Functional properties of fish protein hydrolysates from Cuttlefish (*Sepia pharaonis*) muscle produced by two commercial enzymes

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Abstract

Fish protein hydrolysates were prepared from Cuttlefish (*Sepia pharaonis*) muscle using alcalase and protamex methods. By conducting the Cuttlefish protein hydrolysate using alcalase (CPHA), it showed higher degrees of hydrolysis, proximate composition, yield, emulsifying activity index (EAI), emulsion stability index (ESI), foaming activity index (FAI), solubility, water holding capacity (WHC) and Oil absorption capacity (OAC) than cuttlefish protein hydrolysate using protamex (CPHP) but the foaming stability index (FSI) and peptide chain length (PCL) were more in the second treatment. Amino acids profiles of the CPHs were higher in essential amino acids compared to the recommended pattern of requirement by FAO/WHO and NRC standards. Phenylalanine is the first limiting amino acid in the hydrolysate. However, the hydrolysate has the potential for application as an ingredient in formulated diets. Some functional properties such as FAI, FSI and solubility were high in CPHs, thus, they can be used in different food and beverages.

Keywords: Cuttlefish, Alkaline proteases, Functional properties, Fish protein hydrolysates.

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Introduction

Protein hydrolysates have different applications in a variety of industries including pharmaceuticals, human and animal nutrition, food science and cosmetic industries. Protein hydrolysates are also useful as nitrogen sources for the microbial culture media (Safari *et al.,* 2009) and have been widely used to derive the peptides from fish protein, to get proteolytic enzymes from microbes and to develop plant origin (Kristinsson and Rasco, 2000). These peptides exhibit various biological activities such as antioxidant, antihypertensive, antihuman immunodeficiency virus, antiproliferative, anticoagulant, calcium-binding, antiobesity and antidiabetic activities (Ngo *et al.,* 2012; Elavarasan *et al.,* 2014). Various enzymes such as alcalase (a serine endoproteinase with a broad specificity toward native and denatured proteins, and is active under alkaline conditions), bromelain (a cysteine endopeptidase with broad specificity), flavorzyme (mixture of endo and exopeptidases), protamex (bacterial proteases comprising a mixture of endo and exopeptidases) have been used to produce hydrolysate with antioxidant and functional properties (Klompong *et al.,* 2007). Functional properties of food system are mainly affected by the proteins during processing and storage. The functionality of proteins depends on intrinsic factors such as size, shape, amino acid composition and extrinsic factors sequence like pH, temperature and ions presence (Souissi *et al.,* 2007; Elavarasan *et al.,* 2014). Fish protein hydrolysates (FPH) produced from fish processing waste and food fishes have improved surface-active properties like emulsifying and foaming capacity (Souissi *et al.,* 2007; Taheri *et al.,* 2013; Elavarasan *et al.,* 2014).

Cephalopods including cuttlefish (*Sepia pharaonis*), squid and octopus is one of the most important marine invertebrate classes representing an important seafood supply for human consumption worldwide because of their high nutritional and market values and excellent palatability (Ozyurt *et al,* 2006; Thanonkaew *et al,* 2006). Cuttlefish contains essential nutrient that benefits human health with high protein and mineral content (Nurjanah *et al,* 2012). Head and mantle contained 11.90% - 14.91% protein, 0.47% - 0.52% fat, 1.20% - 1.29% ash and 0.64% - 1.87% collagen. Zinc was found as the predominant element in cuttlefish muscle. Cuttlefish muscle consisted of 28.35% - 33.72% sarcoplasmic protein, 53.11% - 58.53% myofibrillar protein, 3.15% - 5.32% alkali-soluble protein and 4.79% - 13.11% stroma protein. Protein in cuttlefish plays an essential role in both nutritional value and sensory properties (Thanonkaew *et al.,* 2006).

The aim of this study was to evaluate the functional properties of cuttlefish protein hydrolysated with two bacterial proteases including alcalase and protamex.

Materials and methods

Fish sampling

Pharaoh Cuttlefish (*Sepia pharaonis*) was prepared from the Persian Gulf coast in Bushehr Province, Iran. The fish was transferred to the laboratory to remove the wastes including head, arms and viscera and then the muscles were minced and finally stored at -20˚C till for further experiments. Prior to the hydrolysis process, samples were thawed overnight in a refrigerator at 4 ± 1˚C.

