International Food Research Journal 22(1): 337-343 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Biochemical and functional properties of fish protein isolate (FPI) from *pangasius hypophthalmus* byproducts as influenced by time and degree of hydrolysis (DH)

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Article history

Received: 15 April 2014
Received in revised form: 20 June 2014
Accepted: 2 July 2014

Keywords

Alcalaze 2.4L
Protein isolate
Pangasius hypophthalmus byproducts
DH
FPI calcium-binding
FPI functional properties

Abstract

This study focused on determining the influence by DH, hydrolysis time to the functional properties (foaming, emulsifying ability), calcium-binding bio-activity of fish protein isolate (FPI) that derived from enzymatic hydrolysis of *Pangasius hypophthalmus* byproducts. The results showed that in the hydrolysis time with maximum values of foaming, emulsifying or calcium-binding abilities have not corresponded to the highest DH. At 75 minutes of enzymatic hydrolysis, FPI obtained the highest emulsifying capacity (20.99 mL oil/ g FPI), with DH = 18.38%. After 90 minutes hydrolysis time, DH = 20.21% and maximum FPI's foaming ability was 94.59%. At 120 minutes of hydrolysis, the DH and FPI's highest calcium-binding possibility were 22.59% and 27.03 mg Ca^{+2}/g FPI respectively. Chemical composition of FPI at different DH has a high protein content (90.01% ÷ 91.34%), very low lipid content (0.94% ÷ 0.98%). Moisture was from 2.86% to 3.81%; and ash content was from 3.04% to 4.94%. Type of the links between Ca^{+2} and proteins in FPI: 91.46% binding through EF hand structure; 2.28% as hydrogen bonding through water bridge; 3.30% as electrostatic links between Ca^{+2} with the starting amino acid of proteins.

Introduction

Pangasius hypophthalmus is a kind of catfish, according to the ichthyologists, it belong to Pangasiidae family. Raising *Pangasius hypophthalmus* is very popular and brought great economic value for farmers in the Mekong River Delta, Vietnam. In recent years, catfish production in Vietnam is constantly increasing, accompanied by the number of byproducts also increases equivalently to a total huge number of about 700,000 tons/year (Vietnam Association of Seafood Exporters and Producers - VASEP, 2014). This urges a reasonable solution for using *Pangasius hypophthalmus* byproduct resources to bring larger benefits for farmers and enterprises.

Biochemical as well as functional properties of proteins can be improved by enzymatic hydrolysis under controlled conditions (Quaglia and Orban, 1990). FPI's functional properties and biological activities depend on its origin and produced methods (Samanta, 2013). In generally, the hydrolysis time of byproducts which derived from fish has been a major influence on the molecular weight, hydrophobicity, forming the interaction or the polarization of the proteins in the final products after hydrolysis (Kristinsson and Rasco, 2000). So it could change the functional properties and bio-activities of FPI. High foaming ability of FPI involves the formation a lot of relatively small molecular weight proteins in hydrolysis solution (Elizabeth, 2005; Anusha, 2010). The foam formation related to the diffusion of soluble proteins to air/water inter-surface (Cecilia *et al*., 2012). Emulsifying ability of FPI is determined by the number of proteins with medium or relatively big molecular weight (Vilailak *et al*., 2007). So the very high DH reduces the FPI's emulsifying ability (Sitthipong *et al*., 2011). Calcium-binding is one of the most important biological functions of FPI which derived from Pangasius hypophthalmus byproducts (Tai, 2013). At a relatively high DH followed by a long hydrolyzing time will create a large number of very small molecular weight peptides. The proteins have molecular weight <3 kDa accounted for 60% or more would had a decisive role in the formation of FPI's calcium-binding ability (Laurent *et al*., 2010; Hoa *et al*., 2012). However, almost functional properties (such as foaming, emulsifying…) or calcium-binding bioactivity of FPI have not usually reached the maximum value at the highest DH in enzymatic hydrolysis (Nabil *et al*., 2007; Balaswamy

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et al., 2011; Tai, 2013).

