting that no complete hydrolysis was occurred in trypsin added samples. It might be due to the presence of trypsin inhibitory compounds of protein in the acid-aided extract of *P. pardalis* protein or else due to the complex enzymatic hydrolysis process including the pH, temperature and time lag of hydrolysis.

There were no clear demarcations of protein bands in pepsin hydrolysis (Plate 3). Absence of protein bands and presence of smears revealed that all the proteins have been hydrolysed properly converted to FPH by the action of pepsin enzyme. In comparison, the Plate 4 carries no protein bands but the smears in the last three lanes (Lane 5, 6, 7) while the lanes 2,3 and 4 appeared similar to lane 1. Accordingly, papain 9 h and pepsin 0 h were determined as the best hydrolysing time lags for respective two enzymes. Since the enzymes are substrate specific, the time required to complete hydrolyzation of extracted proteins vary with different enzymes (Hu *et al*., 2013). Thus, only the fish protein hydrolysates derived from pepsin (0 h) and papain (9 h) hydrolysis were used for the determination of bioactive properties.

Bioactive properties of FPH

Antioxidant activity of FPH

In the DPPH assay, protein hydrolysates reduce the DPPH radicals into a yellow-coloured compounds due to capturing an electrons or hydrogen by the DPPH radicals to become a stable diamagnetic molecule (Liu *et al*., 2010). Based on this principle, the antioxidant activity of a compound can be expressed as its ability to scavenge the DPPH radical (Centenaro *et al*., 2011). Accordingly,

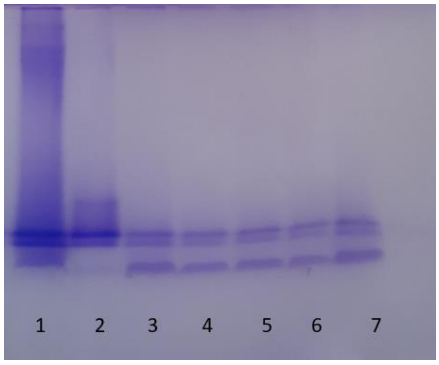

Plate 2. 15% SDS-PAGE image of trypsin treated hydrolysates. Note: Lane 1: Control (FPE), Lane 2: 0 hour (FPH), Lane 3: 3 hours (FPH), Lane 4: 6 hours (FPH), Lane 5: 9 hours (FPH), Lane 6: 12 hours (FPH), Lane 7: 24 hours (FPH)

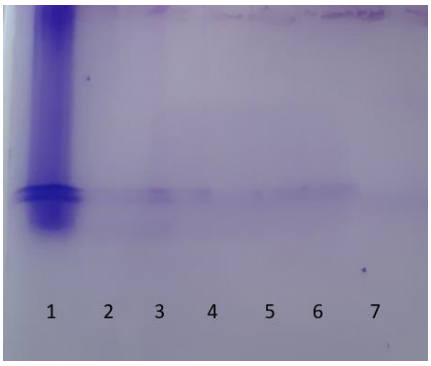

Plate 3. 15% SDS-PAGE image of pepsin treated hydrolysates. Note: Lane 1: Control (FPE), Lane 2: 0 hour (FPH), Lane 3: 3 hours (FPH), Lane 4: 6 hours (FPH), Lane 5: 9 hours (FPH), Lane 6: 12 hours (FPH), Lane 7: 24 hours (FPH)

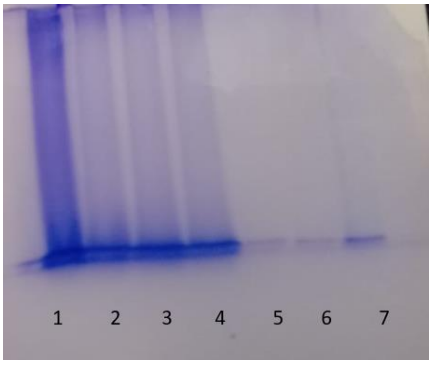

Plate 4. 15% SDS-PAGE image of papain treated hydrolysates. Note: Lane 1: Control (FPE), Lane 2: 0 hour (FPH), Lane 3: 3 hours (FPH), Lane 4: 6 hours (FPH), Lane 5: 9 hours (FPH), Lane 6: 12 hours (FPH), Lane 7: 24 hours (FPH)

Figure 2 shows the DPPH radical scavenging capacity of the pepsin and papain treated FPH in comparison to ascorbic acid where the highest DPPH radical scavenging activity ($P<0.05$) was reported in pepsin treated hydrolysates.