Enzymes

Alcalase® (with a confirmed activity of 2.4 AU/g and a density of 1.18 g/mL) and protamex (1.5 AU/g, combined proteases) as bacterial endopeptidase extracted from *Bacillus licheniformis* and *Bacillus subtilis,* respectively were purchased from Novozymes, Bagsvaerd, Denmark and stored at 4˚C. Other chemical reagents were used for analytical grade of experiments.

Preparation of cuttlefish protein hydrolysates (CPHs)

The minced muscles of cuttlefish were heated in water bath shaker (Akhtarian, Iran) at 85˚C for 20 min to inactivate endogenous enzymes. The cooked muscles were mixed with sodium phosphate buffer 1:2 (w:v) and homogenized in a Moulinex® blender for about 2 minutes at an ambient temperature. The pH of mixture was adjusted at 8.5 and 7.5 for the optimum activity of alcalase and protamex, respectively. Alcalase and protamex were added to the substrate at 1.5% of total protein of cuttlefish muscle. All reactions were performed in a shaking water bath (Akhtarian, Iran) with constant agitation (150 rpm) at 55˚C for 2 hours. The termination of reactions were done by heating the solution at 95˚C for 5 minutes to inactivate the enzyme. The hydrolysates were cooled with ice to room temperature (22-25˚C) and centrifuged at 6700g at 4˚C for 20 minutes (H-103NR Kukusan, Japan). The collected supernatants were dried with a freeze drier (Vaco 2 Zirbus, Germany) and kept at 4 ˚C for further analysis (Bhaskar *et al.,* 2008; Safari *et al.,* 2009; Ovissipour *et al.,* 2009a; Ovissipour *et al.,* 2009b; Safari *et al.,* 2011).

Chemical composition

The moisture content was determined by drying the samples in an oven (Behdad, Iran) at 105˚C to reach a constant weight (AOAC, 2005). The total crude protein determination (N ×6.25) in raw materials was performed using the kjeldahl method (AOAC, 2005). Total lipid in sample was determined by soxhlet extraction (AOAC, 2005). Ash content was specified by charring in a predried sample in a crucible at 600˚C until a white ash was formed (AOAC, 2005). Soluble protein in the fish hydrolysates was measured by Biuret method in the supernatant (Layne, 1957).

Degree of hydrolysis (%), average peptide chain length and yield (%)

Hydrolysis degree determination was performed based on Hoyle and Merritt (1994) method. One volume of 20% trichloroacetic acid (TCA) was added to the supernatant followed by centrifugation at 6700×g at 4˚C for 20 minutes to collect the 10% TCA-soluble materials. The degree of hydrolysis (DH) was computed as:

%DH= (10%TCA, soluble N_2 in the sample / total N_2 in the sample) ×100

Average peptide chain length (PCL) was calculated by the method as described by Adler-Nissen and Olsen (1986) from DH % as follows:

$PCL = 100/DH\%$

Where, *PCL*, average PCL; and *DH*, degree of hydrolysis.

The yield was calculated as percentage ratio of meat weight in gram to the weight of the hydrolysates obtained in g.

Yield (%) = Weight of the hydrolysate g / Weight of the total Meat taken for hydrolysis (%)×100

Solubility

To determine the protein solubility, 250 mg of CPHs samples were dispersed in 20 mL of deionized water and pH of mixture adjusted to 4, 6, 7. The mixture was then stirred at room temperature for 30 minutes and centrifuged at 7500g for 15 minutes. Protein content in the supernatant was determined using Biuret method (Taheri *et al.,* 2013). Total protein content in the sample was determined after solubilization of the sample in 0.5 N NaOH. Protein solubility was calculated as follows:

Solubility (%) = protein content in supernatant / total protein content in sample × 100

Amino acid composition

Sample preparation was performed by hydrolysis with 6 M HCl at 110˚C for 12 h and derivatisation using phenyl isothiocyanate prior to HPLC (Knauer, Germany) analysis. The total amino acids were analyzed by the Pico Tag method, using a Pico Tag column (3.9 × 150 mm) at a flow rate of 1 mL min with UV detection. Breez® software was applied for data analysis (Ovissipour *et al.,* 2009a).