Materials and methods

Material

By-products (the spine and head) of Pangasius hypophthalmus were received from Can Tho Fish Join Stock Company (CAFICO) - Mekong River Delta, Vietnam. Then it had been refrigerated, transported to the laboratory, divided into small unit for each experiment and stored at - 20^0C until used. Enzymes Alcalase 2.4L (declared activity 0.5 IU/g subtract) was purchased from EAC Co., Ltd. (sole-exclusive agent for Novozyme in Ho Chi Minh city, Vietnam). Commercial SPI (SPI4) was purchased from Prestige L.O company (France). All chemical reagents used for the experiments were in analytical grade.

Hydrolysis process

Hydrolyzing the *Pangasius hypophthalmus* by-products by protease (Alcalaze 2.4L) under controlled conditions (see Influence of hydrolysis time, DH to functional features and bio-activities of FPI, below section) for each functional property or bio-activity. After hydrolysis, filtering to seperate the solid and liquid, inactivating enzyme Alcalaze 2.4L by heat treatment at 90^0C/10 minutes as recommendation of Novozymes. Hydrolysed solution was then cooled to 4^0C for a preliminary de-fatting, vacuum filtered through non-ash paper and then centrifuged to de-fat at the speed of 15,000 rpm for 20 minutes. The solution obtained after centrifugation was brought to freeze-dry to get FPI powder. FPI powder is used to study the calcium-binding possibility as well as its foaming and emulsifying abilities. At each hydrolysis time 30, 45, 60, 75, 90, 105, 120, 135, 150 minutes, determining simultaneously each pair of values: DH and FPI's calcium-binding; DH and FPI's foaming; DH and FPI's emulsifying.

Determining the DH

Degree of hydrolysis (DH - %) was determined by pH-stat method. DH is calculated as a percentage of the peptide bonds that were broken off to the total number of peptide bonds (total nitrogen-N), and in each case calculated using the base volume, according to the formula:

$$DH(\%) = \frac{V_B \times C_B}{m_p} \times \frac{1}{\alpha} \times \frac{1}{N_{total}} \times 100$$

Where: V_B is the volume (liter) of base used (NaOH) to keep the pH constant during the reaction; C_B is the concentration molar; m_p is the total protein (Nx6.25);

α is the degree of dissociation of the α-NH_2 released during hydrolysis:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}}$$

In particular, pH and pK are dissociation values during controlled hydrolysis condition. The total number of peptide bonds (N_{total}) in FPI is regarded as 8.6 meq/g

Chemical analysis of FPI

The moisture and ash content were determined according to the AOAC standard methods 930.15 and 942.05 respectively. Total nitrogen content of FPIs was determined by using the Kjeldahl method. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

Determination of FPI's calcium-binding possibility

Calcium-binding possibility of FPI is determined by the method of Flame Atomic Absorption Spectrophotometric (FAAS). For binding Ca^{+2} ions to proteins in FPI, 1g FPI powder was dissolved in 1 liter of sodium phosphate buffer solution (pH = 7.8); added 1.11 g $CaCl_2$ (20 mμ) to FPI solution; adjusted the temperature to about 20 - 22^0C; stirred for 30 minutes at speed of 100 rpm; preliminarily centrifuged at 6000-7000 rpm, adjust pH = 7 with bicarbonate buffer solution; super-speed re-centrifuged at 26,000 rpm. Calculation the calcium-biding possibility of FPI (mg Ca^{+2}/g FPI). Vaporizing and atomization samples by gas flame. The gas clouds will absorb the monochromator radiation beam. Using the spectrometer for collecting the entire separation spectrum beams and select a calcium spectral absorption line in order to measure its intensity. In a certain limit of concentration (C) of substances to be determined. The value of this intensity depends linearly on the concentration (C) according to the following equation:

$A = a.C$; $A = \lg I_0/I_t = e.l.C$

Where: A is the intensity of the spectral absorption; a is experimental constant; C is concentration of substances to be determined (mg/g)

Testing the links between Ca^{+2} and proteins in FPI, after Ca^{+2} associated with proteins in FPI, to ensure that Ca^{+2} were binded to proteins by ionic bonds, static links, or hydrogen bonds through the intermediate bridge of water etc… (Not a mechanical bond). The solution obtained after ultracentrifugation at 26000 rpm will added 25 ml LYSIS buffer solution (Consists of 50 mμ Tris-H_3PO_4; 2 mμ EDTA, 1 mμ phenyl methyl sulfonyl fluoride (PMSF), 1microgam

leupeptin). The solution was put into a magnetic field; cooled at 4^0C for 5 minutes to ensure the complete removal of $CaCl_2$ that were stick or not binded to a protein in FPI; Adjusted the pH to 7.5; brought the solution to monochromatic radiation environment. The spectral lines of calcium ions will be magnified 20000 times to know the density of calcium-binding to the FPI's proteins.

