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Research Article

A comparative study on some properties and antioxidant activity of *Hypophthalmichthys molitrix* (Valenciennes, 1844) protein hydrolysates produced by different hydrolysis methods

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Abstract

Some functional, physicochemical and antioxidant properties of *Hypophthalmichthys molitrix* protein hydrolysates produced by alcalase (SCPH-alcalase), acid (SCPH-acidic) and alkaline (SCPH-alkaline) were investigated. No significant difference was observed for degree of hydrolysis (DH), L*, a* and b* values among all types of hydrolysate samples ($p>0.05$). The highest protein recovery, protein and essential amino acids content were observed in SCPH-alcalase. All protein hydrolysates displayed antioxidant activities, that enhanced by increasing the hydrolysates concentration. The chemical hydrolysates had the highest DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity, while SCPH-alcalase showed the highest ABTS (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical-scavenging activity and FRAP (Ferric reducing antioxidant power) ($p<0.05$). The highest solubility and the lowest fat adsorption were observed with the SCPH-alcalase ($p<0.05$). Increased numbers of hydrophilic groups of SCPH-alcalase are supported by FTIR spectra. The results showed the significant effect of different hydrolysis conditions on hydrolysates properties and indicated alcalase enzyme might be more suitable to produce hydrolysates with desirable functional, nutrition and antioxidative properties.

Keywords: Fish protein hydrolysate, *Hypophthalmichthys molitrix*, Chemical hydrolysis, Enzymatic hydrolysis, Antioxidant capacities

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Introduction

Hydrolysis converts proteins into smaller peptides with different sizes, containing 2-20 amino acids, thereby making hydrolysates the most available amino acid source for various physiological functions of human body (Halim *et al.,* 2016). Hydrolysis of proteins leads to numerous alterations in protein functional characteristics that are important, particularly if they are used as ingredients in food products (Van der Ven *et al.,* 2002). Many studies exhibited fish protein hydrolysates with high nutritional and desirable functional properties (Kristinsson and Rasco, 2000a; Gbogouri *et al.,* 2004; Wasswa *et al.,* 2007), as well as antioxidant activity of peptides derived from natural marine sources like round scad (*Decapterus maruadsi*) (Thiansilakul *et al.,* 2007); smooth hound (*Mustelusmustelus*) (Bougatef *et al.,* 2009); grass carp (*Ctenopharyngodon idella*) (Li *et al.,* 2012); anchovy sprat (*Clupeonella engrauliformis*) (Ovissipour *et al.,* 2013); whitecheek shark (*Carcharhinus dussumieri*) (Alinejad *et al*., 2017). The incorporation of synthetic antioxidants in foodstuffs is restricted and there is a growing interest in using natural antioxidants, including peptides derived from hydrolyzed food proteins, as alternatives to synthetic ones (Ovissipour *et al.,* 2013).

During the past few decades, laboratory to pilot/industrial-scale produced of fish or other protein hydrolysates are conducted using chemical (acid or alkaline) and biological hydrolysis (alcalase) methods. Chemical hydrolysis has been the method of choice for the industry primarily in the past because it is relatively inexpensive and quite simple to conduct. This process is carried out under high temperature and pH, which result in reduction of nutritional qualities, poor functionality of fish protein hydrolysate (FPH), and restricted use as flavor enhancers (Kristinsson and Rasco, 2000b). Enzymatic processes overcome these disadvantages by using lower temperature over a pH range of 5-8, which have received more attention recently in different fish species. The high cost of enzymes is a barrier for applying enzymatic processes in the food industry (He *et al.,* 2013). However, this is an effective method for improving the functional properties of protein without affecting its nutritive value, compared with chemical hydrolysis (Li *et al.,* 2012). On the other hand, by applying enzyme technology for protein recovery in fish processing, it may be possible to produce a broad spectrum of food ingredients to improve and upgrade the functional and nutritional properties of protein (Ovissipour *et al.,* 2013). Thus, enzymatic hydrolysis of protein can provide more marketable and value-added products of fish protein hydrolysates (Hu *et al.,* 2013).

Silver carp (*Hypophthalmichthys molitrix*) is a major cultured fresh water species, which was used for the production of protein hydrolysates. Dong *et al.* (2008) and Zhong*et al.*(2011) investigated antioxidant and some functional properties of protein

hydrolysates from silver carp muscle and by-product by enzymatic hydrolysis, respectively. Nevertheless, no comparative information regarding antioxidant activities, degree of hydrolysis, protein recovery, protein content, solubility, fat adsorption, color, amino acid composition and FTIR spectra of protein hydrolysates obtained from silver carp muscle under three different hydrolysis methods, including acid, alkaline and alcalase treatments has been reported. This study offers better understanding about some functional, physico-chemical and antioxidant properties of silver carp protein hydrolysates produced from enzymatic and chemical hydrolysis processes.