Chemical score

The chemical score of the CPHs was measured according to Bhaskar *et al.* (2008), relative to the essential amino acid (EAA) profile in a standard protein as described by FAO/ WHO (1990). Briefly, the chemical score was calculated using the following equation:

Chemical score = EAA in test protein (g 100 g $^{-1}$) / EAA in the standard protein (g 100 g^{-1})

Foaming properties

Foaming activity index (FAI) and foaming stability index (FSI) were determined according to the method by Souissi *et al.* (2007). A mass of 250 mg of sample of CPHs was added to 25 mL of distilled water. The pH of mixture was adjusted to 4.0, 6.0, or 7.0 with 0.1 N HCl and then whipped at a speed of

13,500 rpm for 1 min with an ultra homogenizer (Ika, Germany) at room temperature. The whipped sample was then poured into a graded cylinder immediately and the volume of the water that had drained from the foam phase was measured after 30s. The FAI was calculated by the following equation:

$$FAI\ (\%) = \frac{\text{Volume after whipping} - \text{Volume before whipping}}{\text{Volume before whipping}} \times 100$$

The whipped sample was allowed to stand at 20˚C until the foam was disappeared and the volume of whipped sample was then recorded. The FSI was calculated as follows:

$$FSI\ (\%) = \frac{\text{Volume after standing} - \text{Volume before whipping}}{\text{Volume before whipping}} \times 100$$

Foaming properties of CPHs were compared with standard bovine serum albumin (BSA) (Sigma Chemical Co. St. Louis, MO).

Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) were measured using the procedure described by Klompong *et al.* (2007) and Pearce and Kinsella (1978), with slight modification. Twenty five hundred milligrams of freeze-dried samples were dissolved in 50 mL of 0.1 N NaCl and then 25 mL of soybeans pure oil was added. The mixture was homogenized by an ultra-homogenizer for 120 sec at 10,000 rpm for making an emulsion. Aliquot of the emulsion was homogenized and 15 µL were pipetted from the container bottom at 0 and 10 minutes after homogenization. Afterward the sample was mixed with a 5 mL of 0.1% sodium dodecyl sulphate solution. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer (Hitachi, Japan). This was used to calculate EAI and ESI using the method suggested by Pearce and Kinsella (1978):

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

$$ESI\ (min) = A10 \times Dt / DA$$

Where A_0 is the absorbance at 0 minutes following homogenization; A10 is the absorbance at 10 minutes following homogenization; Dt = 10 min; and $DA = A_0 - A10$.

Oil absorption capacity (OAC)

The capacity of the CPHs to absorb fat was determined as described by Souissi *et al.* (2007) with a slight modification. Briefly, 250 mg of sample was mixed with 10 mL of corn oil in 50-mLcentrifuge tube. The sample was mixed for 10 minutes at room temperature. The mixture was then centrifuged for 10 minutes at 4300rpm and the volume of the supernatant was weighed. Fat absorption was expressed as the volume (mL) of fat absorbed per 1 g of protein hydrolysate. Fat adhesion to the walls in the tube was measured in an empty tube.

Water holding capacity

The water holding capacity (WHC) of the CPHs was determined by the method that has been established by Tounkara *et al.* (2013). 250mg of

sample was then mixed with 10 mL of distilled water in 50 mL centrifuge tube. The sample was mixed for 10 minutes at room temperature. The mixture was then centrifuged for 15 minutes at 4300rpm and the volume of the supernatant was weighed. The difference between initial volumes of distilled water was added to the protein sample and the volume obtained after filtration was determined. The results were reported as mL of water absorbed per gram of protein sample.

Statistical analysis

Statistical analysis was done using SPSS version. 18 (SPSS Inc., Chicago, Il, USA). ANOVA and Duncan's new multiple range test were considered to determine the significant difference of the means at 5% probability level.

Results

Degree of hydrolysis, PCL and yield

The DH%, mean of PCL and yield (%) of Cuttlefish protein hydrolysates (CPHs) are shown in Table 1. At the optimum condition, DH% and yield were found at 19.29 and 13.89 and 9.41 and 5.31% for alcalase and protamex, respectively.

Proximate composition

Proximate composition of raw material of cuttlefish and freeze dried CPHs are shown in Table 2. Raw material protein, ash, fat and moisture and CPHs were statistically different (p<0.05). The moisture, fat and ash in raw material were higher than those for the CPHs, while CPHs showed higher protein content than do the raw fish.

