RESEARCH ARTICLE

ENZYMATIC HYDROLYSIS OPTIMIZATION OF PANGASIUS HYPOPHTHALMUS BY-PRODUCTS TO OBTAIN FISH PROTEIN ISOLATE (FPI) WITH FOAMING FUNCTION

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Abstract:
Fish protein isolate (FPI) from Pangasius hypophthalmus by-products has high protein content (>90%). Almost its proteins are in small molecular weight. FPI can be applied in food industry due to its functional properties, such as: solubility, gelling, emulsifying, foaming, film-forming... In particular, one of its typical ones is foaming capacity. Our study provided the results of FPI’s foaming ability from Pangasius hypophthalmus byproducts. The experiments were carried out during enzymatic hydrolysis by protease (Alcalase 2.4L) at different hydrolysis conditions and optimized by response surface methodology (RSM) to obtain FPI that has the highest possibility of foaming. By the way, we simultaneously examine the molecular weight of proteins it contains by HPLC analysis method. Results showed that the foaming ability of FPI from Pangasius hypophthalmus by-products highest reaches 94.92% with hydrolysis conditions as follows: enzyme/substrate (E/S) ratio of 0.2% (v/w); hydrolysis temperature is 64°C; hydrolysis time is 92 minutes. The protein percentage by molecule weight of FPI as follows: <3 kDa: 48.23%; from 3 kDa to <7 kDa: 13.90%; from 7 kDa to <10 kDa: 20.02%; from 10 to 20 kDa: 8.25%.

Key Words: Protein isolate, foaming ability, Pangasius hypophthalmus by-products, optimizing, RSM.

1. INTRODUCTION

Protein isolate has been used as foaming and durable foaming agent in cakes, bread, ice-cream, meat products, desserts, and salad dressings, soft-drinks... (Javier Vioque et al., 2000). The foam formation involves the diffusion of soluble proteins to air/water inter-surfaces (Cecilia Abirached et al., 2012). At that surface, protein focused and stretched out immediately for increasing solubility and surface activity of soluble proteins (Mohamed Beva KelfalaFoh et al., 2012; J.G. Rocha-Estrada et al., 2010). The absorption of 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 (Amiza, M. A. et al., 2011; C. W. Coffmann et al., 2007). FPI from Tilapia (Oreochromis niloticus) has good foaming ability as well as durable foam capability, even much better foaming ability of soy protein isolate - SPI (M.B.K Foh et al., 2011). This is similar to what Taheri A. et al., (2012) showed when studying the foaming ability of the FPI derived from rainbow trout (Onchorhynhus mykiss) by-products. The foaming ability of FPI depends on its origin and produced method: Foaming ability of FPI from surimi of Sardinella reaches from 87.69% to around 89% while foaming ability of FPI from by-products of Sardinella aurita is 68.21% (Tai M.V at al., 2013; Nabil Souissi et al., 2007). The highest foaming ability of FPI gained in conditions of small protein ratio (with molecular weight less than 3 kDa) is more than 30% (Tai M.V., 2013). The FPI’s foaming ability from by-products of Catla catla, Jatropha curcas, Silver carp is equivalent to the one of whey protein isolate - WPI (K. Elavarasan at al., 2013; Mohsen Azadian at al., 2009; Cecilia Abirached et al., 2012). In Vietnam, total production of Pangasius hypophthalmus reached 1.17 million tonnes with export turnover of $1.8 billion in 2013. In the period 2007-2013, the average yield of Pangasius hypophthalmus is 1.2 million tons (Vietnam Association of Seafood Exporters and Producers - VASEP, 1/2014). While processing of catfish (mainly frozen fillets), an average of 2.6 kg of raw materials will generate to 1 kg of final product. Thus, the annual catfish by-products amount of Vietnam could reach 700, 000 tons. This is really huge, if it is rational used will bring enormous revenues to farmers and enterprises.
2. MATERIALS AND METHODS

2.1. 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°C until used. Enzymes Alcalase 2.4L were purchased from EAC Co., Ltd. (sole-exclusive agent for Novozyme in Ho Chi Minh city, Vietnam). All chemical reagents used for the experiments were in analytical grade.