Materials and methods

Production of fish protein hydrolysates

Silver Carp (weight 1000-1200 g/fish) were purchased from Sari local fish market (Mazandaran, Iran) and transferred in ice to the laboratory within 30 min. Fish were filleted and ground in a moulinex® blender (Labtron, LS100, Thailand). FPHs were prepared according to Ovissipour *et al.*(2012). Minced meat was mixed with distilled water 1:2 (w/v). To deactivate the endogenous enzymes, samples were heated at 85°C for 20 min in a water bath (W614-B, FaterRizpardaz, Tehran, Iran). After providing optimum enzymatic hydrolysis conditions (temperature 50°C, pH 8.5) obtained by Ovissipour*et al.*(2009), alcalase (2.4AU/g, Novozymes Co, Bagsvaerd, Denmark) was added to the substrate based on enzyme activity per kilogram crude protein (34 AU/kg protein). The hydrolysis was performed at 50°Cfor 120 min. The reaction was terminated by heating the mixture at 95°C for 20 min. Acidic and alkaline hydrolysis were done, at 70°C for 6h at pH 4 and 11, respectively (Ovissipour *et al.*, 2012). In order to terminate the acidic and alkaline reactions, neutralization was carried out, using 2 N NaOH and 2 N HCl, respectively.

The samples were cooled and then centrifuged at 8,000×g for 20 min using a centrifuge (Hermle labor technik GmbH z206A,Germany) and hydrolysates were freeze-dried using a freeze dryer (FDU, Operon, 8624, South Korea).

Crude protein

Total crude protein (N × 6.25) in raw material and FPHs was determined using the Kjeldahl method (AOAC, 2002).

Protein recovery

Protein of fish hydrolysates were measured by the Biuret method in the supernatant following centrifugation, using a liquid solution of bovine serum albumin (BSA, 4 g/dL according to the manufacturer, Zistchimi, Tehran, Iran) as a standard (Layne, 1997). Absorbance was measured at 540 nm in a UV/vis spectrophotometer (T80^{+}UV-VIS, PG Instruments Ltd, Leicester, UK). Protein recovery (%) was calculated as the amount of protein present in the soluble fraction of the hydrolysate by Biuret method relative to the initial amount of protein present in the reaction mixture by Kjeldahl (Ovissipour *et al.*, 2009).

Degree of hydrolysis

Degree of hydrolysis was determined according to the method of Hoyle and Merritt (1994) as described by Ovissipour *et al.* (2009). The 20% trichloroacetic acid (TCA) was added to the same volume of the supernatant to obtain 10%TCA. Then the mixture was centrifuged at 8000×*g* for 20 min. The degree of hydrolysis was calculated as follows:

%DH= (10%TCA souble protein in the sample/total protein in the sample) × 100

Amino acid composition

To estimate the amino acid composition, FPHs was hydrolyzed with 6M HCl at 110 C for 22h in a capped glass test tube. Derivatisation was done using phenyl isothiocyanate (PITC, Fluka, Hanover, Germany), prior to HPLC analysis (Smartline 1000, Knauer, Berlin, Germany) using the method of Antoine*et al.*(1999). The amino acids were analyzed using a 250×4.6mm C18 column (Knauer) at a flow rate of 0.5 mL/min with a Smartline UV detector 2500 (Knauer) at 254 nm.

Chemicalscore

The chemical score of FPHs was computed to study the nutritional value of silver carp protein hydrolysates, which is related to the essential amino acid (EAA) profile in standard protein as described by FAO/WHO (1985).

Solubility

The hydrolysate solutions (10 mg/mL) were prepared by dissolving hydrolysates in distilled water at a wide range of pH values from 1.0 to 10.0 with 6 N HCl or 6 N NaOH. The mixture was stirred for 30 min and centrifuged at 8000×g for 15min (Dong *et al.,* 2008). The Protein content of the supernatant was determined using the Biuret method (Layne, 1997) and the solubility was estimated using the following equation:

%Solubility = (Protein content in the supernatant /Total protein content in the sample) ×100 (1)

Fat Adsorption

Fat adsorption was measured according to the method of Shahidi *et al.* (1995) with slight modifications. A 10mL of sunflower oil (Nina, FRICO, Tehran, Iran) was added to 500mg sample in a 50mL centrifuge tube and the mixture was Vortexed (Vortex, Labtron, LS100, Tehran, Iran) for 30 s every 10 min to 30 min. Then centrifuged at 1000×g for 30 min. Free oil was decanted and the fat adsorption of the sample was determined from the weight difference.

Color measurement

Color Flex spectrocolorimeter (Hunter Lab Reston, VA, USA) was used to determine the color of the samples. The color parameters of the hydrolysates were expressed as

L^*(lightness/brightness), a^* (redness/greenness), and b^* (yellowness/blueness) values.