Solubility

The solubility of CPHA and CPHP in three different pHs of 4, 6 and 7 were shown in Fig.1. Solubility of CPHA was more than CPHP, but there was no significant difference between two enzymes. The highest and least solubility of CPHA and CPHP were in pH = 7 and pH = 4, respectively.

Water holding capacity (WHC)

In this study, CPHP exhibited higher WHC (3.32 mL/g hydrolysate) than CPHA (2.87 mL/g hydrolysate). CPHs have an excellent water holding capacity (WHC).

Oil absorption capacity (OAC)

The OAC value was also higher in CPHP (6 mL/g hydrolysate) than CPHA (4.34 mL/g hydrolysate).

Amino acid profile

The amino acid composition of CPHA and CPHP are shown in Table 3**.** In this research, computed chemical scores are based on the reference protein of FAO/WHO for adults, and amino acid requirements of juvenile common carp, as listed by NRC.

Table 1: Protein (mg/mL), DH (%), Yield (%) And PCL in CPHs by alcalase and protamex

Factors Samples	Moisture	Fat	Protein	Ash
Cuttlefish muscle	58.17±2.11[a]	10.03±0.8[a]	29.82±1.17[b]	1.27±0.4[a]
CPHA	4.11±0.04[b]	0.9±0.05[b]	92.35±1.44[a]	1.25±0.71[a]
CPHP	5.87±0.12[b]	1.21±0.07[b]	89.67±2.21[a]	2.45±0.32[a]

Values represent means ± SE (n = 3)
E/S : Enzyme / Substrate, DH : Degree of hydrolysis, PCL : Peptide hhain length
Value in the same column with different superscripts are significantly different at p<0/05
CPHA: Cuttlefish protein hydrolysate by alcalase
CPHP: Cuttlefish protein hydrolysate by protamex

Table 2: Proximate Composition (%) of the freeze dried of CPHs and the raw cuttlefish (N = 3).

Paramaters Enzymes	Protein(mg/mL)	DH (%)	Yield (%)	PCL
CPHA	32.99+2.03[a]	19.29+2.19[a]	9.41+0.15[a]	5.37+1.44[a]
CPHP	23.59+2.11[a]	13.89+1.69[a]	5.31+0.13[b]	7.45+1.98[a]

Values represent means ± SE (n = 3)
Value in the same column with different superscripts are significantly different at p<0/05.

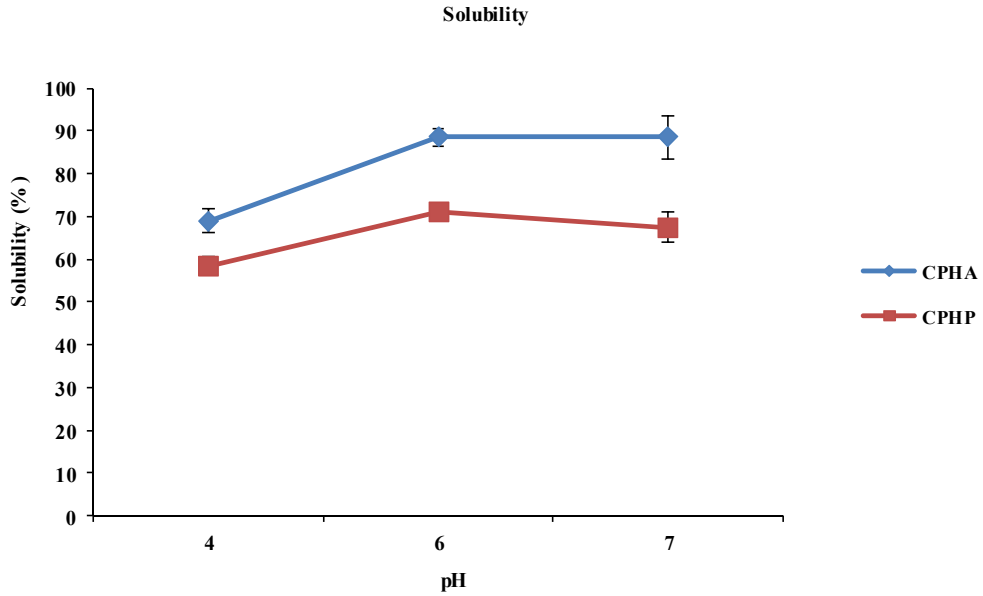

Figure 1: the solubility of Cuttlefish protein hydrolysates (CPH) prepared by Alcalase (CPHA) and protamex (CPHP) as influenced by different pHs.