2.2. Methods

2.2.1. Hydrolysis process:

By-product was cut in size of 5 mm, added water (ratio: water/by-product was 2/1), heated at 95°C in 15 minutes, cooled to proper temperature, adjusted pH=7 by phospat-citratbuffer solution, added enzyme Alcalase 2.4L, hydrolyzed the by-product in difference conditions of temperature, E/S ratio, hydrolysis time.

After hydrolysis, filtering to separate the solid and liquid, inactivating the protease (Alcalaze 2.4L) by heat treatment at 90°C/10 minutes as recommendation by Novozymes. Hydrolysed solution was then cooled to 4°C 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-drying to get FPI powder. FPI powder is used to study the foaming ability as well as its protein’s molecular weight.

The molecular weight of the proteins in FPI were determined by high pressure liquid chromatography (HPLC).

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:

\[
DH = \frac{V_B \times C_B \times \frac{1}{\alpha} \times \frac{1}{N_{total}} \times 100}{m_p}
\]

Where: \( V_B \) is the volume (liter) of base used (NaOH) to keep the pH constant during thereaction; \( C_B \) is the concentration molar; \( m_p \) is the total protein (N\times6.25); \( \alpha \) is the degree of dissociation of the \( \alpha \)-NH\(_2\) released during hydrolysis.

2.2.2. Optimization of hydrolysis the *Pangasius hypophthalmus* by-products.

Experimental planning: Response Surface Methodology (RSM) with 2 fold rotation plan centered (star distance \( \alpha=1.682 \)) is applied to optimize the conditions of hydrolysis. In particular, preliminary experimental has previously been selected as the basis for experimental optimization design. The parameters include: E/S ratio, temperature, hydrolysis time.

2.2.3. Determination of FPI’s foaming ability

Foaming ability of FPI was determined by the method of Kazunobu Tsumuraa et al (2004): 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:

\[
F(\%) = \frac{V_f-V_w}{V_i} \times 100
\]

Where, \( V_f \): total volume in foaming phase; \( V_w \): volume of separated water; \( V_i \): volume of initial mixture
3. RESULTS AND DISCUSSION

3.1. Optimizing of enzymatic hydrolysis

Before optimizing, we conducted preliminary experiments to identify the influence of each individual factor (ratio E/S, the temperature and time of hydrolysis) to FPI's foaming ability. The results of preliminary experiments were used as the basis for determining the simultaneously impact by all of three above factors to FPI’s foaming ability. When designing the optimization modal, the ratio E/S (X1, % v/w), hydrolysis temperature (X2, °C), hydrolysis time (X3, minute) were experimented simultaneously for 2 purposes: (1) to building the regression equation that describe the relationship among the hydrolysis factors that influence to the FPI's foaming ability from Pangasius hypophthalmus by-products. (2) Finding out the hydrolysis conditions in order to achieve the maximum FPI’s foaming ability. The experiment levels of independent factors in optimization as follows (Table 1):

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels of factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- α</td>
</tr>
<tr>
<td>Ratio E/S (X1, % v/w)</td>
<td>0.12</td>
</tr>
<tr>
<td>Hydrolysis temperature (X2, °C)</td>
<td>52</td>
</tr>
<tr>
<td>Hydrolysis time (X3, min.)</td>
<td>46</td>
</tr>
</tbody>
</table>

The coded levels of variables: above level (+1); database level (0); below level (-1); α = 1,682

The experimental number were calculated as follows: \[N = 2^k + 2n_0 = 2^3 + 2 \times 6 = 20\] (k: number of experimental factors (k = 3), n0: number of experiments in center or mind (n0 = 6). experimental matrix shows the simultaneously of the factors is presented in Table 2.

<table>
<thead>
<tr>
<th>N0</th>
<th>Coded levels of variables</th>
<th>Real variables</th>
<th>Observed foaming ability (%)</th>
<th>Expected foaming ability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>x1</td>
<td>x2</td>
<td>x3</td>
<td>X1</td>
<td>X2</td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>0.15</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>0.25</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>7</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Regression equation is a second-order polynomial as below:

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \]

Where, \( Y \) is the dependent variable (foaming ability in real value); \( X_i \) and \( X_j \) are the levels of the independent variable (experimental factor) which represent the influence of \( X_1 \), \( X_2 \), \( X_3 \) on the response factor (foaming ability); \( \beta_0 \) is constant; \( \beta_i \), \( \beta_{ii} \), \( \beta_{ij} \) are the coefficients of the regression equation.