Antioxidant activity

DPPH radical scavenging assay

The 2, 2-diphenyl-1-picrylhydrazyl(DPPH) radical scavenging activity was determined by following the method of You *et al.*(2009), with slight modifications. 2mL of hydrolysates solution with different concentrations of FPHs (1-10 mg protein/mL) was mixed with 2mL of 0.1 mM DPPH in methanol and incubated for 30min in the darkness at room temperature. The absorbance of solutions was measured at 517 nm ($T80^+$UV-VIS, PG Instruments Ltd, Leicester, UK). The control was measured in the same manner, except that distilled water was used instead of sample. The DPPH radical scavenging activity (DRSA) was calculated as follows:

$$DRSA\ (\%) = [1-(A_s-A_0/A_c)] \times 100 \quad (2)$$

Where, A_s, A_0 and A_c are absorbance of FPHs mixed with DPPH solution, FPHs mixed with methanol, and distilled water mixed with DPPH solution, respectively.

ABTS assay

The ABTS radical (2, 20-azinobis-(3-ethylbenzothiazoline-6-sulfnic acid)) scavenging activity of the samples was measured according to the method of Gómez-Guillén*et al.*(2010). A stock solution of ABTS radical included in 7mM ABTS in potassium per sulfate 2.54mM, was kept in the dark at room temperature for 16h. An aliquot of stock solution was diluted with distilled water to prepare the working solution of ABTS radical with an absorbance of 0.70±0.02 at 734nm. A 20µL of sample containing various concentrations (1 to 10 mg protein/mL) of hydrolysates and distilled water (as control) were mixed with 980µL of ABTS radical working solution. They were incubated at 37°C for 30min in the dark. Then the reduction of absorbance at 734nm was measured. The ABTS radical scavenging activity was calculated according to the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = [(B-A/B)] \times 100 \quad (3)$$

Where A and B are the absorbance of the sample and blank, respectively.

Ferric reducing antioxidant power (FRAP) assay

The FRAP method described by Gómez-Guillén *et al.* (2010), was used to determine the ferric ion reducing capacity of the samples. It is based on the increase in absorbance at 593nm due to the formation of the complex tripyridyltriazine (TPTZ)-Fe (II) in the presence of reducing agents at 37°C. A 100µL of different concentrations of hydrolysates (1-10mg/mL) was incubated (37°C) with 3mL of FRAP reagent (containing TPTZ and $FeCl_3$). Absorbance was measured at 593nm after 30min in the dark. The results were expressed as µmol $FeSO_4$:$7H_2O$

equivalents/L of FPH based on a standard curve of $FeSO_4.7H_2O$, which relates the concentration of $FeSo_4$:H_2O (μmol) to the absorbance at 593 nm.

Fourier transforms infrared (FTIR) spectroscopy

The FTIR spectra were obtained from discs containing 10 mg dried sample in approximately 90 mg potassium bromide (KBr). All spectra were analyzed using a FTIR spectroscopy (Nicolet Nexus 870 FT-IR, USA). The spectra were recorded in the range of 400 to 4000 cm^{-1}, with 8 scans and at a resolution of $4cm^{-1}$.

Statistical analysis

All experiments were carried out in triplicates (n=3), except for determination of amino acid composition (n=1) and FTIRspectra (n=1). Data were analyzed using SPSS statistical software (Version 16.0.0; IBM Institute Inc., USA). One-way ANOVA and the Duncan's test were used for mean comparisons. Data are presented as means±standard deviation and Significance was determined at a 95% probability (p<0.05).

Results

Degree of hydrolysis (DH), Protein content and protein recovery

The DH obtained by different hydrolysis treatments is presented in Table 1. DH values of hydrolysates did not differ markedly in all cases (p>0.05). Table 1 shows protein content and protein recovery of the SCPHs. The results showed a significant increase for SCPH-alcalase, and no considerable difference between both hydrolysates prepared by chemical hydrolysis methods.

Table 1: Degree of hydrolysis (DH), protein recovery (PR)and protein content of silver carp protein hydrolysates prepared using different hydrolysis methods.

Sample	DH (%)	PR (%)	Protein content (%)
SCPH-alcalase	16.02±0.82[a]	66.00±2.80[a]	74.26±0.62[a]
SCPH-acidic	15.43±0.97[a]	41.55±3.04[b]	38.68±1.64[b]
SCPH-alkaline	14.00±1.13[a]	38.37±2.77[b]	36.40±0.92[b]

Different letters in the same column indicate significant differences (p<0.05).
Values were given as mean ± standard deviation.