The amino acid composition in this study with reference proteins 1 and 2 showed that the amino acid profile of the CPHs was higher in essential amino acids, compared with the recommended pattern of requirement by FAO/WHO for adults and NRC standard for common carp except phenylalanine for both enzymes and threonine for protamex treatment .

Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) of CPHs were 32.7 ± 0.43 and 33.81 ± 2.32 % (for alcalase) and 30.45 ± 0.35 and 31.67 ± 1.72 % (for protamex), respectively. EAI of CPHA was significantly higher than CPHP (p<0.05).

Foaming properties

The results of foaming activity index (FAI) and foaming stability index (FSI) of CPHs are shown in Figs. 2 and 3. CPHA had higher FAI and FSI than the CPHP. The highest FAI and FSI were found at pHs 6 and 7 for both CPHA and CPHP, while foaming properties decreased at acidic pH (pH = 4) (p<0.05).

Discussion

Degree of hydrolysis and yield

DH% is the important factor that determines the functional and biological activity of resulted hydrolysates. Alcalase was found to break down more peptide bonds at the optimum condition followed by protamex. Obtained results were in agreement with the report given by Safari *et al.* (2009) and Ovissipour *et al.* (2009b) but Elavarasan *et al.* (2014) reported that DH% in protamex treatment was higher than alcalase treatment for Indian Carp (*Catla catla*). The reported value of protein hydrolysate yield from *Mugil cephalus* was 10-15% (Rebeca *et al.,* 1991). Fish protein hydrolysates yields for different enzymes were dependent on the DH %. Average PCLs of hydrolysates were 5.37 and 7.45 for alcalase and protamex, respectively. The results indicated that the higher the DH %, the lower the average length of peptide chain.

Proximate composition

The maximum protein value of different FPH reported by others is 63.4% to 90.8% (Bhaskar *et al.,* 2008; Ovissipour *et al.,* 2009a). Decreasing lipid content in the CPHs might significantly increase stability of the material toward lipid oxidation, which may also increase the product stability (Ovissipour *et al.,* 2009a).

Solubility

In general, the degradation of proteins to smaller peptides leads to more soluble products (Linder *et al.,* 1996; Gbogouri *et al.,* 2004). CPHA, with higher DH than CPHP, had higher solubility. Gbogouri *et al.* (2004) reported that hydrolysates had an excellent solubility at high degrees of hydrolysis.

Table 3: The amino acid content (g/ 100 g protein) of cuttle fish protein hydrolysates and chemicals and chemical score, compared with FAO/WHO and NRC reference proteins.

Amino acids	CPHA (g/ 100 g)	CPHP (g/ 100 g)	Reference Protein 1[a]	Reference Protein 2[b]	Chemical score for CPHA		Chemical score for CPHP	
					RP_1	RP_2	RP_1	RP_2
Aspartic acid+Asparagin	8.01	8.88	-	-	-	-	-	-
Glutamic acid+Glutamin	14.71	13.72	-	-	-	-	-	-
Hydroxy proline	0.89	1.07	-	-	-	-	-	-
Serin	5.26	5.03	-	-	-	-	-	-
Glycine	3.62	4.34	-	-	-	-	-	-
Threonine	1.21	0.59	0.9	3.9	1.34	0.31	0.65	0.15
Histidine	4.78	3.79	1.6	2.1	2.98	2.27	0.42	0.55
Alanine	4.94	6	-	-	-	-	-	-
Proline	7.57	7.38	-	-	-	-	-	-
Arginine	14.61	14.02	-	1.31	-	11.15	-	0.09
Tyrosine	0.54	0.51	-	-	-	-	-	-
Valine	4.64	3.83	1.3	3.6	3.56	1.28	0.33	0.93
Methionine	4.05	4.35	1.7	3.1	2.38	1.3	0.39	0.71
Cysteine	0.3	1.59	-	-	-	-	-	-
Isoleucine	5.61	5.36	1.3	2.5	4.31	2.24	0.24	0.46
Leucine	8.64	8.32	1.9	3.3	4.54	2.61	0.22	0.39
Phenylalanine	3.98	3.77	-	6.5	-	0.61	-	1.72
lysine	6.63	7.96	1.6	5.7	1.16	4.14	0.21	0.76

RP_1: Chemical score calculated with FAO/WHO reference protein as the base.
RP_2: Chemical score calculated with amino acid requirements as per NRC (1993).
a: Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990).
b: Essential amino acid requirements of common carp according to NRC (1993).