Determination of FPI's foaming ability

Foaming ability of FPI was determined by the method of Kazunobu Tsumuraa *et al* (2005): 0.25 g FPI would be dissolved in 25 ml of distilled water. The mixture was adjusted to pH 7 by 0.5N NaOH and then was stirred by electric mixer to create foam system at room temperature. The sample after stirring was poured into the instrument (flash) for measuring both the total volume in foaming phase and the volume of separated water after 30 seconds. Foaming ability is calculated as follows:

$$FA(\%) = \frac{V_f - V_w}{V_i} * 100$$

Where: V_f: total volume in foaming phase; V_w: volume of separated water; V_i: volume of initial mixture.

Determination of FPI's emulsifying ability

Emulsifying capacity of the samples was measured as described by Rakesh and Metz (1973), with some modification. One gram of each freeze-dried sample was transferred into a 250 mL beaker and dissolved in 50 mL of 0.5 N NaCl and then 50 mL of soybeans pure oil was added. Homogenizing the solution for 120 sec at 10 000 rpm to make an emulsion. The mixture was transferred into centrifuge tubes, kept under a water-bath at 90^0C for 10 min and then centrifuged at 3000 rpm for 20 min. Emulsifying capacity was calculated using the equation:

EC (mL oil/g FPI) = (VA-VR)/ WS

Where: V_A is the volume of oil added to form an emulsion; V_R is the volume of oil released after centrifugation; W_S is the weight of the sample.

Determination the effect of hydrolysis time, DH on FPI's calcium-binding ability

Conducting hydrolysis of Pangasius hypophthalmus byproducts under controlled conditions (optimal ones by experimental planning: Response Surface Methodology - RSM before): pH 7; E/S ratio (w/v): 0.15%; temperature: 55^0C, additional water rate of 200%. At each hydrolysis time of 30, 45, 60, 75, 90, 105, 120, 135 and 150 min., determining simultaneously both the DH and FPI's calcium binding ability.

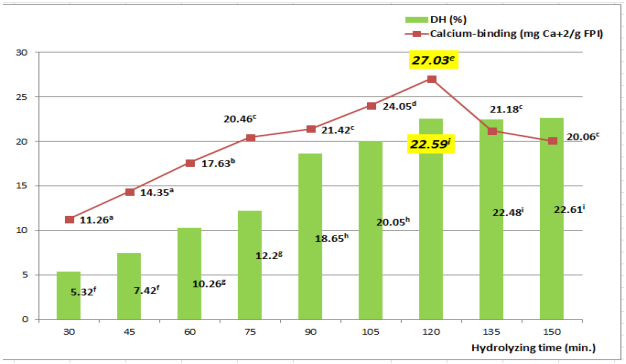

Figure 1. Influence of hydrolysis time and DH on FPI's calcium-binding

(*) Results reported are means of triplicate samples. Values with different superscripts are significant different ($p < 0.05$); Values with same superscripts are insignificant different ($p < 0.05$)

Determination the effect of hydrolysis time, DH on FPI's foaming ability

Conducting hydrolysis of *Pangasius hypophthalmus* byproducts under controlled conditions: pH 7; E/S ratio (w/v): 0.20%; temperature: 64^0C, additional water rate of 200%. At each hydrolysis time of 30, 45, 60, 75, 90, 105, 120, 135 and 150 min., determining simultaneously both the DH and FPI's foaming ability.