Amino acid composition and chemical score

The amino acid composition and the chemical score of silver carp protein hydrolysate are shown in Table 2. The amino acid content of all hydrolysates was generally higher in essential amino acids compared to the suggested amino acid pattern recommended by FAO/WHO (1985) for adult humans except in terms of methionine in chemical hydrolysates. As shown in Table 2, the obtained essential amino acids values were 62.51%, 52.48% and 49.71% in SCPH-alcalase, SCPH-acidic and SCPH- alkaline, respectively. Several amino acids, such as Tyr, Met, His, Lys, and Trp, were generally regarded as antioxidants (Dong *et al.*, 2008). According to the results (Table

2), the total content of the mentioned antioxidant amino acids of SCPH-alcalase, SCPH-acidic and SCPH-alkalinewere20.76%, 19.93% and 19.70%, respectively.

Table 2: The amino acid composition and chemical score of silver carp protein hydrolysate.

Amino Acid	Quantity of Amino Acid (%)			Reference protein [a]	Chemical Score		
	SCPH-alcalase	SCPH-alkaline	SCPH-acidic		SCPH-alcalase	SCPH-alkaline	SCPH-acidic
Alanine	3.02	5.08	5.19		-	-	-
Arginine*	18.75	15.02	18.15	-	-	-	-
Aspartic acid	5.14	8.48	7.02	-	-	-	-
Cystine	0.27	0.72	0.04	-	-	-	-
Glutamic acid	11.69	11.60	10.18	-	-	-	-
Glycine	4.74	11.25	11.79	-	-	-	-
Histidine*	6.50	9.52	9.22	-	4.06	5.94	5.76
Isoleucine*	4.15	2.62	2.45	1.6	3.19	2.01	1.88
Leucine*	8.08	4.25	3.97	1.3	4.25	2.23	2.08
Lysine*	8.29	7.54	7.52	1.9	5.2	1.45	1.32
Methionine*	2.90	1.27	1.65	1.6	1.70**	0.74**	0.97**
Phenylalanine*	4.60	3.28	3.08	1.7	-	-	-
Proline	5.01	5.73	5.79	-	-	-	-
Serine	3.81	3.60	3.21	-	-	-	-
Threonine*	4.22	2.30	2.47	-	4.69	2.56	2.74
Tyrosine	3.07	1.38	1.53	0.9	-	-	-
Valine*	5.01	3.92	3.98	-	3.85	3.01	3.06
Hydroxyprolin	0.75	2.44	2.76	1.3	-	-	-

[a] Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1985)
*Essential amino acids
**The apparent rate limiting amino acid shown

Solubility

The solubility of hydrolysates obtained chemical and biochemical hydrolysis at different pH values are shown in Figure 1. All hydrolysates had solubility above solubility of SCPH-alcalase was 90% at pH ranging from 1.0 to 10.0, with lower values at pH 5. Also, protein solubility of SCPH-alcalase was significantly higher than that of the other hydrolysates.

Fat Adsorption

As shown in Table 3, SCPH-alcalase showed lower fat adsorption (p<0.05).

Color parameters

The color parameters of the FPHs did not differ markedly (p>0.05) (Table 4). However, lower L*-values, higher redness (a*-value) and yellowness (b*-value) were found in SCPH-alcalase, when compared with hydrolysates prepared with chemical treatments.

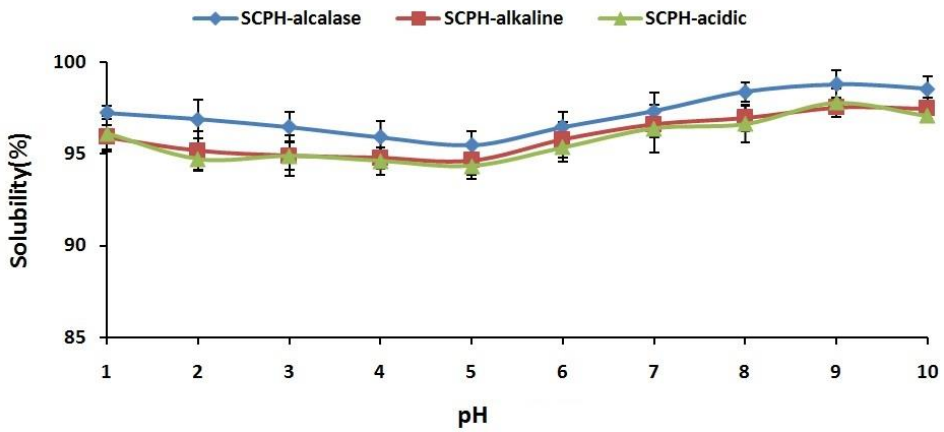

Figure 1: Solubility of silver carp protein hydrolysates prepared under different condition hydrolysis. Bars represent standard deviations from triplicate determination.

Table 3: Fat adsorption of different silver carp protein hydrolysates.