TBARS assay is widely used in scientific researches to quantify the level of lipid peroxidation by means of measuring malondialdehyde concentration (MDA), which is formed as the end product of oxidation of polyunsaturated fatty acids caused by free radical-induced reactions (Oakes and Van Der Kraak, 2003). As shown in Table 1, there was a significant difference ($P<0.05$) in TBARS values among hydrolysates, where the highest lipid peroxidation was observed in the papain treated hydrolysates. TBARS values of all the hydrolysates were significantly higher ($P<0.05$) than the control sample (oil emulsion).

These observations revealed that the pepsin (0 h) treated FPH contains high antioxidant properties compared to the papain (9 h) treated FPH. Since these FPHs are less efficient in retardation of malonaldehyde formation, they should not be incorporated into high lipid-containing foods. Because of derived peptides of hydrolysates is lack in exhibiting antioxidative properties, it might cause lipid oxidation followed by development of rancid aroma due to formation of secondary products including malonaldehyde, ketones, esters and aldehydes. The first known scientific study of antioxidant activity of FPH was reported in 1995. Since then, many studies demonstrated the antioxidant activity of peptides isolated from various FPH (Shahidi *et al.*, 1995).

Metal chelation properties

Metal chelating activity of peptides is beneficial in increasing of bioavailability of minerals, which retards oxidation of food (Hebert *et al.*, 2010). It is useful in the food and pharmaceutical industry as a carrier of micronutrients, which facilitate their absorptions in the digestive system (Guo *et al.*, 2014). As demonstrated in Figure 3, no significant difference was observed between metal chelating activity of two hydrolysates produced from pepsin (0 h) (52.31 % ± 0.93) and papain (9 h) (50.95 % ± 1.17) ($p>0.05$). Besides, pepsin treated FPH showed comparatively high iron (Fe (II)) chelating activity. The results indicated that protein hydrolysates had a noticeable capacity for iron-binding strength.

Antioxidant capacity of bioactive peptides can be attributed to their metal ion chelation properties, radical scavenging and inhibition of lipid peroxidation of peptides. Sarmadi and Ismail, 2010 proposed that the structure of the resulting peptides, its amino acid sequence and the size can affect the antioxidant properties. According to them, the chelating activity was positively correlated with the histidine content of the purified

Table 1. TBARS assay of FPH

Hydrolysates	TBARS value† (MDA mg/ L)
Pepsin	0.087 ± 0.05[b]
Papain	0.117 ± 0.09[a]
Control	0.045 ± 0.09[c]

†Values with the same letters are not significantly different at $p<0.05$.

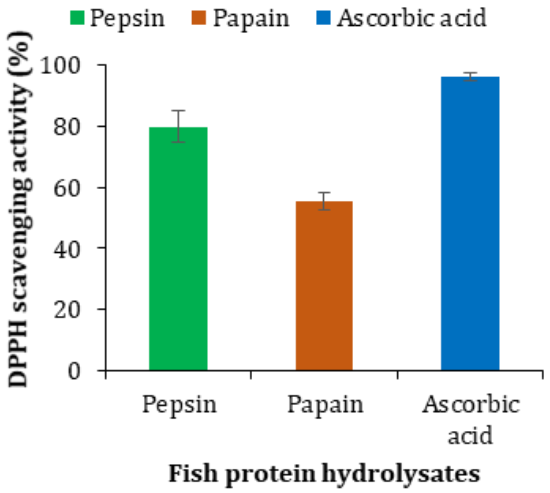

Figure 2. DPPH Radical Scavenging activity of fish hydrolysates produced from pepsin (0 h) and papain (9 h) enzymatic hydrolysis

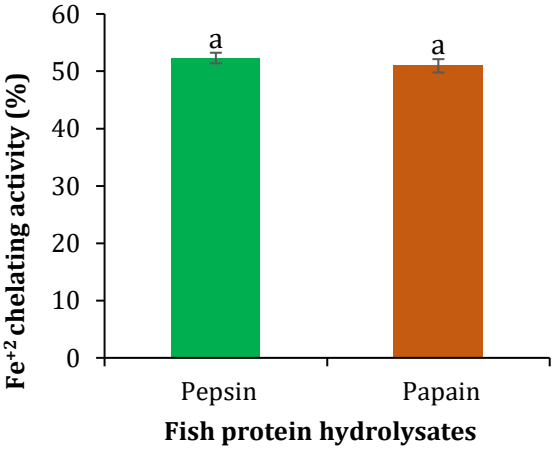

Figure 3. Ferrous (Fe (II)) chelating activity of fish hydrolysates produced from pepsin (0 h) and papain (9 h) enzymatic hydrolysis