In order to determine the effect of hydrolysis time, DH on the emulsifying ability

Conducting the hydrolysis under controlled conditions: pH: 7.4; ratio E/S (w/v): 0.19%, temperature: 62^0C, additional water rate of 200%. At each hydrolysis time of 30, 45, 60, 75, 90, 105, 120, 135 and 150 min., determining simultaneously both the DH and FPI's emulsifying ability.

Statistical analysis

All analytical determinations were carried out in triplicate and mean values with standard deviation (SD) are presented. Results were analyzed statistically by ANOVA using SPSS 15.0 to ascertain whether differences were significant at $p<0.05$.

Results and Discussion

Influence of hydrolysis time, DH to functional features and bio-activities of FPI

Calcium-binding bio-activity

The results are presented in Figure 1. During hydrolysis time from 30 to 120 minutes, while the time was prolonged, both FPI's calcium-binding and DH increased linearly with hydrolysis time ($p<0.05$). Specifically, at the hydrolysis time 30 min, the lowest FPI's calcium binding ability was 11.26

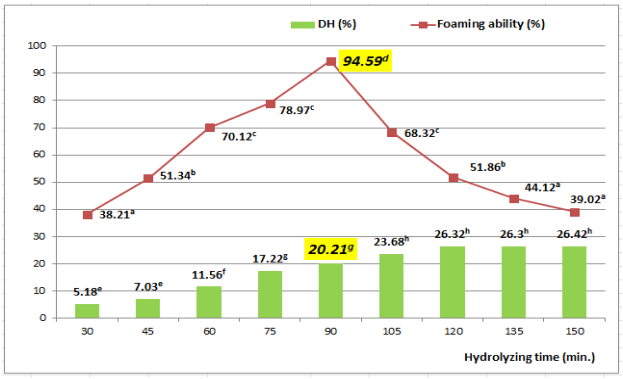

Figure 2. Influence of hydrolysis time and DH on foaming ability of FPI

(*)Results reported are means of triplicate samples. Values with different superscripts are significant different ($p < 0.05$); Values with same superscripts are insignificant different ($p < 0.05$)

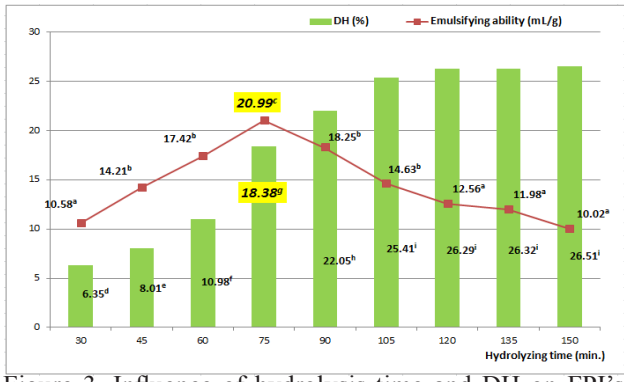

Figure 3. Influence of hydrolysis time and DH on FPI's emulsifying ability

(*)Results reported are means of triplicate samples. Values with different superscripts are significant different ($p < 0.05$); Values with same superscripts are insignificant different ($p < 0.05$)

mg/g, corresponding to the lowest DH = 5.32%. In the hydrolysis time 120 minutes, the maximal FPI's calcium binding ability reached (27.03 mg/g) corresponding to DH = 22.59%. Thus, in this period, FPI's calcium binding ability and DH increased 140.05% (from 11.26 mg Ca^{+2}/g FPI to 27.03 mg Ca^{+2}/g FPI) and 324.62% (from 5.32% to 22.59%) respectively. However, after 120 min. of hydrolysis, the DH was hardly increased (very small fluctuation from 22.48% to 22.61%), while the calcium-binding decreased significantly. The FPI's calcium-binding ability decreased 25.79% from 27.03 mg/g (at 120 minutes of hydrolysis) to 20.06 mg/g (at 150 minutes of hydrolysis).

This result was explained as follows: when prolonged hydrolysis time, the proteins in *Pangasius hypophthalmus* byproducts was hydrolyzed more deeply, so the DH increased; the size of the protein molecules smaller and smaller. The small molecular weight proteins determined the FPI's calcium-binding ability. FPIs with the number of proteins that molecular weights from 2 to 8 kDa accounted for approximately 70% of the total proteins in FPI will have the highest calcium-binding (Laurent *et al*., 2010; Tai, 2013). If hydrolysis time was too long, leading to the formation of a lot of free amino acids. Although DH did not change significantly, the amount of smaller molecular weight proteins reduced significantly, causing the decreasing of FPI's calcium-binding possibility.