Sample	SCPH-alcalase	SCPH-acidic	SCPH-alkaline
Fat adsorption (mL/g of protein)	1.71±0.14[b]	2.44±0.16[a]	2.32±0.2[a]

Different letters indicate significant differences ($p< 0.05$).
Values were given as mean ± standard deviation.

Table 4: Color parameters of different silver carp protein hydrolysates.

Sample	Color parameters		
	L^*	a^*	b^*
SCPH-alcalase	88.35±0.1[a]	-1.19±0.07[a]	15.06±0.63[a]
SCPH-acidic	88.50±0.95[a]	-1.19±0.03[a]	14.09±1.21[a]
SCPH-alkaline	88.49±0.80[a]	-1.19±0.08[a]	13.80±0.96[a]

Different letters in the same column indicate significant differences ($p < 0.05$).
Values were given as mean ± standard deviation.

Antioxidant activity

DPPH radical-scavenging assay

Figure 2a shows the DPPH radical-scavenging activity of the SCPHs at various concentrations. As seen, these results indicated that all protein hydrolysates exhibited scavenging activity. The reducing power of all hydrolysates increased with protein concentrations (1-7mg protein/mL) (p<0.05) and afterward remained almost unchanged. Also, chemical SCPHs exhibited higher DPPH radical scavenging activity than enzymatic hydrolysates at the same concentrations from 1 to 10mg protein/mL (p<0.05).

ABTS radical scavenging activity

As shown in Figure 2b, all hydrolysates exhibited the ABTS radical scavenging activity. It is obvious that the hydrolysates obtained with alcalase had the highest ABTS activity value, which increased with protein concentration. The ABTS activity values of hydrolysates produced by Alcalase, acidic and alkaline hydrolysis improved from 33.93%, 7.58% and 2.08% at the lowest concentration (1mg protein/mL) to a maximum value of 96.48%, 77.26% and 47.12%, at concentration of 4, 7 and 7mg/mL, respectively.

Thereafter, a slight increase in ABTS radical activity was observed to 10 mg protein⁄mL.

FRAP assay

The FRAP assay of SCPHs was shown in Figure 2c. This method such as the ABTS assay showed a similar increasing trend. The SCPH-alcalase showed significantly higher reducing power than other hydrolysates (p<0.05).