To build the mathematical description as a regression equation, the coefficients of the equation must be determined. Its coefficients have the following values:

- \( b_0 = 94.00; b_1 = 0.79; b_2 = 1.69; b_3 = 0.91; b_{11} = -1.48; \)
- \( b_{22} = -0.83; b_{33} = -0.92; b_{12} = -0.36; b_{13} = -0.95; b_{23} = -0.48 \)

Two of coefficients: \( b_{12} (P=0.114652 > 0.05) \) and \( b_{23} (P=0.0530999 > 0.05) \) have no statistical significance. Thus, these coefficients \( (b_{12} \) and \( b_{23} \)) are removed from the regression equation. The regression equation takes the following form:

\[ Y = 94.00 + 0.79 X_1 + 1.69 X_2 + 0.91 X_3 - 1.48 X_1^2 - 0.83 X_2^2 - 0.92 X_3^2 - 0.95 X_1 X_3 \]

Testing the compatibility of the regression equation and experimental results shows that three experimental factors \( (X_1, X_2, X_3) \) have a strong influence \( (P<0.05) \) on the foaming ability of FPI from *Pangasius hypophthalmus* by-products during hydrolysis. The compatibility of the regression equation (Lack of fit) is checked with the Modde5.0.

After checking, the "Lack of fit" is not statistically significant (Lack of it has \( P = 0.053, P > 0.05 \)). Thus the regression equation has high compatibility with experiments (*Kun-Nan Chen, 2008*).

**Table 3.** Testing results the compatibility of the regression equation

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom (DF)</th>
<th>Sum of Squares (SS)</th>
<th>Mean Squares (MS)</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>9</td>
<td>190.427</td>
<td>21.1585</td>
<td>28.4675</td>
<td>0.000</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>7.43252</td>
<td>0.743252</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

11
The influence of the experimental factors on the foaming ability of FPI from *Pangasius hypophthalmus* by-products are shown on the response surface in Figure 1.

Overall, the foaming ability of FPI increased along with the rising of experimental factors to a limited value. However, then the foaming ability was stability and it is tended to decrease when the values of factors exceeded the limits value. Figure 1A shows the effects of pair of elements (X₁: ratio E/S and X₂: hydrolysis temperature) on FPI’s foaming ability. Results indicate that foaming ability rises up about 8.6% plus, along with the increasing of the E/S ratio (to 0.2% v/w) and hydrolysis temperatures (to 64°C), DH=18.6%. However, the foaming ability reduces when hydrolysis was carried out in condition of higher E/S ratio and temperatures. The influence of E/S ratio and hydrolysis time (X₁ and X₃) on FPI’s foaming ability is shown in Figure 1B. During hydrolysis time from 80 to 92 min. (DH=18.73%), foaming ability tends to increase gradually (from 88.4% to 94.23% respectively). However, the hydrolysis time further increased to 100 min., DH reached 24.3%, foaming ability falls to 92.2%. The same rule is observed when considering the impact of the simultaneous hydrolysis temperature and hydrolysis time (figure 1C). The foaming ability increases until the hydrolysis temperature (X₂) reaches 64°C and hydrolysis time (X₃) reaches to 92 min. (DH=18.31%). This ability descended when the temperature and hydrolysis time value continues to increase.

This is explained as follows: When increasing both E/S ratio and hydrolysis temperature, the Degree of Hydrolysis (DH) increased; up to a certain limit, the enzyme was “saturated” and DH tended to be constant. On the other hand, when the temperature rises higher and higher can lose catalytic activity of protease. 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 M.V, 2013), so the FPI’s foaming ability gains maximizing value. However, then any increasing in temperature and hydrolysis time in conditions optimal E/S ratio, the proteins in by-products were gradually hydrolyzed in depth (DH increased). The amounts of amino acid as well as the number of proteins in very, very small molecular weight were generated. This reduces significantly the FPI’s foaming ability.