Our results are similar to the ones of previous studies (Heinz *et al*., 2001; Ruiyan *et al*., 2014) on calcium-binding ability of FPI which derived from other fishes. The highest calcium-binding ability of FPI from Nile Tilapia *(Oreochromis niloticus)*; Common carp *(Cyprinus carpio)* were reached at DH from 19.20% to 24.18%. In addition, the increasing amino acid content of hydrolysis solution will lead to significantly decreasing calcium-binding ability of FPI from byproducts of rainbow trout *(Onchorhynchus mykiss)* (Taheri *et al*., 2012).

FPI's foaming capability

The results of study were displayed in Figure 2. According to results showed in Figure 2: The DH increased linearly with hydrolysis time ($p < 0.05$) during hydrolysis. The highest DH at the longest hydrolysis time of 150 minutes was 26.42%; but at this highest DH value, the FPI's corresponding foam ability have not reached the maximum one (only 39.02%). Foaming ability of FPI from *Pangasius hypophthalmus* byproducts reached the maximum value (94.59%) in the hydrolysis time of 90 min., corresponding to DH after 90 minutes of hydrolysis was 20.21%. In the period from 30 to 90 minutes, the foaming capacity raised when both DH and hydrolysis time increased. Specifically, the hydrolysis time prolonged from 30 to 90 minutes, foaming ability and DH increased 38.21%÷94.59% and 5.18%÷20.21% respectively. When the hydrolysis was proceeded in longer periods (from 90 to 150 minutes): DH still raised from 20.21% to 26.42% but foam ability decreased from 94.59% to 39.02%.

This is explained as follows: When increasing hydrolysis time, the peptide bonds were cleaved/cut by the Alcalase 2.4L enzyme, leading to the increasing of DH. The DH increased up to a certain limit, the enzyme was "saturated" and DH tended to be constant. When the hydrolysis time prolonged, the maximum number of proteins with small molecular weight released. These proteins (less than 7 kDa in molecular weight) have good ability to create foam (Tai, 2013), so the FPI's foaming ability gains maximizing value. The foam formation involves the diffusion of soluble proteins to air/water inter-surface. At that surface, protein focused and stretched out immediately for increasing solubility and surface activity of soluble proteins. The absorption of proteins

Table 1. Chemical composition(*) of the FPIs

Source	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)
FPI_1 (DH=22.59%)	90.14 ± 1.60^a	0.94 ± 0.18^a	2.86 ± 0.90^a	4.94 ± 0.16^a
FPI_2 (DH=18.38%)	92.01 ± 1.68^a	0.97 ± 0.44^a	3.81 ± 0.75^b	3.07 ± 0.06^b
FPI_3 (DH = 20.21%)	91.34 ± 0.88^a	0.98 ± 0.12^a	3.81 ± 0.37^b	3.04 ± 0.09^b
Commercial SPI	90.08 ± 1.43^a	0.39 ± 0.08^b	4.18 ± 0.42^c	4.45 ± 0.24^c

(*) Results reported are means of triplicate samples ± standard deviation. Values in the same column with different superscripts are significant different at $P < 0.05$

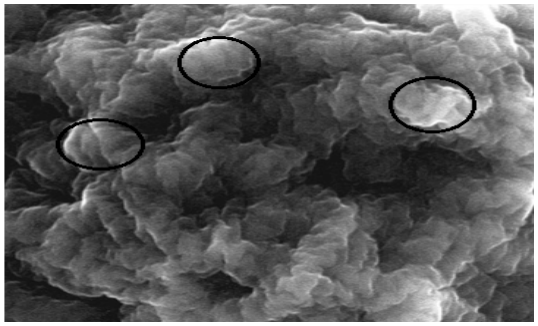

Figure 4. The links between Ca^{+2} and FPI's proteins

to the foams was made through the hydrophobic region. Therefore, foaming ability is based on both molecule weight and structural features of the protein. So when prolonged the hydrolysis time to 90 minutes will lead to reduce the foaming ability of FPI, although DH still increasing.