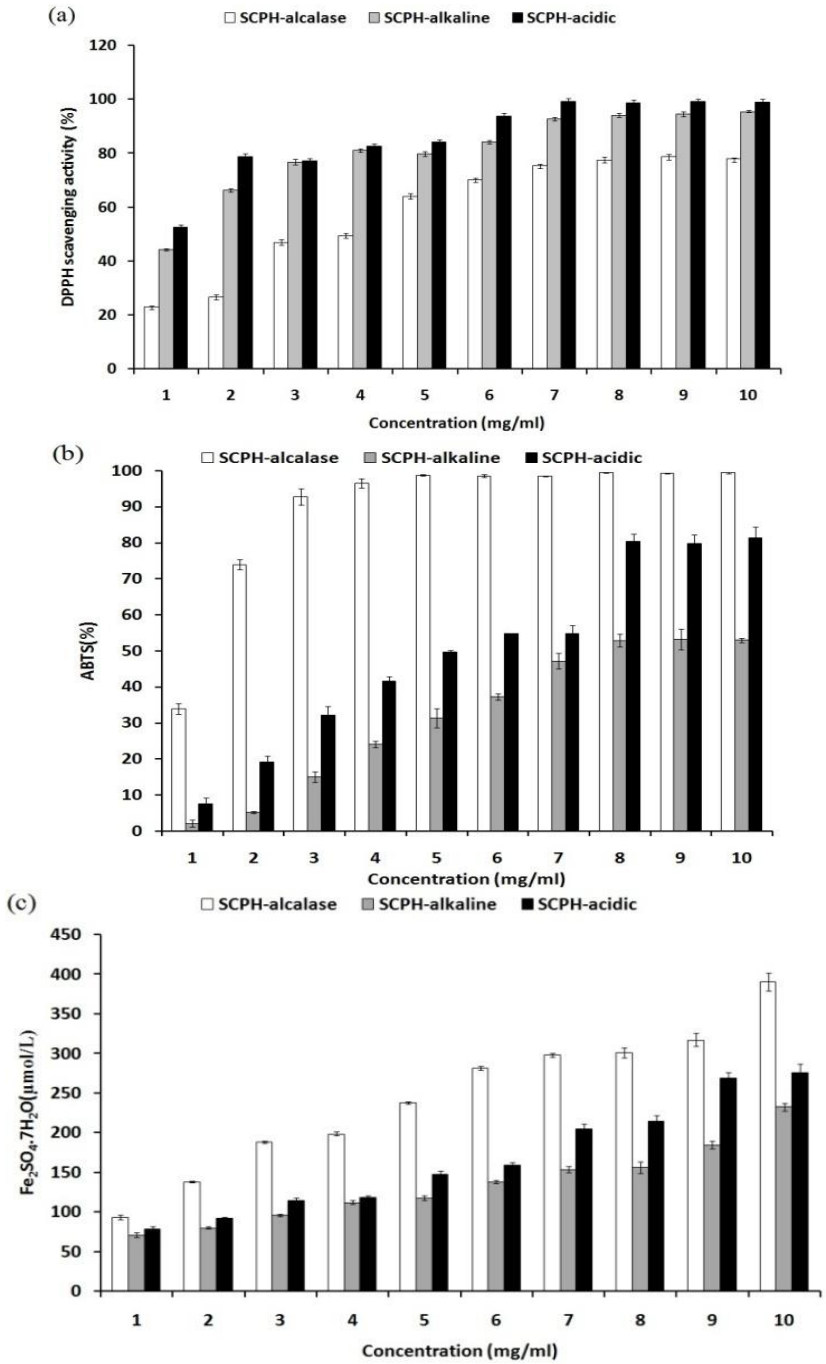

Figure 2: DPPH (A) ABTS (B) FRAP (C) radical scavenging activity of silver carp protein hydrolysates produced with different hydrolysis methods. Bars represent standard deviations from triplicate determinations.

FTIR spectroscopy

The FTIR spectra of SCPH extracted with different hydrolysis methods are shown in Figure 3. The spectra of all FPHs had the major characteristic peaks in the amide region, including similar bands with different peak intensities and amide displacements, depending on the different hydrolysis methods. As can be seen in Figure 3, silver carp hydrolysates produced with alkaline and acid had lower amplitude of peaks and higher wavenumbers, when compared to hydrolysates made with alcalase. For example, the amide-A band, in these three hydrolysates were mainly observed at 3288 cm^{-1} (SCPH-alcalase), 3433 cm^{-1} (SCPH- alkaline) and 3442 cm^{-1} (SCPH- acidic).

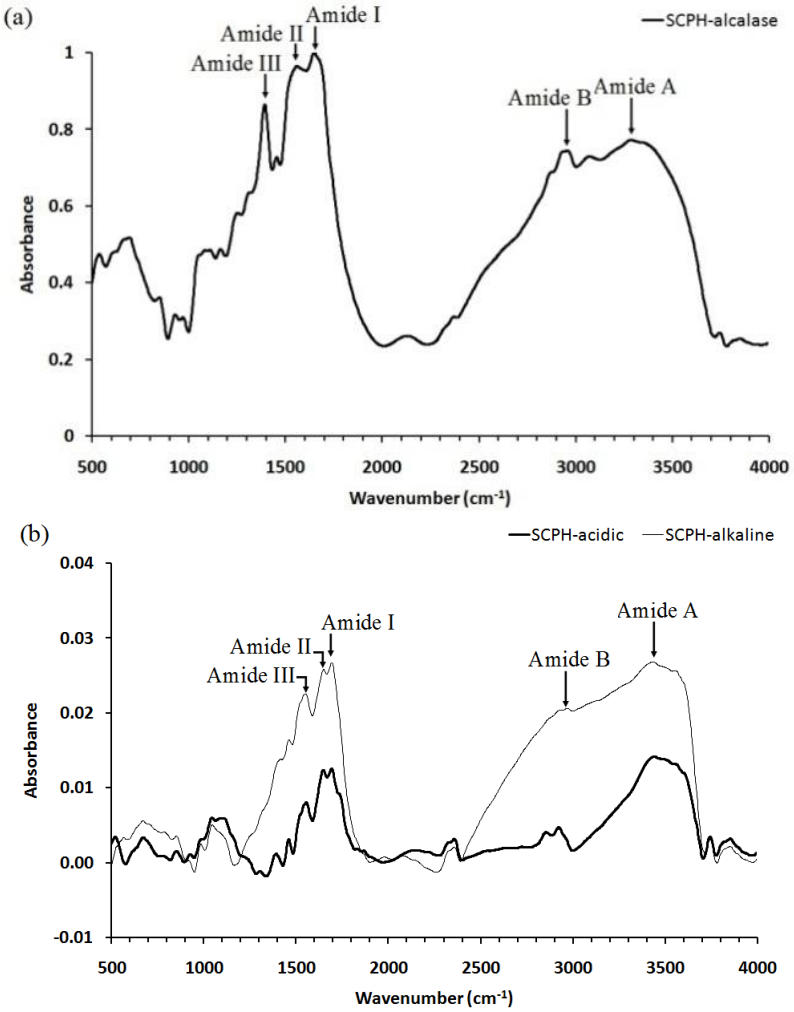

Figure 3: FTIR spectra of silver carp protein hydrolysates obtained using biochemical hydrolysis (A) and chemical hydrolysis (B).