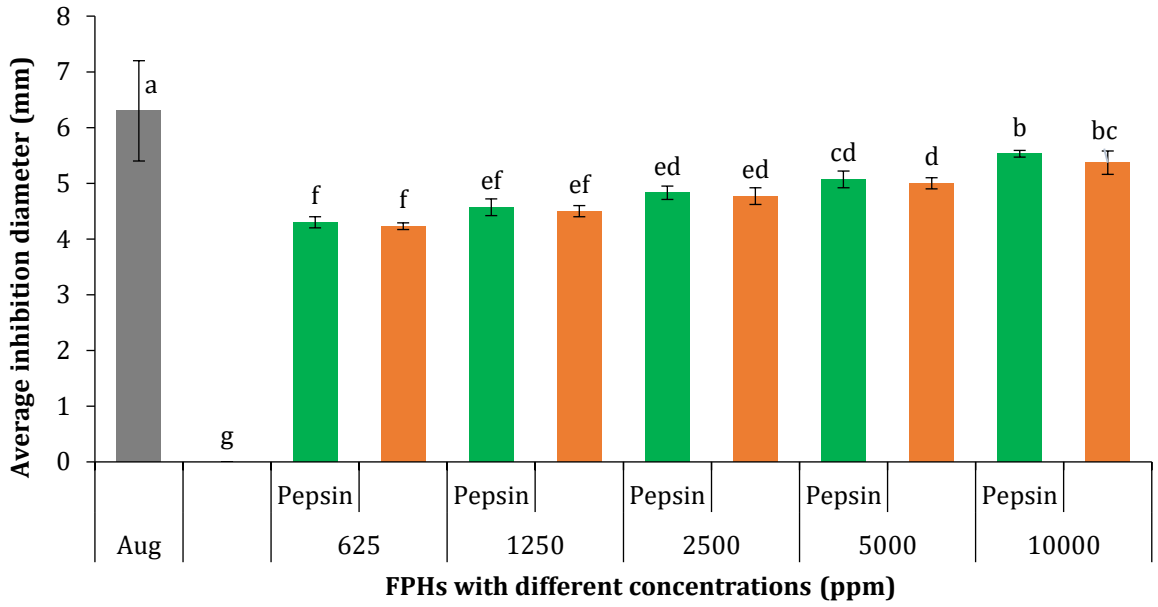

Figure 4. Antimicrobial activity of FPHs. Note: Aug: Augmentin (Positive control), DW: Autoclaved distilled water (Negative control)

fractions of a chickpea protein hydrolysate, and the small peptide size is with the best chelating activities.

In the current study, it was revealed that the considerably low iron binding ability of both FPHs derived from acid soluble P. pardalis FPE. Hence, the results of TBARS assay and Fe (II) chelating activity are similar in the present study. As transitional metal ions, such as Fe (II) can catalyze the generation of reactive oxygen species that oxidize unsaturated lipids (Stohs and Bagghi, 2005).

Antimicrobial properties

Fish protein-derived peptides demonstrate different types of antimicrobial properties. These peptides are positively charged, short amino-acid-chain molecules that are involved in host defense mechanisms and play a vital role in natural immunity by interacting directly with bacteria and killing them (Zhang *et al*., 2008). According to the results of the present study, all the concentrations of FPHs exhibited clear inhibition zones even at the lowest concentrations of both the treatments. The two treatments (enzymes) at all the concentrations were not significantly different ($p>0.05$) but were significantly different from positive control ($p<0.05$). Among the treatments, the highest concentration of pepsin treated FPH demonstrated the highest antibacterial activity. Diameter of inhibition increased with the increasing concentration of both treatments though there was no significant difference between the treatments. Antimicrobial activity of derived FPH was illustrated in Figure 4.

CONCLUSION

The FPH produced by enzymatic hydrolysis (papain (9 hours), pepsin (0 hour)) of acid extracted *P. pardalis* demonstrated strong antibacterial and antioxidant activities as favorable bioactive properties. Further, FPHs derived from *P. pardalis* showed a ferrous chelating activity which could indirectly strengthen the demonstrated antioxidant properties.

The results of the present study are compatible with the results of a previous study on antibacterial activity against *E. coli* and *Listeria innocua*, of FPHs produced using *Sepia pharaonis* (cuttlefish) ink extract and *Scomber scombrus* (Atlantic mackerel) by-products using commercial enzymes including papain, neutrase, flavoenzyme and protamex (Ennaas *et al*., 2015)

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