The balance of hydrophilic and hydrophobic forces of peptides are another crucial parameters for solubility increments. The higher solubility of CPHA is due to the low molecular weight of the peptides, which are also rich in hydrophilic amino acids. This condition was weaker in CPHP. Salmon by-product hydrolysates showed the lowest solubility at pH = 4 (Gbogouri *et al.*, 2004).

Due to the high solubility of the muscle hydrolysates over a wide pH range, it was supposed that FPHs had a low molecular weight and they were hydrophilic in nature (Sorgentini and Wagner, 2002).

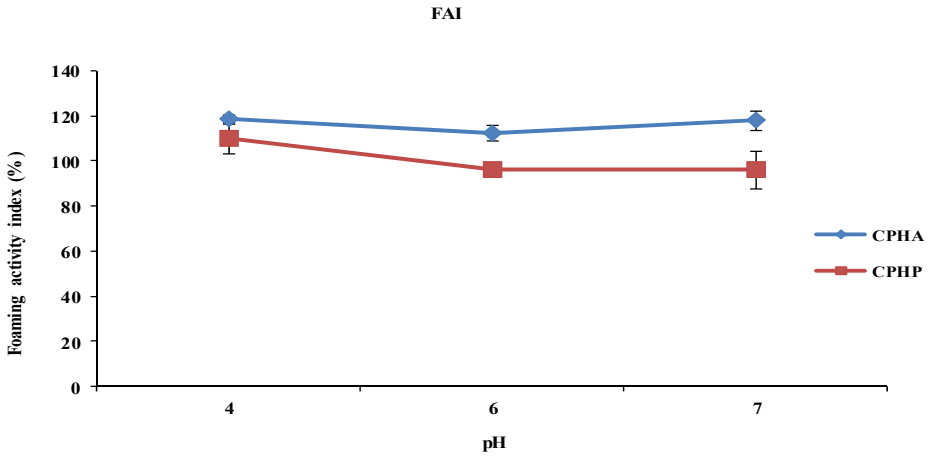

Figure 2: Foaming activity index (FAI) of Cuttlefish protein hydrolysates (CPHs) muscle prepared by alcalase (CPHA) and protamex (CPHP) at different pHs.

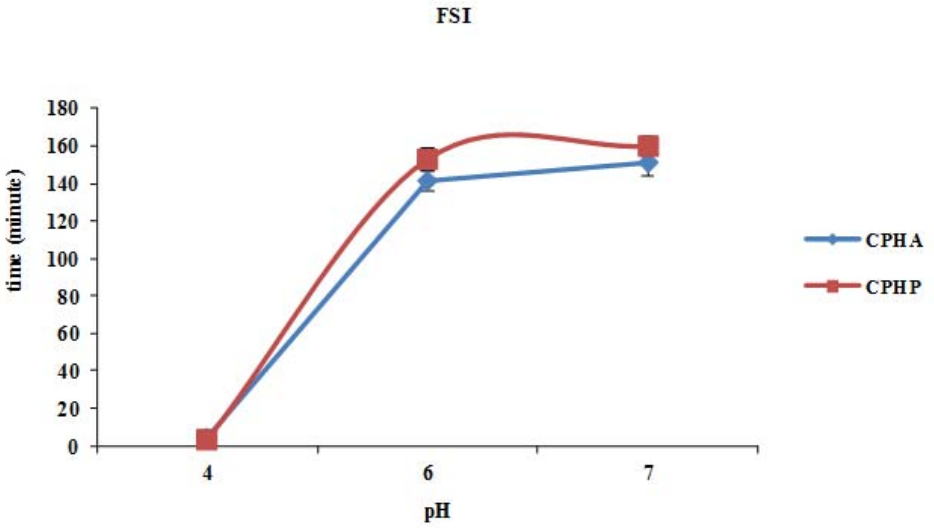

Figure 3: Foaming stability index (FSI) of Cuttlefish protein hydrolysates (CPHs) muscle prepared by alcalase (CPHA) and protamex (CPHP) at different pHs.