Discussion

The effect of hydrolysis is shown by the degree of hydrolysis (DH), which is defined as the percentage of broken peptide bonds. This is an important indicator used for comparison among different protein hydrolysates (Giménez *et al.*, 2009). Dong *et al.*(2008) and Li *et*

al.(2012) reported that the antioxidant and functional properties were closely related to DH of fish protein hydrolysates derived using Alcalase. Also, You *et al.*(2009) noted that different DH led to different peptide chain lengths and exposure of terminal amino groups, which greatly affect the antioxidant activities of the hydrolysates. Therefore, DH values remained almost the same in all hydrolysates. Chemical hydrolysis at 70˚C required more time (6h) than alcalase (2h), to reach approximately the similar DH.

The obtained protein content and protein recovery values confirmed the study performed by Ovissipour *et al.*(2012), which reported that the highest recovery and protein content of hydrolysates are related to alcalase hydrolysis of Persian sturgeon visceral protein.

Nutritional value and functional properties of hydrolyzed proteins are affected by the amino acid composition (Dos Santos *et al.*, 2011). The chemical score has been used to evaluate nutritive value of a protein by comparing the amount of essential amino acids in the test with reference proteins (Ovissipour *et al.*, 2012). The results showed that the protein hydrolysates obtained in this study can effectively supply nutritional requirement of an adult human. So, it can be considered as a source of protein to human requirements with Met as the rate limiting amino acid among those measured. These results are in agreement with our previous study on yellowfin tuna (*Thunnus albacores*) viscera hydrolysates (Motamedzadegan *et al.*, 2010). As shown in our results, based on the essential amino acids values, the hydrolysates would be ranked in the following order: SCPH-alcalase> SCPH-acidic > SCPH-alkaline. Also, the total content of the mentioned antioxidant amino acids of SCPH-alcalase was higher than those of the hydrolysates made by chemical hydrolysis. Generally, the differences in amino acid composition of hydrolysates depended on the existing differences in enzyme specificity and hydrolysis conditions, leading to the various antioxidative activities (Khantaphant *et al.*, 2011).

The high solubility of SCPH may be related to smaller peptide size and to the balance of hydrophilic and hydrophobic forces of the peptides. The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and lead to greater solubility. In addition, insoluble protein fractions were removed by centrifugation and collected soluble peptides before freeze drying, which are other important reasons for increased solubility (Kristinsson and Rasco, 2000a). Generally, higher protein solubility of SCPH-alcalase than the other hydrolysates in our study is probably because of the higher numbers of amino and carboxyl groups generated in the enzymatic hydrolysis, which leads to more hydrophilicity of proteins.

Fat adsorption is an important functional characteristic that is required for meat and confectionery industry (Kristinsson and Rasco, 2000a). Lower fat adsorption

of SCPH-alcalase might be explained by the exposure of more polar groups on the surface of SCPH-alcalase, which is resulted in increased water solubility (Figure 1), hydrophilicity (Fig. 2) and decreased fat adsorption of these hydrolysates. On the other hand, the fat binding capacity of hydrolysates may be influenced by surface hydrophobicity of FPH (Kristinsson and Rasco, 2000a; Šližyté*et al.*, 2005; Wasswa *et al.*, 2008). All samples exhibited a light yellowish color. Dong *et al.*(2008) reported that the dark color of fish protein hydrolysate resulted from lipid oxidation and the Maillard browning reaction. Based on our findings, it is likely that the color of the protein hydrolysate is positively influenced by hydrolysis conditions.

Due to the diversity of oxidation processes and antioxidant mechanisms of protein hydrolysates, the use of a single method to evaluate the antioxidant activity cannot provide a clear idea about its real antioxidant potential (Bougatef *et al.*, 2009). Therefore, the antioxidant activities of silver carp hydrolysates produced under chemical and biochemical hydrolyses were evaluated by several methods, including DPPH radical-scavenging activity, ABTS radical-scavenging activity and FRAP.

The results showed that DPPH radical-scavenging activity of the SCPHs increased with protein concentrations. Similar results have been reported by Bougatef *et al.*(2009), Jemil *et al.*(2014) and Alinejad *et al*. (2017), who observed that the DPPH scavenging activity increased with the concentration of protein hydrolysates. DPPH radical scavenging activity of chemical SCPHs was higher when compared to enzymatic hydrolysates. It seems that the radical scavenging ability of protein hydrolysates would be related to the peptides length and the different amino acids composition (Jemil *et al.*, 2014). Moreover, DPPH radical-scavenging activity is affected by the increased hydrophilicity of the shorter peptides that makes it more difficult to react with the lipid-soluble DPPH radicals (Binsi *et al.*, 2016). From results, the total content of hydrophobic amino acids of SCPH-acidic was slightly higher than that of other hydrolysates. This confirms higher DPPH radical scavenging of SCPH-acidic, when compared with that of other SCPHs. The results were in agreement with Klompong *et al.*(2007), Theodore *et al.*(2008), You *et al.*(2009),Li *et al.* (2012) and Morales-Medina *et al.* (2016). They explained that the higher level of DPPH activity is associated with high hydrophobic amino acids content which would make the peptides more accessible to the DPPH radicals and allow them to trap the radical more easily.