Our studying results have been in appropriate with previous publishing researches about the foaming ability of FPI from *Sardinella aurita* by-product, *Pollachius virens*, *Silver catfish*... High foaming ability of FPI is explained by the relatively small size of its peptides. The foaming ability of FPI byproducts of *Sardinella aurita*, *Pollachius virens* can reach from around 89% to 100.3% (Nabil *et al.*, 2007; Gholam *et al*., 2012; Tai, 2013) at DH from 17.68% to around 25%. Foaming ability of FPI from *Pacific Hake (Merluccius productus)* was 107.7% at DH = 43% (Anusha, 2010).

FPI' emulsifying ability

The results(*) of study were displayed in Figure 3. According to the results showed in Figure 3, emulsifying ability of FPI reaches a maximum value at 75 min. of hydrolysis time (20.99 mL oil/g FPI), corresponding to an medium DH level (18.38%). In the hydrolysis period from 75 to 150 minutes, when the DH increased, FPI's emulsifying ability decreased linearly ($p < 0.05$). The DH reached the highest value at 150 min. of hydrolysis time (26.51%), but at that time the emulsifying ability of the FPI is lowest (only 10.02 mL oil/g FPI). In contrast, in the initial period of hydrolysis (from 30 to 75 minutes), the increasing of FPI's emulsifying ability linearly depended on

Table 2. Results of testing the links between Ca^{+2} and proteins in FPI

Area (%)	Ca^{+2} - binding to protein	Comment
91.46	Binding constants of Ca^{+2} Ion to Calcium-binding protein in high density	*EF-hand effects predicted*
2.28	Binding constants of Ca^{+2} Ion to Calcium-binding protein in medium density	*Hydrogen bonding via H_2O*
3.30	Binding constants of Ca^{+2} Ion to Calcium-binding protein in low density	*Peptide stated by no loop, -C-C- structure*
2.96	not identified	

the increasing of DH ($p<0.05$). This is because of emulsifying ability of FPI also relevant to the molecular weight of the protein molecules in FPI.

The explanations for these: emulsifying ability of FPI was significantly related to the molecular weight as well as structures of the proteins in FPI. The emulsifying capacity has been determined by the maximum amount of oil that can be emulsified in a given volume of emulsifier solution of known concentration (Cheftel *et al*., 1989). The proteins with molecular weight from 7 to 20 kDa have an important role in the emulsifying process. In which the ones from 7 kDa to <10 kDa have a decisive role in emulsifying ability of FPI. The percentage of proteins with molecular weight from 7 kDa to <10 kDa in FPI accounts for 47%÷60% will have the best emulsifying ability (Tai, 2013).

The study results are consistent with the research of Rodriguez-Huezo *et al*. (2010) on emulsifying ability of FPI from *Hypophthalmichthys nobilis*. Emulsifying ability of FPI from *Hypophthalmichthys nobilis* reached 19.05 mL oil/g FPI, at DH=21.32%. The emulsifying ability of FPI that originated from Sardinella *(Sardinella aurita)* was 20.00 ± 0.18 mL oil/g FPI at DH = 17.43 (Nabil, 2007). FPI from silver catfish (*Pangasius* sp.) had emulsifying ability of 21.30 mL oil/g FPI (Amiza *et al*., 2013).

Chemical composition of FPI

The chemical composition of FPIs from Pangasius hypophthalmus at difference DH were determined and compared to that of a commercial soy protein isolate - SPI (SPI4 , purchased from Prestige L.O. company, France); as shown in Table 1. Basing on results showed in Table 1, the protein content of FPIs in all of three DH values (DH=22:59%; DH=18:38%; DH=20:21%) was higher than 90%. FPI from Pangasius hypophthalmus byproducts was a very high protein products and have no significant difference ($p<0.05$) at 3 different degree of hydrolysis. This content is equivalent to the one of soybean protein isolates - SPI ($p <0.05$). High protein content reflected the quality of the FPI. Our studying results had been similar to the findings of other

investigators whom reported protein content ranging around from 78% to 93% for lyophilized hydrolysate or FPI samples from Salmon (Kristinson *et al*., 2000); Pacific whiting muscle (Pacheco-Aguilar, 2008); Catla catla (Balaswamy *et al.*, 2011) and Pollachius virens (Gholam *et al*., 2012).