Water holding capacity (WHC)

CPHs have an excellent water holding capacity (WHC) and it can increase the cooking yield. Increasing the polar group concentration such as -COOH and -NH_2 that is caused by enzymatic hydrolysis has a substantial effect on the amount of absorbed water (Kristinsson and Rasco, 2000). The result of WHC in this study was similar to Balti *et al.* (2010) report (2.5- 5.5 mL/g). The species of cuttlefish studied by Balti *et al.* (2010) was *Sepia officinalis* while the surveyed species in this study was *Sepia pharaonis*. The glutamic acid and aspartic acid content in CPHs are higher than other sources and these residues can bind to water almost 3 times more than non-ionizable polar groups. These results also suggest that the hydrolysates could be used as an additive in intermediate-moisture (IM) food to bind water and improve texture (Deeslie and Cheryan, 1988).

Oil absorption capacity (OAC)

OAC showed that the quantity of oil is bound by the protein and it is an important functional characteristic for the meat and confectionary industries (Gbogouri *et al.,* 2004). Hydroxyproline content affects OAC , and a powder containing higher amounts of charged amino acids, such as aspartic acid, glutamic acid, lysine and arginine is able to absorb more fat (Slizyte *et al.,* 2009). The OAC values obtained in the current study are higher than those found for grass carp skin hydrolysates (2.4 - 3.6 mL oil in per g hydrolysate) (Wasswa *et al.,* 2007) but similar to those reported for red salmon head (Sathivel *et al.,* 2003).

Amino acid profile

The chemical score provides an estimation for the nutritive value of a protein. This factor compares values of essential amino acids with the test and standard proteins. The results of the common carp chemical score showed that phenylalanine is the most limiting amino acid, while other amino acids are present in adequate or excess qualities. Based on the CPHs amino acid composition, and the FAO/WHO (1990) and NRC (1993) standards, the hydrolysates can provide both human and common carp requirements.

Emulsifying properties

Proteins have the ability for stabilizing food emulsions. The obtained EAI and ESI in this study were lower than previous reported values. Based on the study of Chobert *et al.* (1988), peptides with low molecular weight may not be amphiphilic enough to exhibit good emulsifying properties. However, Kristinsson and Rasco (2000) reported that there is no clear relationship between peptide size and emulsifying. Physical and chemical characteristics of the peptides may play an important role in functional properties. With a limited degree of hydrolysis, the hydrolysates have exceptional emulsifying activity and stability (Kristinsson and Rasco, 2000; Elavarasan *et al.,* 2014). On the other hand, by increasing DH, the EAI

and ESI showed a decrease (Gbogouri *et al.,* 2004). Low value of EAI and ESI was due to increase the DH in CPHA and CPHP.

Foaming properties

Low foam stability in pH=4 was related to the poor solubility of CPHs at acidic pH. Produced CPH peptides in this research were efficiently absorbed and denatured in order to reduce the interfacial tension and form the viscoelastic film that is necessary for an effective foaming agent. The pH has a major effect on foam stability and decreasing of foam stability at acidic pH may be due to the repulsion of peptides (Klompong *et al.,* 2007; Elavarasan *et al.,* 2014). A protein may have an excellent foam ability, but it may not necessarily produce stable foam (Wilde and Clark, 1996). There was a direct relationship between FAI and FSI in this study. FAI was very high in Three pHs (4, 6 and 7) but FSI was not stable in pH = 4 (4 minute) while in pHs 6 and 7, it was stable for 130- 150 minutes. In this study, CPHP exhibited a superior foam stability rather than CPHA. The size and charge of peptides may be different from CPHs produced by various enzymes. CPHP most likely including larger peptides (lower DH than CPHA) could form flexible film around the air bubbles, as evidenced by higher foam stability.

Generally, it can be concluded that proximate composition and DH and yield in CPHA were higher than CPHP. Although properties of proteins depend on the type of enzymes used, the functional characterization of CPHA and CPHP were almost similar to each other. The prepared CPHs in this study had high FAI and FSI, thus they can be used as foaming agent in different food and beverages. Based on the CPHs amino acid composition, and compared with the FAO/WHO and NRC standards, the hydrolysates can provide both human and common carp requirements. Further investigations needs to be carried out on antioxidants properties, isolation, purification and characterization of the peptides responsible for this multifunctional nature of cuttlefish protein hydrolysates.

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