The results obtained for ABTS radical activity in this study were similar to that reported for grass carp (*Ctenopharyngodonidella*) protein hydrolysates prepared with alcalase and papain (Li *et al.*, 2012). Chalamaiah *et al.*(2015) reported that low molecular mass peptides (<10 kDa) present in the protein hydrolysates might be responsible for the exhibited radical inhibiting activity. Based on our results,

gel electrophoresis indicated that the weight of these peptides was <10 kDa (data not shown). Thus, all hydrolysates contained antioxidant peptides that could react with radicals to convert them to more stable products, and terminate the radical chain reaction. Despite of the relative similarity in DH, SCPH-acidic exhibited higher ABTS radical scavenging activities than SCPH-alkaline. This might be attributed to the employed hydrolysis conditions influencing the antioxidant activity of protein hydrolysates. Also, during hydrolysis, a wide variety of smaller peptides and amino acids are generated (Klompong *et al.*, 2007).

Furthermore, the results of FRAP assay of SCPHs indicated that peptides generated from diverse hydrolysis methods, had different capacities of providing electron to the radicals.

Generally, the amide-A band in FTIR spectra is associated with the N-H stretching vibration that had the reducing manner in SCPH-acidic, SCPH-alkaline and SCPH-alcalase, respectively. Its position is shifted to lower frequencies when the involvement of N-H group of shorter peptide fragments in hydrogen bonding occurs (Nikoo *et al.*, 2014). This behavior for amide-A spectra also reported by Gómez-Guillén*et al.*(2010) for squid skin hydrolysate which is attributed to a greater hygroscopicity in this hydrolysate due to more hydrophilic nature of its amino acid composition, in comparison with the tuna skin hydrolysate. The lower wavenumber (2959 cm^{-1}) for the amide-B peak corresponds to asymmetric stretch vibrationof =C-H as well as $-NH_3^+$ observed in SCPH-alcalase (Gómez-Guillén *et al.*,2010). Hoque *et al.*(2011) observed higher amplitudes of amide-A and amide-B peaks of film prepared from gelatin with partially hydrolyzed, compared with gelatin film without hydrolysis. They noted higher amount of $-NH_2$ or $–NH_3^+$ group obtained from hydrolysis process more likely resulted in the higher amplitude of amide-A and amide-B peaks obtained. This is in agreement with our findings which observed the amplitude of amide-B peak 0.0047, 0.02 and 0.074 for SCPH-acidic, SCPH-alkaline and SCPH-alcalase, respectively. The amide I band (associated with C=O stretching vibration), between 1600 and 1700 cm^{-1}, is the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Nagarajan *et al.*, 2012). Exact location depends on the hydrogen bonding and the conformation of the protein structure (Nikoo *et al.* 2014). In this study, the amide-I peak was observed at 1651 cm^{-1} and 1694 cm^{-1} for hydrolysates made by enzymatic and chemical hydrolysis, respectively. The amide II band is less sensitive to secondary structure than the amide I, but because of its nature (in-plane NH bending and CN stretching vibration); it is very much influenced by hydration (Gómez-Guillén *et al.*, 2010). Peaks at wavenumbers of 1200-1480 cm^{-1} (amide-III) assigned to the vibrations in plane of C-N and N-H groups (Zhang *et al.*, 2011). The higher amplitude of peaks and lower wavenumbers of

SCPH-alcalase is associated with more hydrophilicity in these hydrolysates. This can be confirmed by the increase in solubility of SCPH-alcalase. Thus, it can be concluded that the secondary structure of hydrolysates obtained from silver carp was affected by different hydrolysis processes.

Generally, protein hydrolysates obtained by different methods of hydrolysis have more advantages in comparison with intact protein. All hydrolysates possessed antioxidant activities, which increased with the concentration of protein. This indicates that they have the potential to prevent lipid oxidation by radical scavenging activity. The SCPH prepared by alcalase had the highest potential as nutrition and antioxidative ingredients in human diets, due to higher levels of essential amino acids, protein recovery, protein content, solubility, FRAP and ABTS radical-scavenging. Also, FTIR spectra showed the highest hydrophilicity of SCPH-alcalase. Therefore, hydrolysis method has notable influence on physicochemical, functional and antioxidant properties of FPHs, which is important for their food and pharmaceutical applications.

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