Lipid content in FPIs at three DH values (DH=22.59%; DH=18.38%; DH=20.21%) was equivalent to each other ($p < 0.05$) and less than 1%. FPI from *Pangasius hypophthalmus* byproducts is a very low lipid content product compared to other ones derived from animals. This lipid content is higher than the one of SPI because *Pangasius hypophthalmus* belonged to fat fish group while SPI is derived from soybean (vegetable). Protein and Lipid contents of FPI did not show remarkable variation between three different levels of DH. However, the moisture content in the FPI decreased with increasing degree of hydrolysis and the values were statistically different for samples. The ash content in FPI fluctuated from 3.04% to 4.94% and had the significant different ($p < 0.05$) with the one of SPI. Ash and moisture content in FPI from *Pangasius hypophthalmus* byproducts were equal to ones of the FPI from Silver catfish (3.99% ÷ 5.61% and 3.33% ÷ 4.45% respectively) (Azima *et al*., 2013).

The links between Ca^{+2} and proteins in FPI from Pangasius hypophthalmus *by-products*

Testing results of the links between Ca^{+2} with FPI's proteins are shown in Table 2 and Figure 4. Figure 4 express the links between calcium ions and proteins in FPI at DH=22.59% (the DH that FPI's calcium-binding ability reached a maximum value of 27.03 mg/g). At this DH, the small molecular weight proteins (mainly from 1 to 3 kDa) play a key role in FPI's calcium-binding ability accounted for great numbers. At DH = 22.59%, there were 91.46 % Ca^{+2} in solution binded to proteins via forming of EF hand structure; 2.28 % linkages between Ca^{+2} with the proteins were hydrogen bonds through the "bridge" of water; 3.30 % links among Ca^{+2} and proteins are associated by electrostatic bonds (between Ca^{+2} with the proteins that started by no loop amino acid and beginning –C–C– structure (Table 2). This result is similar to findings of Zhengjin *et al*, 2003; Ann, 2005; Rong *et al*., 2011; Tai, 2013. The authors confirmed that the calcium-binding ability of FPI derived from freshwater fish *Blunt Snout Bream (Megalobrama amblycephala)*, by-product of *Sardinella aurita* or surimi of some catfish, mostly formed through the structure of the EF hand (over 79%); about 6% Ca^{+2} binded to proteins via electrostatic bonds between Ca^{+2} and amino acids in protein's structure.

Conclusion

Protein isolate obtained from enzymatic hydrolysis of *Pangasius hypophthalmus* byproducts at different DH had good functional features and good calcium-binding bio-activity. However, when prolonged hydrolysis time, at maximum DH, all the foaming, emulsifying, calcium-binding abilities of FPIs had not reached the highest values. The highest foaming ability of FPI from *Pangasius hypophthalmus* byproducts was 94.59% at hydrolysis time of 90 minutes, DH=20.21%. The highest FPI's emulsifying ability reached 20.99 mL oil/g FPI with hydrolysis time of 75 minutes, DH=18.38%. The hydrolysis time prolonged to 120 minutes, DH and the maximum calcium-binding ability were 22.59% and 27.03 mg Ca^{+2}/g FPI respectively. Chemical compositions of FPI were as follows: Protein: 90.01 ÷ 91.34%; lipid: 0.94 ÷ 0.98%; moisture: 2.86% ÷ 3.81%; and ash: 4.94% ÷ 3:04%. Type of the links between Ca^{+2} and proteins in FPI: 91.46% binding through EF hand structure; 2.28% as hydrogen bonding through water bridge; 3.30% as electrostatic links between Ca^{+2} with the starting amino acid of proteins.

Acknowledgements

We would like to express our thanks to Prof. David Dunker, University of Minnesota, USA for his invaluable helps in FAAS analysis and testing the links between Ca^{+2} and proteins in FPI.

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