Optimization results show that the foaming ability of FPI from *Pangasius hypophthalmus* by-products reaches the highest of 94.92% in terms of hydrolysis factors: temperature is 64°C, the E/S ratio is 0.2% (v/w), hydrolysis time is 92 minutes.

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 peptids. The foaming ability of FPI byproducts of *Sardinella aurita, Pollachius virens* can reach from around 89% to 100.3% (Nabil Souissi et al., 2007; Gholam Reza Shaviklo at al., 2012; Tai M.V., 2013) during enzymatic hydrolysis (using protease Alcalase) in optimum conditions: pH=7, hydrolysis temperature 58°C, hydrolysis time 120 minutes, E/S ratio 0.3%. Foaming ability of FPI from *Silver catfish, Pacific Hake* (*Merluccius productus*) was 107.7% at DH = 43%, hydrolysis temperature 62°C, hydrolysis time 90 min, pH 7 (Amiza, M. A., 2013; Anusha Perera Geethangani Samaranyaka, 2010). The study results of foaming ability of

<table>
<thead>
<tr>
<th>Lack of Fit</th>
<th>Pure Error</th>
<th>Total Corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>6.17118</td>
<td>1.26133</td>
<td>197.859</td>
</tr>
<tr>
<td>1.23424</td>
<td>0.252267</td>
<td>10.4137</td>
</tr>
<tr>
<td>4.89258</td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td>insignificant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FPI from *Pangasius hypophthalmus* by-products are similar to the findings of Diniz, A. M. (1997); Klompong, V. et al (2007) on the foaming ability of FPI from *Squalus acanthias, Selaroides leptolepis, Grass carp*.

### 3.2. The analytical results of the molecular weight of proteins in FPI

Analytical results of the molecular weight of proteins in FPI that derived from *Pangasius hypophthalmus* by-products in the hydrolysis conditions after optimization (E/S ratio is 0.2% w/v, hydrolysis temperature is 64°C, hydrolysis time is 92 minutes) to have the highest foaming ability 94.92% are shown in Table 4 and Figure 2.

**Table 4.** The ratio distribution basing on molecular weight of proteins in FPI

<table>
<thead>
<tr>
<th>No</th>
<th>Molecular weight (kDa)</th>
<th>Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt; 20</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10 - 20</td>
<td>8.25</td>
</tr>
<tr>
<td>3</td>
<td>7 - 10</td>
<td>20.02</td>
</tr>
<tr>
<td>4</td>
<td>3 - 7</td>
<td>23.5</td>
</tr>
<tr>
<td>5</td>
<td>&lt; 3</td>
<td>48.23</td>
</tr>
</tbody>
</table>

*Figure 2.* HPLC analysis results determining the molecular weight distribution of proteins in FPI

According to results in Table 4, when the hydrolysis time to reach 92°C, the proteins in FPI in the molecular weight of 7 kDa or smaller than are dominant (71.71%, in which the protein has a molecular weight less than 3 kDa accounting for 48.23%). This group of peptide plays a decisive role to foaming ability of FPI. The rate of proteins in molecular weight <3 kDa accounted for approximately 40% of the FPI’s total protein would have an important role in improving the foaming ability of FPI. This result is similar to studying of Tai M.V. (2013); Mohsen Azadian at al (2012); Ann Elizabeth Theodore (2005). They confirmed that foaming ability of FPI obtained from *Sardinella aurita* or *Silver cap* or other catfish by-products as *Afromastacembelus, Aethiomastacembelus* reached the highest value when quantity of protein with molecular weight from 2 kDa to 8 kDa around 72%; in the hydrolysis time from 75-95 minutes, hydrolysis temperature 59°C.
4. Conclusion
FPI derived from *Pangasius hypophthalmus* by-products through enzymatic hydrolysis has good foaming ability. The foaming ability achieves maximum value 94.92%, corresponding to the hydrolysis conditions as follows: E/S ratio is 0.2% (v/w), hydrolysis time is 92 minutes, and hydrolysis temperature is 64°C. Ratio of proteins in different molecular weights in FPI: <3 kDa accounted for 48.23%, from 3 kDa to <7 kDa accounted for 23.50%, from 7 kDa to <10 kDa accounted for 20.02%, from 10 kDa to 20 kDa accounted for 8.25%.

Acknowledgements
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References


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